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Colonization ability of autochthonous *Saccharomyces cerevisiae* strains in mixed culture fermentation and in vineyard environment

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ABSTRACT

Yeast is the main protagonist of the alcoholic fermentation and, together with the grape juice quality, determines the final wine characteristics. Responding to the consumer requests of more "natural" wine products without additives that keep sensory characteristics of the production area, the most challenging enology is focused to perform spontaneous fermentation in which the interactions among autochthonous microorganisms present in must determine better sensory characteristics, traditionally related to the production area.

In this context the first part of this thesis project was developed. This involved the inoculum of autochthonous *Saccharomyces cerevisiae* strains, isolated in the winemaking area of Prosecco Superiore di Conegliano Valdobbiadene DOCG, in two vineyards belonging to this production area, one cultivated using conventional practices and the other grown under organic farming methods. Their ability to colonize the vineyard was assessed and spontaneous fermentations with grapes coming from the inoculated vine rows were performed. The results of barks sampling performed for two consecutive years showed that the presence of inoculated strains in the vineyard was very poor and limited to the first six months after treatment, regardless of the cells concentration of the inoculated suspension. Similar results were obtained when the strains present in the fermenting grape musts, during spontaneous vinifications performed in the two vintages, were genetically characterized. A very small percentage was identified as to be some of the inoculated strains. Surprisingly, the fermenting grape musts were colonized mainly by commercial strains, used from the local wineries, present on grape bunches at harvest.

With the aim to understand the interaction mechanisms that occur among different *S. cerevisiae* strains during alcoholic fermentation, eight autochthonous strains (among them the five yeast released in vineyard were present) were pairwise inoculated in synthetic must. The kinetics produced by the co-fermentations not always reproduced those of the dominant strains when evaluated in single-strain fermentations, but on the contrary, the presence of a second strain deeply influences the fermentation kinetics, improving or sometime worsening the fermentation trend. The best competitor was a strain with neutral killer phenotype. These results highlight the existence of other factors than the killer character involved in must colonization.

In the last part of this thesis work the hypothesis if the must colonization ability could depends on yeast nutrient request that influence the nutrient availability to the rest of microbiota, was tested. Therefore the nitrogen request of the wine industrial strain QA23 was investigated in comparison with those of three autochthonous *S. cerevisiae* strains chosen among the yeasts previously tested in co-fermentations in synthetic must. In order to investigate if different nitrogen needs could influence colonization ability during must fermentation, pairwise strain fermentations were performed in synthetic must with high and low nitrogen level. Results suggested a strong implication of nitrogen assimilation ability on must colonization. The strain with highest nitrogen demand is the one that strongest opposed to QA23 colonization. During the fermentation its colonization ability increased, indicating better performance when nitrogen was depleted.

The knowledge of the specific phenotypic characteristics of the strains, besides the genetic characterization, proved to be crucial to understand and control the composition of the microbial flora presents in the must and in the vineyard environment. The management of the colonization dynamics is proposed as powerful tool in the hands of winemakers and valid alternative to the use of additives in winemaking.

The yeast *Saccharomyces cerevisiae* after about 7000 years remains the main protagonist of winemaking and the maintenance of its biodiversity proved to be the key to guarantee the production of wines with new sensory characteristics.

RIASSUNTO

Il lievito è il protagonista principale della fermentazione alcolica ed assieme alla qualità del mosto di partenza determina le caratteristiche finali del vino. In risposta alla richiesta da parte dei consumatori di vini "naturali", prodotti senza aggiunta di additivi, e con profili sensoriali caratteristici della zona di produzione, la sfida enologica è orientata all'ottenimento di fermentazioni spontanee in cui le interazioni che avvengono tra i microrganismi autoctoni presenti nei mosti determinano caratteristiche sensoriali migliori e tradizionalmente legate al territorio di produzione.

In questo contesto si inserisce la prima parte del progetto di questa tesi che ha previsto l'inoculo di ceppi autoctoni (ecotipici) di *Saccharomyces cerevisiae* isolati nell'area del Prosecco Superiore di Conegliano Valdobbiadene DOCG, in due vigneti appartenenti a questa zona di produzione, uno coltivato con tecniche agronomiche biologiche e l'altro gestito in modo convenzionale. È stata valutata la loro capacità di colonizzare l'ambiente vigneto e di avviare una fermentazione spontanea nei mosti ottenuti con l'uva proveniente dai filari inoculati. I risultati dei campionamenti di porzioni di ritidoma eseguiti per due anni consecutivi dimostrano che la presenza dei ceppi inoculati in vigneto è molto bassa e limitata ai primi sei mesi dal trattamento, indipendentemente dalla concentrazione cellulare della sospensione inoculata. Anche durante il processo di fermentazione del mosto, durante le microvinificazioni condotte con l'uva raccolta dai due vigneti nei due anni di sperimentazione, si osservano percentuali di presenza molto basse di alcuni ceppi inoculati. Nelle fermentazioni si sono imposti lieviti commerciali usati dalle cantine dell'areale di produzione considerato in questo studio, e che sono presenti sui grappoli d'uva in epoca di raccolta.

Con lo scopo di comprendere i meccanismi d'interazione tra ceppi appartenenti alla specie *Saccharomyces cerevisiae* durante la fermentazione alcolica, otto ceppi autoctoni (tra cui quelli usati nell'esperienza di rilascio in vigneto) sono stati inoculati a coppie in mosto sintetico. Dall'analisi delle performances fermentative e della loro abilità di colonizzare il mosto si osserva che non sempre la cinetica di fermentazione prodotta dal co-inoculo riproduce quella del ceppo dominante, ma al contrario, la presenza di un secondo ceppo influenza la cinetica di fermentazione, migliorandola o peggiorandola. Il ceppo di

lievito che maggiormente è riuscito ad imporsi nelle prove di fermentazione non presenta fenotipo killer dimostrando che altri fattori, oltre al carattere killer, condizionano la colonizzazione del mosto.

Per testare l'ipotesi che la capacità di "dominare" le fermentazioni derivi dalla capacità di sottrarre nutrienti agli altri ceppi, l'ultima parte di questo lavoro di tesi ha riguardato lo studio delle richieste di azoto di un ceppo commerciale QA23 e di tre ceppi di vigneto (scelti tra quelli già studiati nelle precedenti prove di co-fermentazione). Inoltre è stato studiato il ruolo della disponibilità di azoto nelle dinamiche di colonizzazione alle stendo prove di co-inoculo in mosto sintetico con alta e bassa concentrazione di azoto. Dai risultati si osserva che il ceppo che presenta maggiori richieste di azoto si oppone maggiormente alla colonizzazione del ceppo commerciale, e la sua capacità di colonizzare il mosto aumenta in condizioni di basse concentrazioni di azoto.

La conoscenza delle caratteristiche fenotipiche specifiche dei ceppi, oltre che la caratterizzazione genetica, si dimostrano fondamentali per comprendere e controllare la composizione della flora microbica presente nei mosti e nell'ambiente vigneto. La gestione delle dinamiche di colonizzazione si propongono come valido strumento a disposizione dell'enologo e valida alternativa all'uso di additivi in vinificazione.

Il lievito *Saccharomyces cerevisiae* dopo circa 7000 anni si conferma il principale protagonista dell'arte enologica e il mantenimento della sua biodiversità si rivela la chiave per garantire la produzione di vini con sempre nuove caratteristiche sensoriali.

CHAPTER 1

Introduction

1.1 GRAPE BERRY YEAST COMMUNITIES

The earlier work of Louis de Pasteur, by the last quarter of the XIXth century, already showed that the microorganisms responsible for wine fermentations are yeasts present on the grapes. Since then a huge amount of information has been gathered on yeast dissemination in wine associated invironments but ecological relationships are still to be fully understood.

Worldwide surveys seem to indicate that apparently sound grapes are colonised by a wide variety of yeast species. However, this variety may be reduced to relatively few groups of similar physiological characteristics. Some authors (Barata A. *et al*., 2012) propose to systematise the microbiota of grape berries into three main yeast groups, characterised by similar behaviour on grape berries: (i) oligotrophic, oxidative basidiomycetous yeasts, the yeast-like fungi *A. pullulans*, and lactic acid bacteria (*Lactobacillus* spp., *Oenococcus oeni*); (ii) copiothrophic, oxidative ascomycetes (several *Candida* spp.); weakly fermentative apiculate (*Hanseniaspora* spp.), filmforming (*Pichia* spp.), fermentative (*C. zemplinina, Metschnikowia* spp.) yeasts; (iii) copiotrophic strongly fermentative yeasts (*Saccharomyces* spp., *Torulaspora* spp., *Zygosaccharomyces* spp., *Lachancea* spp. and *Pichia* spp.) and the obligate aerobic acetic acid bacteria (*Gluconobacter* spp., *Gluconoacetobacter* spp., *Acetobacter* spp.). The balance among these groups, after véraison, is particularly dependent on nutrient availability on berry surface.

The first group is composed by species favoured by the nutrient poor environment of truly sound berries. In vineyards, soil, leaves and bark also characterized by the dominance of basidiomycetous oxidative yeasts and the yeast-like fungi *A. pullulan.*

The increase in the proportion of oxidative or weakly fermentative ascomycetous species (*Hanseniaspora, Candida, Metschnikowia* and *Pichia* spp.), may occur during ripening. The mechanisms underlying this succession are not clear, species interaction may

occur (Fleet, 2003), but the main factor should be related to nutrient availability. In fact, when approaching maturity, berries begin to behave differently from plant leaves, probably because of cuticle softening and release of volatile organic compounds (VOC's). The emergence of these species is probably the result of juice release, even in visually intact berries, as suggested by the effect of diffuse powdery mildew which injury is invisible to the naked eye.

The proliferation of the third yeast group is explained by the high nutrient availability resulting from grape damage. Damaged grapes possess, besides much higher cell counts, wider species diversity than sound grapes. Basidiomycetes may be still present in numbers similar to those of sound grapes but their proportion is strongly decreased by the proliferation of ascomycetous species. *H. uvarum* and *C. zemplinina* may be present in higher numbers but their relative proportion also decreases in favour of the fermentative yeasts (e. g. *Pichia* spp., *Zygosaccharomyces* spp., *Zygoascus* spp., *Torulaspora* spp.), which may occasionally dominate the overall microbiota. Moreover, the frequency of occurrence of *S. cerevisiae* was found about 0.05 to 0.1% in sound berries and 25% in damaged berries, usually with numbers of about 10^5–10^6/berry (Mortimer and Polsinelli, 1999). The genera *Zygosaccharomyces* spp. and *Torulaspora* spp. were detected at higher frequencies in grapes affected by noble rot, sour rot and honeydew, suggesting their adaption to conditions of reduced water activity and presence of weak organic acids (Barata et al., 2008).

1.1.2 Factors influencing species diversity

The microbial communities on grapes may be affected by a large number of factors. Moreover, all factors commonly described as influencing grape microbiota (rainfall, wind, temperature, diseases, pests, viticultural practices, etc.), affect primarily skin integrity and so their impact will be discussed further taking into account the expected changes induced by berry damage.

The climatic and microclimatic conditions include the effect of temperature, UV exposure, rainfall, sunlight and winds. Several studies mention that diversity and quantity of microbial populations are dependent on these conditions. For instance, rainy vintages lead to higher use of phytochemicals, higher fungal proliferation and higher berry damage, in conjunction with lower UV irradiation. Concerning total yeast counts, Combina et al.

(2005) found that years with increased rainfall yielded higher counts, probably due to increase in berry volume allowing release of juice in joint areas such as the area between the pedicel and the berry, and higher exosmosis leading to nutrient release on the grape surface. On the contrary, Comitini and Ciani (2006) found 10 times less total counts in years with high rainfall. The reports on species diversity are also not conclusive. Large scale works do not demonstrate any relation between climatic conditions and yeast diversity. The authors of those studies stated that, when a large number of vintages are analysed, appearance/ reappearance cycles of certain strains have no obvious explanation.
The main vineyard treatment studied is related with the use of pesticide treatments, mainly those against fungi (downy mildew, powdery mildew and grey rot). Viviani-Nauer et al. (1995) found that pesticides decreased yeast population and diversity in fermenting musts. Cabras et al. (1999) reported the absence of effect on fermentation of *S. cerevisiae* by 6 different fungicides while fermentation by *K. apiculata* was stimulated. More recent works concern the differences from organic and conventional farming systems and they are tempting to conclude that organic farming leads to higher biodiversity, both in *S. cerevisiae* and in non- *Saccharomyces* yeasts (Cordero-Bueso et al., 2011).

The association between yeasts and invertebrates is well-known in general ecological studies. Few data available from vineyards showed that bees and wasps carry yeasts for the grapes. A study conducted by Stefanini et al. (2012) demonstrate the role of social wasps as vector and natural reservoir of *S. cerev*isiae during all seasons. They provide experimental evidence that queens of social wasps overwintering as adults (*Vespa crabro* and *Polistes* spp.) can harbor yeast cells from autumn to spring and transmit them to their progeny. This findings indicate that wasps are a key environmental niche for the evolution of natural *S. cerevisiae* populations, the dispersion of yeast cells in the environment, and the maintenance of their diversity. Regarding birds, a recent survey evidenced that swabs of bird beaks and the initial part of the digestive tract are reservoirs of several grape contamination species, like *H. uvarum* (Francesca et al., 2010).

There are few reports on the interactions between microbial populations that possibly include killer toxin, antibiotic and quorum sensing mechanisms (Fleet, 2003; Golubev, 2006). Yeast–yeast interactions are mostly studied regarding the killer effect, but this activity is probably not relevant in natural populations (Sangorrín et al., 2001). In

particular, Van der Westhuizen et al. (2000) found that killer activity did not affect the distribution of *S. cerevisiae*.

There is a broad line of thought considering that the grape microflora is dependent on the vineyard location, grape variety and other vineyard related factors. The wish for this belief is that wine tipicity may be, at least partially, dependent on the specific grape microbiota of the producing region. The ultimate goal is to find yeasts according to each wine "terroir". Regard geographic location some authors (Van der Westhuizen et al. (2000), Khan et al. (2000), Schuller et al. (2005), Schuller and Casal (2007) and Valero et al. (2005, 2007)), on different hemispheres, found essentially the same results. They observed that indigenous *S. cerevisiae* populations on grapes were subjected to natural fluctuations of periodical appearance/ disappearance, but no attempt to define "terroir" strains was made because no strain common to all sites, in one region, was found. Furthermore, the studies of Schuller et al. (2005), Schuller and Casal (2007) and Valero et al. (2005, 2007) also concluded that *S. cerevisiae* from commercial starters were only detected near water running off the winery and that there was no influence of starter utilisation in the biodiversity of *S. cerevisiae* populations in the vineyards. Pretorius et al. (1999), describing close vineyards with equal climate, stated that intra-annual variations should be attributed to other vineyard factors like age and size.

The presence of *Saccharomyces* spp. strains is associated to other natural resources, such as oak and other broad-leafed trees. Sampaio JP & Gonçalves P (2008) in a recent work report consistent isolation of *Saccharomyces* spp. from oak bark and soil beneath oak trees located in the Mediterranean area. In other works was reported the isolation of *S. cerevisiae* and *S. paradoxus* from bark and soil associated with oaks in North America (Sniegowski P. D. *et al.,* 2002) and isolation of *S. paradoxus* from *Quercus robur* trees in England (Johnson L. J. *et al*., 2004). Taken together, these three studies provide very strong evidence that tree bark, and particularly the bark from certain oaks, is a habitat for *Saccharomyces* yeasts. In particular, the frequencies of isolation from certain trees are strikingly high compared with those reported for any other natural sample, including grape berries. The detection of the presence of simple sugars in bark samples of the trees exhibiting the highest frequencies of isolation, suggests that it could be an important factor for the maintenance of *Saccharomyces* in this environment.

1.2 ALCOHOLIC FERMENTATION AND WINE YEAST POPULATIONS

Saccharomyces cerevisiae is so closely associated with humans it is rarely found in environs removed from human habitation. In fact, its evolutionary success can probably be explained by its relationship with humans, particularly in the production of alcoholic beverages, an activity that has been with us for at least 7000 years. It is likely that the first alcoholic fermentations were "happy accidents": harvested grapes were not eaten quickly enough and began to rot, *Saccharomyces* spp "moved in" and took advantage of the free sugary meal and the first wines were made. These early wines presumably tasted good and had an interesting, pleasing, psychotropic effect. One can only assume that early farmers learned from this experience and repeated the "accidents" of previous "vintages" (Pretorius IS *et al*., 2012).

The alcoholic fermentation is the main activity by which yeasts make a positive contribution to wine flavour (Henschke, 1997). They do this by several mechanisms: (i) utilizing grape juice constituents, (ii) producing ethanol and other solvents that help to extract flavour components from grape solids, (iii) producing enzymes that transform neutral grape compounds into flavour active compounds, (iv) producing many hundreds of flavour active, secondary metabolites (e.g. acids, alcohols, esters, polyols, aldehydes ketones, volatile sulphur compounds), and (v) autolytic degradation of dead yeast cells (Cole and Noble, 1997; Lambrechts and Pretorius, 2000). These reactions, especially the production of secondary metabolites, vary with the species and strain of yeast. Thus, the uniqueness and individuality of the flavour contribution by yeasts depends on the species and strain ecology of fermentation and the many factors that determine this ecology (Fleet and Heard, 1993; Fleet, 2001). In recent years, there have been major advances in understanding the yeast ecology of wine fermentation (Fleet, 2001). Grape juice fermentation presents a complex ecosystem that involves the interactive growth and biochemical activities of a mixture of yeast species and strains. These yeasts originate from the flora of the grapes, the flora associated with the surfaces of winery equipment and the winery environment (e.g. air, insects). The surfaces of winery equipment are easily colonized and become locations for the development of a resident or winery yeast flora. The *Saccharomyces cerevisiae* strains resident on the winery surfaces are much more abundant than those that might come from the grapes or vineyard. (G. Beltran *et al*. 2002).

1.2.1 Factors influencing species diversity

Many factors affect the occurrence and growth of yeasts during alcoholic fermentation. These include the initial population and diversity of species and strains in the grape juice, inoculation of the juice with selected starter cultures, chemical composition of juice including any fungicide/pesticide residues, processing conditions such as concentration of sulphur dioxide addition and temperature of fermentation (Fleet, 2001). Furthermore the ecological outcome is determined by. interactions between individual microorganisms. They are neutralism, commensalism, synergism, antagonism, parasitism, and competition. These interactions can enhance or inhibit the growth of any particular species or strain. Within the wine ecosystem, there are numerous mechanisms whereby one yeast may influence the growth of another yeast. Early growth of yeasts in grape juice decreases or strips it of nutrients, making the resultant wine less favorable as an environment for any further microbial growth. Furthermore yeasts growth generates metabolites, some of which will be toxic to other species ,as ethanol and short chain fatty acids. Some species may produce inhibitory peptides, proteins or glycoprotein, such as killer toxins, and enzymes that destroy other species by lysis of their cell walls. Carbon dioxide production and oxygen removal limit the growth of aerobic species. (Fleet, 2003)

1.2.2 Yeasts involved in spontaneous fermentations

Generally, species of *Hanseniaspora (Kloeckera)*, *Candida* and *Metschnikowia* initiate the fermentation, and largely originate from the grapes. Sometimes, species of *Pichia, Issatchenkia* and *Kluyveromyces* may also grow at this stage. These yeasts grow to about 10^6–10^7 cfu/ml but, by mid-fermentation, begin to decline and die off. At this time, *S. cerevisiae* becomes predominant (10^7–10^8 cfu/ml) and continues the fermentation until its completion. However, quantitative studies on grape juice fermentation have shown that *Kloeckera apiculata* and *Candida stellata* can survive at significant levels during fermentation, and for longer periods than thought previously (Ciani *et al.,* 2010).

1.2.3 The use of selected starter cultures of *S. cerevisiae*

Until about the 1980s, the contribution of yeasts to wine production was seen as a relatively simplistic concept. Essentially, grape juice underwent a natural or a spontaneous

alcoholic fermentation that is characterized by the activity of different yeast species/strains, even if almost invariably it is dominated by strains of the yeast *S. cerevisiae*. Although this yeast diversity can contribute to the wine complexity and can produce unique-flavoured wines (Combina et al., 2005; Zott et al., 2008), the dynamics of a spontaneous fermentation is often unpredictable and some non-*Saccharomyces* species can also produce undesirable compounds. Risks associated with spontaneous fermentations include both slow or arrested fermentations and the proliferation of contaminant yeasts. Therefore, in the wine-making industry the growth of undesirable yeasts is controlled by addition of sulphur dioxide to musts and inoculation with selected strains of *Saccharomyces*, mainly *S. cerevisiae* (Henick-Kling et al., 1998).

Use of active dry yeast in fermentation has become one of the most common practices in winemaking because it ensures a reproducible product and reduces the lag phase and the risk of wine spoilage. However, the winemaking community is still widely divided about this practice because of a widespread belief that native yeast strains give a distinctive style and quality to wine. In fact, the use of active dry yeast reduces the number of different indigenous *Saccharomyces cerevisiae* strains due to the starter imposition, but does not completely prevent them from growing until several days after the inoculation. During this time, wild strains may have an important effect on wine flavour and characteristics (Querol *et al*., 1992).

The effect of the common practice of inoculation upon the diversity of indigenous *S. cerevisiae* strains and the development of alcoholic fermentation was studied by Beltran et al. (2002). They analysed the yeast population in a new winery (and therefore with new equipment and no yeast resident flora) for six consecutive years. Non-*Saccharomyces* yeasts, particularly *Hanseniaspora uvarum* and *Candida stellata*, dominated the first stages of fermentation. In all the cases, *S. cerevisiae* took over the process in the middle and final stages of fermentation. The analysis of the *S. cerevisiae* strains showed that indigenous strains competed with commercial strains inoculated in other fermentation tanks of the cellar. The continuous use of commercial yeasts reduced the diversity and importance of the indigenous *S. cerevisiae* strains. A natural origin of the indigenous strains isolated could be proposed but a repeated appearance of these strains in different years pointed out to a colonization of the winery environment with yeasts that go through generations and

generations at each vintage. This hypothesis is supported by the analysis of the inoculated, commercial strains. The presence of these strains, even when not inoculated in the same vintage, competing with "natural" strains, in the spontaneous fermentations prove the establishment of these strains as residents of the winery surfaces.

1.2.4 *Saccharomyces cerevisiae* associated to spontaneous fermentations

The use of commercial starters could mask the distinctive properties that typify some local wines (Romano et al., 2008). When these properties are crucial in the commercial success of a wine, the selection of autochthonous yeast strains for their use as starters is considered an advantageous approach. Wine quality is strongly influenced by the yeasts involved in the fermentative process: body, viscosity, colour, flavour and aroma of wines are strongly determined by the yeasts (Rainieri and Pretorius, 2000). During alcoholic fermentation, genetically distinct *Saccharomyces cerevisiae* strains release various aroma compounds, which influence the organoleptic quality of wines, and different yeast strains contribute differently to the wine quality.

In a recent study, Capece *et al*. (2012), showed that autochthonous *S. cerevisiae* strains can be different not only at the genomic level, but also significantly in their metabolic profiles under the same experimental conditions. The production level of secondary compounds by *S. cerevisiae* strains isolated from Bosco must, was more variable in the must of origin of isolates than in "Greco di Basilicata" must. This suggest that in the isolation grape must the strains are able to express their own characteristics probably because they are better adapted to metabolize the precursor present in this grape must. Otherwise, by inoculating the strains in a grape must different from isolation source, the strains have to adapt the metabolic activity for a better utilization of precursors present in the must.

In the case of particular yeast cultures designed to impose a special character or style on the final product, dominant growth of the inoculated strain would be required. Several works have evidenced that the dominance of the starter is not always guaranteed (Lopes et al., 2007; Barrajón et al., 2009) The capacity of a selected yeast to take over industrial fermentations represents an additional feature to be evaluated in all wine yeast selection programs.

CHAPTER 2

Technological features of eight *Saccharomyces cerevisiae* strains used for the experiments

2.1 INTRODUCTION

The wine technologists gathered the basic properties required for the definition of a ‘‘selected *S. cerevisiae* strain for wine making’’ in two categories (Reed G. and Chan SL., 1979): (1) primary or fitness traits, defined as those strictly associated with the formation of ethyl alcohol by fermentation, and (2) secondary or quality traits, defined as those related to the production of compounds that affect other parameters, such as the body of a wine, the higher alcohols complex (bouquet), and the appearance of undesirable off-flavors. Main primary and secondary traits are summarized in table 1.1, where some further traits, more specific and functional to the type of desired wine, are also listed (Pretorius IS., 2000).
Some of the requirements listed in Table 1.1 are complex and difficult to define genetically without a better understanding of the involved biochemistry and physiology. To date, no wine yeast present on the market has all the characteristics listed, and it is well established that wine yeasts have different behaviour concerning their winemaking abilities. Although this phenomenon can be ascribed to fermentation conditions that are hardly reproducible, the major source of variation can be attributed to the genetic constitution of the wine yeasts (Pretorius IS., 2000).

2.1.1 Fitness traits

The technological traits influence the efficiency of the fermentation process. *S. cerevisiae* strains generally possess the technological characteristics required to perform an efficient fermentation. The determination of these traits is, however, necessary, since most of these characteristics are strain specifics.

Table 2.1.1 Main desirable characteristics of wine yeast

Fitness traits	
Fermentation properties	**Technological properties**
Rapid initiation of fermentation	High genetic stability
High fermentation efficiency	High sulphite tolerance
High fermentation rate	Low sulphite binding activity
High ethanol tolerance	Low foam formation
High osmotolerance	Flocculation properties
Low temperature optimum	Copper resistance
Moderate biomass production	Resistance to desiccation
	Killer properties
	Proteolytic activity
	Low nitrogen demand
Quality traits	
Flavour characteristics	**Metabolic properties with health implications**
Low volatile acidity production	Low sulphite formation
Moderate higher alcohol production	Low biogenic amine formation
Low sulphite/DMS/thiol formation	Low ethyl carbamate (urea) potential
Liberation of glycosylated flavour precursors	
No phenolic off-flavours production	
High glycerol production	
Modified esterase activity	
Enhanced autolysis	
Hydrolytic activity	

2.1.1.1 Main fermentation properties

The rate of fermentation and the amount of alcohol produced per unit of sugar during the transformation of grape must into wine is of considerable commercial importance. The fermentation efficiency is intended as the uppermost concentration of ethanol obtainable by fermentation from an excess of sugar. The fermentation rate (vigour) is the measure of the ability of a starter to bring the fermentative process to a fast completion. It is normally represented as grams of CO_2 developed in 24 h, calculated as the average of a 3-day measurement period (Martini A., 2003). During wine yeast glycolysis, one molecule of glucose or fructose yields two molecules each of ethanol and carbon

dioxide. However, the theoretical conversion of 180 g sugar into 92 g ethanol (51.1%) and 88 g carbon dioxide (48.9%) could only be expected in the absence of any yeast growth, production of other metabolites and loss of ethanol as vapour (Boulton B., *et al*. 1996).
The ethanol production and fermentation rate are closely linked to ethanol tolerance: in fact while ethyl alcohol is the major desired metabolic product of grape juice fermentation, it is also a potent chemical stress factor that is often the underlying cause of sluggish or stuck fermentations. Apart from the inhibitory effect of excessive sugar content on yeast growth and vinification fermentation, the production of excessive amounts of ethanol, coming from harvest of over-ripe grapes, is known to yeast growth rate, viability and fermentation capacity: cell growth stops at relatively low ethanol concentrations, and fermentation stops at relatively higher ones. Decreases in the rate of ethanol production are related to decreases in viable cell count. Cell growth inhibition by ethanol is noncompetitive and has been described as either a linear or an exponential function of ethanol concentration (Boulton B., *et al*. 1996, Benitez T., *et al.* 1996).

Generally, sugar catabolism and fermentation proceed at a rate greater than desired, and are usually controlled by lowering the fermentation temperature (Fleet GH. And Heard GM. 1993). Occasionally, wine fermentation ceases prematurely or proceeds too slowly. The commercial implications of sluggish or incomplete wine fermentations are usually attributed to inefficient utilization of fermenter space and wine spoilage resulting from the low rate of protective carbon dioxide evolution and high residual sugar content. Conversely, financial losses through `runaway' wine fermentations arise from the fact that fermentor space is reduced because of foaming and volatile aroma compounds are lost by entrainment with the evolving carbon dioxide. Thus, yeast behaviours towards temperature are also very important in wine making control: a wide range of growth temperatures is suitable for wine strains, and fermentation efficiency should not swiftly decrease as small temperature changes happen. Optimal performance of wine yeasts in white wine fermentations, conducted at cooler temperatures (10±15°C) so as to minimize the loss of aromatic volatiles, and red wine fermentations, performed at higher temperatures (18±30°C) to enhance extraction of anthocyanin pigments, is therefore of critical importance to wine quality and costeffectiveness (Henschke PA. 1997).

2.1.1.2 Main technological properties

Several antimicrobial compounds, as well as ethanol, can interfere with yeast fermentation activity. Some of these compounds are usually added to fermentation tanks, as sulphite dioxide; other ones are found in grape must coming from agrochemical treatments as copper and pesticides; finally antimicrobial killer toxins are produced by some yeasts and are lethal to other sensitive ones.

Sulphur dioxide is widely used in enology for its antioxidant activity and as antimicrobial agent towards yeast, acetic and lactic acid bacteria in general. Moreover, *Saccharomyces* is the most resistant yeast among wine-related species, so SO_2 addiction selects for this microorganism inhibiting apiculated ethanol-sensitive species; thus tolerance to sulphite forms the basis of selective implantation of active dried wine yeast starter cultures into grape must. SO_2 addiction, anyway, can affect differently fermentation kinetics and although *S. cerevisiae* tolerates higher levels of sulphite than most unwanted yeasts and bacteria, excessive SO_2 dosages may cause sluggish or stuck fermentations (Boulton B., *et al*. 1996). Wine yeasts strains vary widely in their resistance to sulphite, and the underlying mechanism of tolerance as well as the genetic basis for resistance are still unclear. Within the *Saccharomyces* species, resistant strains are quite frequent (around 30%) and they can develop in presence of 150 ppm of SO_2, while more sensitive strains are inhibited at concentrations such as 100 ppm that mainly causes a prolongation of lag phase (Romano P. 2005).

Wide application of copper-containing fungal pesticides (copper oxychloride) to control downy mildew (*Plasmopara viticola*) and, to a lesser extent, dead arm (*Phomopsis viticola*) and anthracnose (*Gloeosporium ampelophagum*) could lead to copper residues in musts that may cause lagging fermentation and affect wine quality detrimentally (Tromp A. and De Klerk CA. 1988). This phenomenon recently increased due to the diffusion of the organic and integrated cultivations, where copper is widely used to reduce or eliminate the need of other chemical treatments. *S. cerevisiae* species exhibits a significant variability in copper resistance and the acquisition of this trait seems to be the result of an environmental adaptation (Romano P. 2005). Several copper uptake, efflux and chelation strategies have been developed by yeasts to control copper ion homeostasis (Avery SV. *et al*. 1996). In particular, copper sensitive strains do not change the metal concentration in wine, whereas

resistant strains sensibly reduce it accumulating copper inside the cell (Brandolini V. *et al*. 2002).

Killer toxins are proteins produced by some yeasts that are lethal to sensitive wine yeast strains. The killers themselves, however, are immune to these mycovirus associated toxins. It remains controversial whether the growth and zymocidal activity of some wild killer yeasts have the potential to delay the onset of fermentation, cause sluggish or stuck fermentations and produce wines with increased levels of acetaldehyde, lactic acid, acetic acid and other undesirable sensory qualities (Shimizu K. 1993). An unfortunate consequence of ignorance regarding the role of killer yeasts in wine fermentations is that some winemakers use co-cultures to inoculate fermentations, one strain being a killer and the other a sensitive strain. The advantage of using killer or neutral wine yeasts should therefore not be underestimated (Pretorius IS., 2000).

2.1.2 Quality traits

The quality of wine is the outcome of complex chemosensory interactions that are difficult to predict because of the influences of many variables. The chemical composition of wine is the foundation of both sensory response and wholesomeness, and it is determined by many factors. These include the grape variety, the geographical and viticultural conditions of grape cultivation, the microbial ecology of the grape and fermentation processes, and winemaking practices (Owens P. and Noble A. 1997). Microorganisms have a prominent role in determining the chemical composition of wine.

They affect the quality of the grape prior to harvest and, during fermentation, they metabolise grape sugars and other components into ethanol, carbon dioxide and hundreds of secondary end-products that, collectively, contribute to the subtlety and individuality of wine character (Lambrechts MG. and Pretorius IS. 2000, Nykanen L. 1986).

2.1.2.1 Flavour characteristics

Alcoholic beverages contain mainly saturated, straight chain fatty acids. The volatile acid content of wine usually lies between 400 and 1000 mg/l, normally more than 90% of volatile acid consists of acetic acid (Henschke PA. and Jiranek V. 1993). Altough

acetic and latic acid bacteria can be associated with high levels of short chain fatty acid, acetic, propanoic and butanoic acids are by-products of alcoholic fermentation (Ribereau-Gayon P. *et al*. 2000).

Fermentation purity is expressed as of the ratio between volatile acidity (as g acetic acid/l) and ethanol (% volume) produced at the end of the fermentation process. High values of this ratio denote the ability to form few undesirable by-products in the course of fermentation. Wines cannot be commercialized if volatile acidity exceeds one tenth of the ethanol content (Martini A. 2003).

Another fermentation by-product affecting wine quality is glycerol. In a model fermentation, about 95% of the sugar is converted into ethanol and carbon dioxide, 1% into cellular material and 4% into other products such as glycerol. Due to its nonvolatile nature, glycerol has no direct impact on the aromatic characteristics of wine. However, this triol imparts certain other sensory qualities; it has a slightly sweet taste, and owing to its viscous nature, also contributes to the smoothness, consistency and overall body of wine (Scanes KT. *et al*. 1998). Wine yeast strains producing a consistent amount of glycerol would therefore be of considerable value in improving the organoleptic quality of wine [151, 209](Michnick J. *et al*. 1997, Remize F. *et al*. 1999).

Among other yeast metabolites, the formation of sulphite and sulphide by wine strains greatly affects the quality of wine. Sulphur is essential for yeast growth and *S. cerevisiae* can use sulphate, sulphite and elemental sulphur as sole sources. Unlike sulphur dioxide (SO_2), which when properly used, has some beneficial effects, hydrogen sulphide (H_2S) is one of the most undesirable yeast metabolite, since it causes, above threshold levels of 50-80 g/l, an off-flavour reminiscent of rotten eggs (Snow R. 1983).

Even though the compositional variability of musts (i.e., the precursors of bouquet molecules variably distributed within grape varieties) is considered the main source of organoleptic specificity, today the wine technologists re-evaluate the role of yeast metabolism (strain-related by-products of fermentation) in the formation of bouquet and aroma (Martini A. 2003). In fact, the growth, by means of alcoholic fermentation as energy source, is the best way for yeasts to make a contribution to wine flavour**,** as well (Henschke PA. 1997). This phenomenon is carried out by several mechanisms that involves the degrading of grape juice constituents and the production of a great amount of different

compounds: mainly ethanol and other solvents that help to extract flavor components from grape solids, hundreds of secondary metabolites (e.g. acids, alcohols, esters, polyols, aldehydes, ketones, volatile sulphur compounds) that contribute considerably to wine aroma and the products of autolytic activity that characterizes the stationary phase of yeast growth. Moreover a great variety of exoenzymes are normally produced by these microorganisms that can transform neutral grape compounds into flavour active molecules (Owens P. and Noble A. 1997, Lambrechts MG. and Pretorius IS. 2000). These reactions, especially the production of secondary metabolites, vary with the species and strain of yeast. Tables comparing the diversity of metabolite production by different yeasts may be found in Fleet GH. (1998), Lema C. *et al.* (1996), Romano P. (1997), Heard G. (1999), and Lambrechts and Pretorius (2000). Thus, the uniqueness and individuality of the flavor contribution by yeasts depends on the species and strains operating the fermentation (Fleet GH. And Heard GM., 1993, Fleet GH. 2003).

2.1.2.2 Metabolic properties that influence wine safety

Today, it is generally accepted that moderate wine drinking can be socially beneficial, and that it can be effective in the management of stress and reducing the risk of coronary heart disease. In the selection and improvement projects concerning wine yeast strains, it is therefore of the utmost importance to focus on these health aspects and to obtain yeasts that may reduce the risks and enhance the benefits. Likewise, research in several laboratories around the world is directed towards the elimination of suspected carcinogenic compounds in wine, such as ethyl carbamate, and asthmatic chemical preservatives, such as sulphites. It might even be possible to develop wine yeasts that could increase the levels of phenolic and antioxidative substances (e.g. resveratrol) associated with the so-called `French paradox', in which, despite the high dietary fat intake of the cheeseloving population of southern France, the death rate from coronary heart disease is significantly lower than the one found in industrialized countries (Pretorius IS. 2000).

2.1.3 Aims of the work

In this chapter the physiological investigation of eight ecotypical strains has been reported.

2.2 MATERIALS AND METHODS

2.2.1 Yeast strains

For this study ecotypical strains were used. These yeasts were isolated from the vineyards in the winemaking area of Prosecco Superiore di Conegliano Valdobbiadene DOCG shown in Table 2.2.1

Strain	**Species**	**Origin**
P283.4	*Saccharomyces cerevisiae*	Wine region of Conegliano Valdobbiadene
P234.15	*Saccharomyces cerevisiae*	Wine region of Conegliano Valdobbiadene
P254.12	*Saccharomyces cerevisiae*	Wine region of Conegliano Valdobbiadene
P301.9	*Saccharomyces cerevisiae*	Wine region of Conegliano Valdobbiadene
P304.4	*Saccharomyces cerevisiae*	Wine region of Conegliano Valdobbiadene
P301.4	*Saccharomyces cerevisiae*	Wine region of Conegliano Valdobbiadene
P138.4	*Saccharomyces cerevisiae*	Wine region of Conegliano Valdobbiadene
B173.4	*Saccharomyces cerevisiae*	Wine region of Conegliano Valdobbiadene

Table 2.2.1 Yeast strains used in this work

2.2.2 Culture media and growth condition

Media

YM solid agar medium

- 3 g L-1 yeast extract (Oxoid);
- 3 g L-1 malt extract (Oxoid);
- 5 g L-1 vegetatone peptone (DIFCO);
- 10 g L-1 glucose (PROLABO)
- 16 g L-1 Bacto Agar (DIFCO).

Adjust to volume with distilled water. Sterilize by autoclaving at 121 ° C for 15 minutes.

YPD (Yeast Extract/Peptone/Dextrose)

10 g L-1 yeast extract (OXOID)

20 g L-1 vegetatone peptone (DIFCO)

20 g L-1 glucose (PROLABO)

Adjust to volume with distilled water. Sterilize by autoclaving at 121 ° C for 15 minutes.

YEPG:

20 g Glucose

20 g peptone

10 g Yeast extract

Make up to volume with distilled water. Sterilize by autoclaving at 121 ° C for 15 minutes.

PYG (with and without methylene blue):

10 g Glucose

3.5 g Peptone

3.0 g Yeast extract

2.0 g Potassium dihydrogen phosphate: KH_2PO_4

1.0 g Magnesium sulfate: $MgSO_4$. $7H_2O$

1.0 g Ammonium sulfate: (NH_4) $2SO_4$

(0.03 g Methylene Blue)

12 g Agar

Make up to volume with distilled water. Sterilize by autoclaving at 121 ° C for 15 minutes.

Adjust the pH to 4.5 with HCl (hydrochloric acid) after having tared the pH meter.

GPY (Glucose Peptone Yeast) agar

Servings per liter:

10 g yeast extract (OXOID)

20 g Peptone (DIFCO)

20 g Glucose (Prolabo)

20 g Bacto Agar (DIFCO)

Make up to volume with water and add 16 g / l Bacto Agar (DIFCO). Sterilize by

autoclaving at 121 ° C for 15 minutes.

YNB (YeastNitrogen Base) w / o AA and Ammonium Sulphate

Servings per liter:

1.7 g YNB w / o AA and ammonium sulphate (Difco)

5 g Ammonium Sulphate

20 g Glucose (Prolabo)

Make up to volume with distilled water. Sterilize by filtration (0.22 µm).

Wallerstein Laboratory (WL medium) nutrient agar (Green & Gray, 1950).

Suspend 75 g WL nutrient agar (Oxoid) in a liter of distilled water.

Sterilize by autoclaving at 121 ° C for 15 minutes.

Fucsine agar medium

3 g/l yeast extract (Oxoid);

3 g/l malt extract (Oxoid);

5 g/l vegetatone peptone (DIFCO);

10 g/l glucose (PROLABO)

0,002 g/l Fucsine (SIGMA)

16 g/l Bacto Agar (DIFCO).

Adjust to volume with distilled water. Sterilize by autoclaving at 121 ° C for 15 minutes.

Biggy agar medium

1 g/l yeast extract

10 g/l glycin

10 g/l glucose

3 g/l sulphite ammonium

5 g/l bismuth ammonium citrate

16 g/l Bacto Agar

pH 6.8

Suspend 42g in 1 liter of distilled water and bring gently to the boil to dissolve the agar. Allow to cool to 50-55°C. Mix gently to disperse the flocculent precipitate and pour into sterile Petri dishes. Do not autoclave the medium.

Synthetic nutrient medium (MSN) MS300

Macroelements
200 g glucose
0,155 g $CaCl_2 \cdot 2H_2O$
0,2 g NaCl
0,75 g KH_2PO_4
0,25 g $MgSO_4 \cdot 7H_2O$
0,5 g K_2SO_4
0,46 g $(NH_4)Cl$
6 g malic acid
6 g citric acid

Microelements
4 mg $MnSO_4 \cdot H_2O$
4 mg $ZnSO_4 \cdot 7\ H_2O$
1 mg $CuSO_4 \cdot 5H_2O$
1 mg KI
0,4 mg $CoCl_2$
1 mg H_3BO_3
1 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$

Vitamins
20 mg Myo-inositol
2 mg Nicotinic acid
1,5 mg Calcium Panthotenate
0,25 mg Thiamine hydrochloride

0,25 mg Pyridoxine hydrochloride
0,003 mg Biotin

Aminoacids
3,70 g leucine
5,80 g threonine
1,40 g glycine
38,60 g glutamine
11,10 g alanine
3,40 g valine
2,40 g methionine
2,90 g phenylalanine
6,00 g serine
2,50 g histidine
1,30 g lysine
1,00 g cysteine
46,80 g proline
1,40 g tyrosine
13,70 g tryptophan
2,50 g isoleucine
3,40 g aspartic acid
9,20 g glutamic acid
28,60 g arginine

Final pH 3.2
Prepare the amino acids in a 1 litre aqueous solution and use 13,09 ml per litre of must. Dissolve all components in distilled water, adjust the pH with KOH of the resulting solution to pH 3.2.

Synthetic nutrient medium (NSM) (Delfini, 1995)

Macronutrients

0,1 g L-1 $CaCl_2$

0,1 g L-1 NaCl

1 g L-1 KH_2PO_4

0,5 g L-1 $MgSO_4 \cdot 7H_2O$

3 g L-1 tartaric acid 123

Micronutrients

0,2 mg L-1 $NaMoO_4 \cdot 2H_2O$

0,4 mg L-1 $ZnSO_4 \cdot 7H_2O$

0,5 g L-1 H_3BO_3

0,04 mg L-1 $CuSO_4 \cdot 5H_2O$

0,1 mg L-1 KJ

0,4 mg L-1 $FeCl_3 \cdot 6H_2O$

0,4 mg L-1 $MnSO_4 \cdot H_2O$

Vitamins

400 µg L-1 pyridoxine hydrochloride

400 µg L-1 thiamine hydrochloride

2000 µg L-1 Inosite

20 µg L-1 Biotin

400 µg L-1 Calcium pantothenate

400 µg L-1 Nicotinic acid amide

200 µg L-1 P-amino-benzoic acid

Variable components

0,3 g L-1 $(NH_4)_2SO_4$

0,3 g L-1 $(NH_4)_2HPO_4$

200 g L-1 Glucose

0,2 g L-1 Hydrolyzed Casein

Prepare the micronutrients and vitamins in a 100 times concentrated aqueous solution and use the 1%. Dissolve all components in distilled water, adjust the pH with KOH of the resulting solution to pH 3.2. Sterilize by autoclaving at 100 ° C for 5 min.

Solution

Ringer Solution for dilutions (1/4 *strenght*; Dept. of Health & Social Security, 1937). Dissolve one tablet preparation (LAB M, International Diagnostics Group) in 500 ml of deionised water. Sterilize by autoclaving at 121 ° C for 15 minutes.

Growth conditions

The yeast strains were grown at 25°C. The liquid cultures to be used for fermentation trials were subjected to orbital shaking (130 rpm).

2.2.3 Fermentation trials on synthetic must

2.2.3.1 Inoculum preparation and trial setting

The inoculum was prepared resuspending several loopfuls of yeast cells, collected from a fresh GPY plate, in 10 ml of MS300 until OD620 value reached 1.5, which corresponds to approximately 1.5 x 10^7 cells/ml. This volume was used to inoculate an Erlenmeyer flask containing 90 ml of MSN and afterwards sealed with silicon cap and supplied with a bowed glass pipette. The advantage to use the synthetic must than the natural, for a first physiological assessment, is to enable a fully control of the development setting, and to facilitate significantly the daily growth monitoring operations.

The medium was sterilized by autoclaving at 100 ° C for 5 minutes.

2.2.3.2 Monitoration of the fermentation process

The fermentation process was monitored by measuring the weight loss daily from the beginning to the end of fermentation process. The fermentations were considered completed when weight loss was lower than 0,1 g within 24 hours.

2.2.3.3 Foaming ability

For each strain at the end of the fermentation test in MNS the ability to produce foam was evaluated by measuring the foam height in mm.

2.2.4 Killer toxin production

For the assay two commercial yeasts were used, as reference strains: the killer strain EC1118 and the sensitive BDX (Table 2.2.2).

Strain	**Specie**	**Origin**
EC1118	*Saccharomyces cerevisiae*	Isolated from fermenting grapes, Champagne, France (*Lalvin*)
Uvaferm BDX	*Saccharomyces cerevisiae*	Isolated from fermenting grapes, Bordeaux, France (*Lallemand*)

Table 2.2.2 Yeast strains used as reference for the evaluation of killer activity.

2.2.4.1 Inoculum preparation

Yeasts were grown on solid YPD medium at 25 °C for 3 days. A loopful of yeast cells was transfer into 5 ml of liquid YPD medium. The tubes were incubated for 24 hours at 25 °C with shaking at 150 rpm until stationary phase (about 10^7-10^8 cells/ml) was reached. Subsequently, 10 µl of culture were transferred into 10 ml of YPD liquid medium and incubated for 17 hours under stirring at 25 °C. The concentration of yeast cells present in the culture medium was measured by spectrophotometric reading at the optical density of 600 nm (spectrophotometer Shimadzu UV-1601).

The suspension was diluted with deionized water to obtain a concentration of 10^5 cells/ml (OD600 of approximately 0.05).

2.2.4.2 Killer activity

The test proposed by Cavazza et al. (1992) was used. Ten ml of melted PYG medium without methylene blue (0,0003%) were poured into Petri dishes, while 10 ml of PYG

medium with methylene blue was transfered into test tubes and kept at 50° C by immersion in a thermostatic bath (Haake DC10).

Each tube was inoculated with 0.1 ml of cell suspension (containing 10^5 cells/ml) of the "sensitive" strain and the medium quickly poured into the Petri dishes with PYG medium without methylene blue.

After the medium was solidified, a loopful of cells from 24-hour culture of the "killer" strain was transferred on the plate. Finally the plate was incubated at 25°C for 4 days.

For each of the eight strains 9 test assessing killer activity and 9 sensitivity to killer toxin were performed.

2.2.5 Hydrogen sulphide production

Each strain was streacked on Biggy agar medium in order to obtain a single colony growth. The plates were incubated at 25 °C for 4 days, the intensity of the dark colour was evaluated. The chromatic scale considered for the evaluation was as follow: (1) white colour no H_2S production, (2) beige colour low production, (3) brown colour medium production, (4) dark colour high production.

2.2.6 Sulphur dioxide production

Each yeast was streacked on Fucsine Agar medium in order to obtain a single colony growth. The sulfur dioxide produced by the yeast, combined with the magenta dye (pink color) present in the medium leads to the formation of a colorless compound. The intensity of the pink colour of individual colonies was assessed. The chromatic scale considered for the evaluation was as follow: (1) purple colour low SO_2 production, (2) pink colour medium production, (3) light pink colour high production, (4) white colour very high production.

2.2.7. Ethanol resistance

2.2.7.1 Inoculum preparation

The yeasts were grown in YPD medium at 25 °C for 3 days. The cultures obtained were used to inoculate 10 ml of YPD liquid medium. The tubes were incubated for 30 hours at 25 °C under stirring until stationary phase (about 10^7 cells/ml) was reached.

2.2.7.2 Test preparation

Different volumes of ethanol were added to melted YPD agar medium (temperature about 45°C) in order to obtain final alcohol concentrations of 14%, 15%, 16%, 17% on plates. The inoculum was done by adding a drop of liquid culture (20 µl) on the surface of the plate. The growth was observed after 24 hours and 48 hours at 25 °C, recording the presence of a growth-inhibition halo around the inoculum.

2.2.8 Sulphur dioxide resistance

2.2.8.1 Inoculum preparation

The yeasts were grown on YPD solid medium at 25 °C for 3 days. A loopful of yeast cells was transfer into 10 ml of liquid medium YPD. The tubes were incubated for 30 hours at 25 °C under stirring until stationary phase (about 10^7 cells/ml) was reached.

2.2.8.1 Test preparation

The stationary phase cultures were diluted in 2ml-Eppendorf tubes and 300 µl of diluted culture were mixed with 1.7 ml of synthetic nutrient medium. A solution of sulphur dioxide (10 g/l) was diluted 1:10 with synthetic must in a Falcon tube. Liquid medium aliquots containing different SO_2 concentrations were prepared directly in cuvettes for UV spectroscopy previously sterilized with UV radiation adding the volumes of solution and diluted culture reported in Table 2.2.3, in order to obtain an inoculum of approximately 10^5cell/ml for each concentration of sulphur dioxide tested.

Final SO_2 (mg/l)	Synthetic must (ml)	Diluted colture (ml)	SO_2 solution (1 g/l)
0	2,8	0,2	-
50	2,65	0,2	0,15
100	2,5	0,2	0,3
200	2,2	0,2	0,6

Table 2.2.3 Volumes of the SO_2 solution used in the test for the preparation of a growth medium containing scalar concentrations of SO_2. The volumes of the diluted culture used as inoculum are also reported.

2.2.9 Copper resistance

YNB w / o AA and ammonium sulphate with different $CuSO_4$ concentrations

The growth medium YNB with different concentrations of $CuSO_4$ (0; 0.05; 0.1; 0.2; 0.4; 0.8 mM) was prepared by adding an appropriate volume of a $CuSO_4 \bullet 5H_2O$ solution (40 mM) previously filtered.

$CuSO_4$ solution 40 mM

3.19 g of $CuSO_4$ were weighed and dissolved in 500 ml of distilled H_2O.

2.2.9.1 Pre-inoculum preparation

The yeasts were grown on plates containing WL medium at 25 °C for 3 days. A loopful of yeast cells collected from the plate transferred into 10 ml of YPD liquid medium. The tubes were incubated for 24 hours at 28°C (120 rpm) until stationary phase (about 10^7 -10^8 cell/ml) was reached. Test tubes containing 10 ml of YPD liquid medium were inoculated with 100 µl of the overnight culture and incubated 15 hours at 28 °C under orbital shaking (120 rpm).

2.2.9.2 Test preparation

Pre-inoculum cultures were grown until OD600 reached 0.25. For each strain 3% of the pre-inoculum culture was transferred in 5 ml of YNB containing different concentrations of $CuSO_4$ (0, 50, 100, 200, 400, 800 µM $CuSO_4$) in order to obtain the concentration of 1×10^4 cells/ml. The tubes were incubated for 48 hours at 28 °C under shaking (120 rpm). The growth of the strains was evaluated by measuring OD600 after 24 and 48 hours.

2.3 RESULTS AND DISCUSSION

In this work eight indigenous strains were chosen. These yeasts were isolated in the vineyard, in previous selection projects of wine yeasts conducted in the area of the DOCG Prosecco Superiore di Conegliano - Valdobbiadene. The eight strains, P283.4, P234.15, P254.12, P301.9, P304.4, P301.4, P138.4, B173.4, were chosen among those with good fermentative performance, based on their genetic profiles, previously obtained analyzing 18 DNA regions highly variable (microsatellites) (Viel, 2012). The technological traits of the eight strains were further investigated by fermentation tests and plate essays.

2.3.1 Fermentation performance

For each strain the fermentation kinetics was obtained by setting up fermentation test in Erlenmeyer flask, each one containing 100 ml of synthetic must MS300, which mimicks the conditions of a standard must containing 200 g/l of glucose and with pH 3.2. The advantage to use a synthetic must rather than a natural one, for a first physiological assessment, is to enable a fully control of the development setting, and to facilitate significantly the daily monitoring operations to test fermentation process.

For each strain the fermentation test was set up in triplicate. The flasks were inoculated with approximately 10^6 cells/ml and kept at a temperature of 25 °C until the end of fermentation. The fermentation activity was measured by daily monitoring the weight decrease of the flasks, due to the loss of CO_2 produced (Figure 2.3.1) In these conditions, all strains have completed the fermentation, consuming all the fermentable sugar in a comparable time (between 13 days and 17 days), although there are clear differences among the strains.

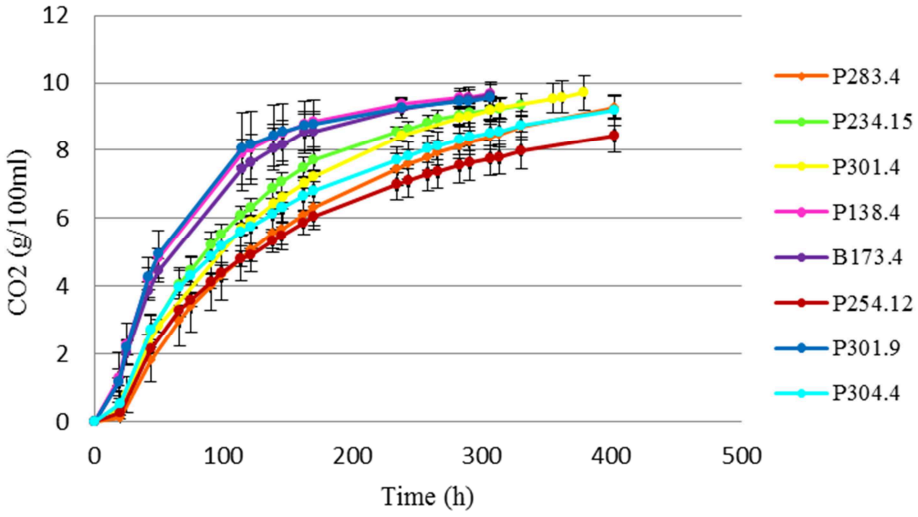


Figure 2.3.1 Fermentation kinetics of the 8 strains tested in synthetic must MS300.

The best fermentation kinetics was obtained by strains P301.9, P138.4 and B173.4 which concluded the fermentation in 13 days. Strains P234.15, P304.4 and P301.4 are characterized by intermediate trends, while the slower strains were P283.4 and P254.12 that conclude the fermentation in 17 days (Table 2.3.1).

Strain	Fermentation time (days)
P283.4	17
P234.15	13
P254.12	17
P301.9	13
P304.4	17
P301.4	16
P138.4	13
B173.4	13

Table 2.3.1. Days of fermentation needed to consume all the reducing sugars.

2.3.1.1 Fermentative vigour

This is an important parameter to evaluate strain fermentation capacity. In particular, it expresses the promptness of yeast fermentation starting at a temperatures between 20 °C and 30 °C (Vincenzini et al., 2005). It is measured as the CO_2 concentration (g/100ml of must) produced after 48 hours form the inoculum of the starter (Zambonelli et al., 1971). The prompt start of the fermentation process is one of the characters that the starter must have regardless of its type of use. Commonly, between oenological yeast, those of the genus *Saccharomyces*, and in particular the species *S. cerevisiae* are the most vigorous (Vincenzini et al., 2005).

Figure 2.3.2 shows the values of CO_2 produced at 48 hours from inoculation. These are in the range between 1,85 and 4,69 g/100 ml. The fermentative vigour is influenced by the adaptation ability of the strain to the oenological environment and therefore to the length of the lag phase. In particular, the adaptation capacity will result in a reduction of the lag phase and therefore a higher fermentative vigour.

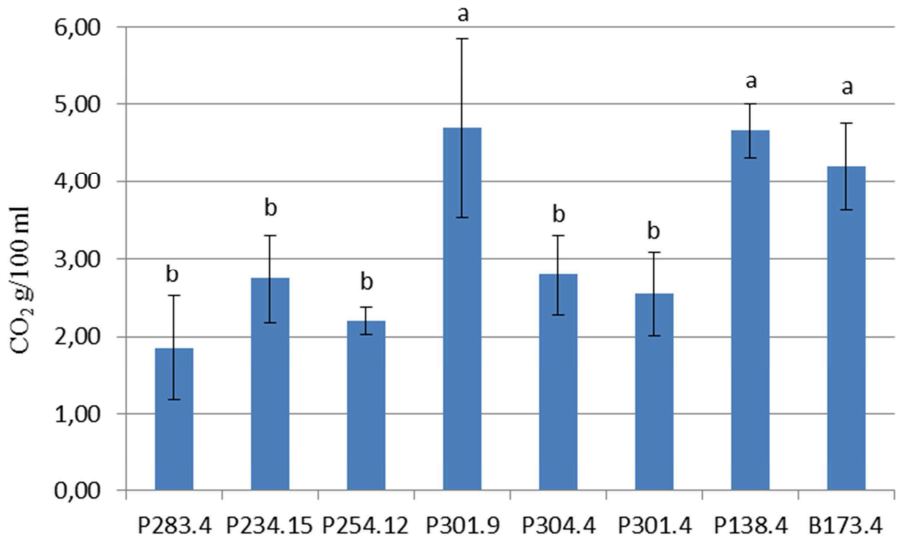


Figure 2.3.2 Fermentative vigour evaluated during tests in synthetic must MS300. Statistical analysis: one-factor ANOVA (p value < 0.05). Different letters indicate the existence of statistically significant differences.

In this experiment the values of the fermentative vigour are perfectly in agreement with the fermentation trend and rate. In fact, the lower fermentative vigour, corresponding to the value of 1.85 g/100 ml of CO_2 produced, is associated with the strain P283.4, which as noted previously, is one of the slower strains to close the fermentation, together with

P254.12 (2.20 g/100ml). Strains P234.15, P304.4 and P301.4 have intermediate values of CO_2 produced (2.74, 2,79 and 2.55 g/100ml) but the difference with the first two values is not statistically significant. The highest values of fermentative vigour (4.69, 4.66 and 4.20 g/100ml) are associated with strains P301.9, P138.4 and B173.4 that conclude more quickly the fermentation.

With regard to the CO_2 production estimated 7 days after inoculation, it may be noted that the results obtained confirm both those observed at 48 hours than those relating to fermentation kinetics. The values, shown in Figure 2.3.3, settle between 6.08 and 8.97 g/100 ml of CO_2 produced and statistical test (ANOVA) distinguishes the three groups of strains, with low (P283.4 and P254.12), medium (P234.15, P304.4 and P301.4) and high (P301.9, P138.4 and B173.4) fermentation rate, except in the case of P304.4.

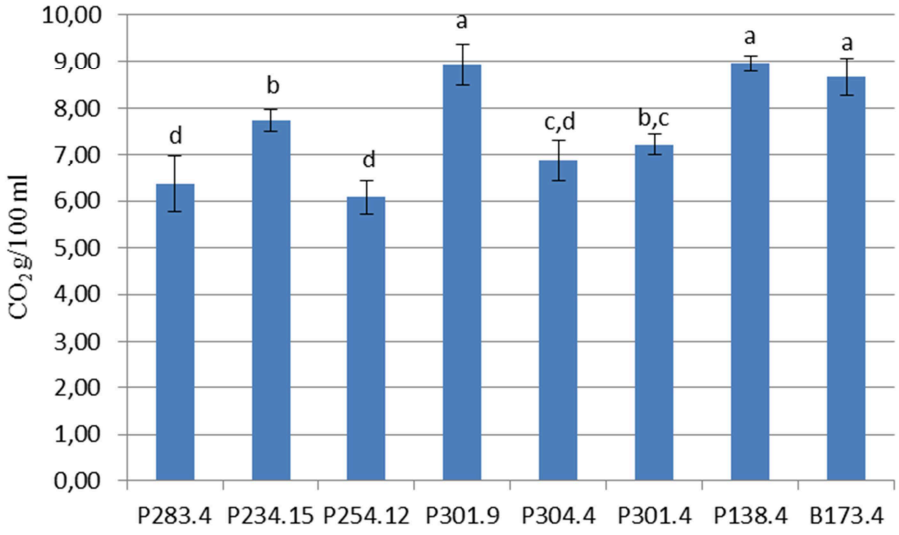


Figure 2.3.3 CO_2 produced by strains after 7 days of fermentation during the tests in synthetic must MS300. Statistical analysis: one-factor ANOVA (p value < 0.05). Different letters indicate the existence of statistically significant differences.

2.3.1.2 Foaming

Another important character is the foaming capacity. The high production of foam generally is a very negative characteristic, technologically impossible to manage in cellar, as it causes the spill of the must from the fermentation vats once started the yeasts growth. It is due to cell hydrophobicity and cell tendency to floating of pulverulent and flocculent strains.

Foaming capacity is considered a positive character in the case of second fermentations during sparkling wine production in autoclave, as in over-pressure conditions it slows the precipitation of the cells that show therefore a greater fermentative vigour (Zambonelli, 2003). In Table 2.3.2 is reported the amount of foam produced by the 8 strains measured at the end of fermentation in synthetic must.

Strain	Foam (mm)
P283.4	10
P234.15	>18
P254.12	7
P301.9	16
P304.4	16
P301.4	14
P138.4	7
B173.4	7

Table 2.3.2 Foam production at the end of fermentation.

Despite the amount of foam produced by most of the strains is technologically acceptable large differences were evidenced among the yeasts. P254.12, P138.4 and B173.4, are those with poor foaming capacity; P283.4, P301.9, P304.4 and P301.4 show an intermediate foam production, while the strain P234.15 is the only one that evidenced high production level.

2.3.2 Analysis of killer activity

The killer phenotype is defined as the ability of a yeast strain to produce a protein, called "killer toxin", which inhibits the development of other yeasts.

The *S. cerevisiae* killer strains (K) produce a specific toxin called K2 active in winemaking conditions against sensitive yeast strains (S) of the same genus. The K2 toxin acts on the membrane permeability influencing the release of protons, potassium cations, ATP and amino acids. The killer character undoubtedly increases the competitiveness, favoring strains K with respect to S, but not to those defined "neutral" (N) which are non–producing insensitive strains. Generally the commercial strains are killer yeasts. The activity is limited

to sensitive wild *Saccharomyces*, without, however, affects the competitiveness of the starter against the indigenous non-*Saccharomyces*.

So the killer factor can be considered an important character, but additional, in the selection of yeast starter for winemaking (Vincenzini et al., 2005).

A previous study carried out on wine yeasts isolated in Conegliano area showed that the killer character is present in 44% of the strains collected and it is positive correlated with the evolutionary process and colonization of oenological environments (Ciani et al., 1997).

For the assessment of the killer activity the test proposed by Cavazza et al. (1992), was used. In this way it was possible to verify together with the killer activity, the presence of strains sensitive to the toxin.

As control strains two commercial yeasts have been used: EC1118 and BDX killer and sensitive reference strains, respectively. The killer activity of the eight strains and of the two reference strains was determined using a cross test in which each strain was considered as a possible producer and subsequently as possible sensitive (Figure 2.3.4).

Each strain was uniformly distributed, with a spatula, on the surface of PYG plates. Subsequently a loopful of cells, from a previous growth, of each strain was placed on the surface of the plate.

After incubation, toxin production was attested, evaluating the presence of a growth-inhibition halo on the plate.

The “killer” strains were defined as those able to inhibit the growth of at least one strain (called “sensitive”) whose cells were spread simultaneously on the plate, thus evidencing an inhibition halo. The "neutral" strains were defined as those, although not showing killer activity, able to growth on the plate in contact with a killer strain (no inhibition halo is present).

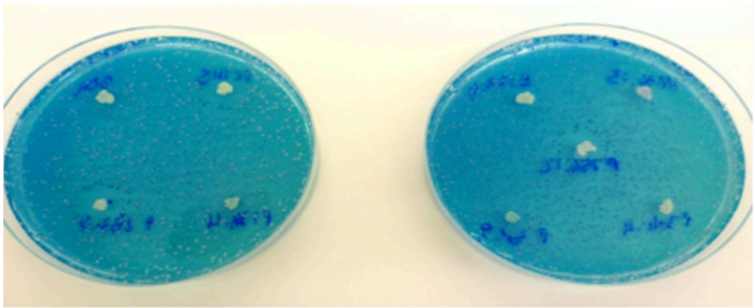


Figure 2.3.4 Killer activity: plate essay.

Strain	Killer character
P283.4	K
P234.15	N
P254.12	K
P301.9	S
P304.4	N
P301.4	S
P138.4	N
B173.4	K

Table 2.3.3 Evaluation of the killer activity, K killer strain, S sensitive, N neutral.

The results of the killer tests are reported in Table 2.3.3.

Strains P283.4, P254.12 and B173.4 produce killer toxins, while P301.4 and P301.9 strains were sensitive to the toxins produced by killer strains. The strains P138.4, P234.15 and P304.4 are neutral, as they are not producers and insensitive to killer toxins produced by the other strains.

2.3.3 Hydrogen sulphide and sulphur dioxide production

The yeast ability to produce hydrogen sulphide (H_2S) and sulphur dioxide (SO_2) are qualitative and technological features very important from the enological point of view, as they affect the quality (H_2S) and healthy (SO_2) aspects related to wine. For this reason, the evaluation of the potential production of the two sulfur compounds was carried out by plate test. Although the test conditions are far from those enological, the test provides information regarding the differences in the two compounds production related to the genetic features of the strains. A series of rapid tests were carried out for the evaluation of the production of H_2S and SO_2, by using growth media containing specific indicators.

For the evaluation of hydrogen sulphide production the Biggy agar medium (Oxoid) was used. Biggy contains bismuth sulphite, which, in the presence of hydrogen sulphide, is converted to bismuth sulphide, giving to the colonies a brown colour whose intensity is proportional to H_2S production.

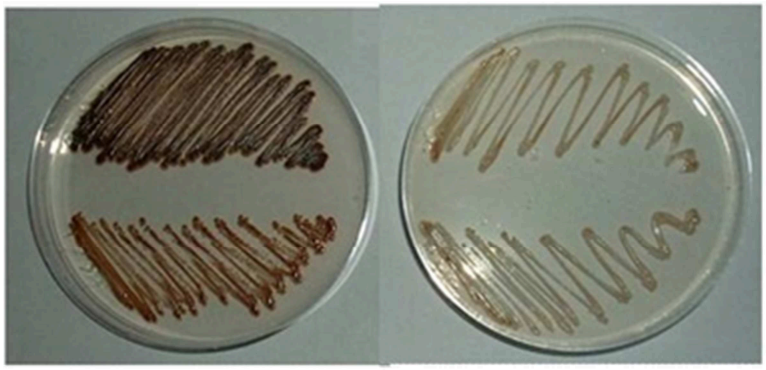


Figure 2.3.5 Yeast growth on Biggy agar medium

Hydrogen sulphide is an undesirable compound in wine as it confer a negative aroma of "rotten eggs". Since it is a volatile compound, mild stirring of the wine strongly reduced its concentration.

For the estimation of sulphur dioxide production a medium containing the basic indicator Fuchsine has been used, which tends to concentrate more within the cells compared to the surrounding growth medium; once inside, SO_2 produced by the yeast, combined with the Fuchsine (magenta color) leads to the formation of a colorless compound, in a quantity proportional to SO_2 production

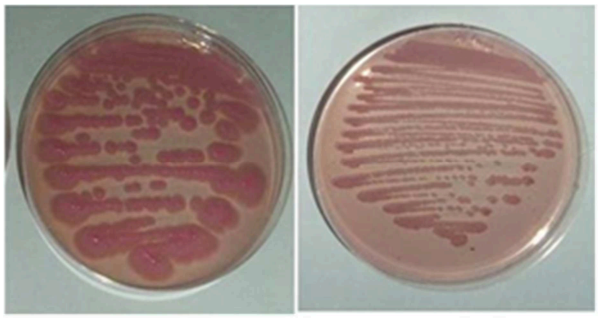


Figure 2.3.6 Yeast growth on Fuchsine agar medium

As sulphur dioxide is toxic for humans, modern oenology constantly tries to reduce its use, also limiting the contribution due to the yeast. For this reason in yeast selection projects strains with low sulphur dioxide production are chosen. Moreover, this character is, with an inverse relationship, linked to the production of hydrogen sulphide.

The results of SO_2 and H_2S production tested on plate assay are reported in table 3.6.

Strain	Biggy agar	Fuchsine agar
P283.4	3	2
P234.15	2	2
P254.12	3	2
P301.9	3	2
P304.4	3	2
P301.4	3	2
P138.4	3	2
B173.4	3	2

Table 2.3.4 Production of hydrogen sulphide and sulphur dioxide on plate assay.
The chromatic scale considered for the evaluation of H_2S production was as follow: (1) white colour no H_2S production, (2) beige colour low production, (3) brown colour medium production, (4) dark colour high production.
The chromatic scale considered for the evaluation of SO_2 was as follow: (1) purple colour low SO_2 production, (2) pink colour medium production, (3) light pink colour high production, (4) white colour very high production.

Regarding hydrogen sulphide production, 7 out of 8 strains are medium producers, while P234.15 produces low levels of H_2S. In relation to the production of sulfites all strains are medium producers. The results obtained were very similar to those usually found in most of the strains isolated in enological environment. The plate assay is a semi-quantitative test giving information about the potential production of the two compounds as the test do not reproduce the winemaking environment, where the variability of redox conditions plays a key role in yeast metabolism. The results confirm that the production of sulphur compounds is a strain-specific character.

2.3.4 Ethanol resistance

This parameter, together with the fermentative vigour, measures the yeast technological attitude to fermentation. In strain, the higher is the ethanol resistance level, the higher the ability of yeast to transform reducing sugars. The test was performed by

transferring an aliquot of a liquid culture of each strain on YPD agar medium supplemented with increasing concentrations of ethanol.

The lowest ethanol concentration tested was 13%, as previous tests showed that generally *S. cerevisiae* strains are resistant to such concentration. The results of ethanol resistant test are shown in Table 2.3.5.

Strain	Ethanol resistance (% Vol)
P283.4	13
P234.15	14
P254.12	15
P301.9	13
P304.4	15
P301.4	15
P138.4	15
B173.4	14

Table 2.3.5 Evaluation of ethanol resistance.

The ethanol resistance level of the eight strains ranged from 13% up to 15% indicating a medium-to-high ethanol tolerance.

All these strains are certainly good candidate to be used in vinification, although the level of resistance is different. In particular, the strains P254.12, P304.4, P301.4 and P138.4 shows a remarkable resistance level and thus may be very convenient in non conventional vinification such as in the production of sweet wine and during second fermentation.

2.3.5 Sulphur dioxide resistance

Sulphur dioxide resistance is the ability to keep unchanged or sufficiently high the fermentation speed in the presence of selective doses of SO_2. The antiseptic effect of sulfur dioxide added to musts reduces the develop of bacteria and non *Saccharomyces* yeasts present in the must, resulting in a delay of the beginning of the alcoholic fermentation leaded by technological yeasts. High doses can prolong the microbial lag-phase, establishing slow fermentation process, which may lead to fermentation stuck causing

severe damage to winemaking. Yeasts belonging to the species *Saccharomyces cerevisiae* possess several detoxification systems from SO_2 that are more efficient respect to those found in other yeasts species, thus ensuring a limited lag phase; the shorter is the lag-phase at the beginning of the fermentation, the higher is the resistance level of the strains towards sulfur dioxide.

The tests were carried out using as growth medium the synthetic must proposed by Delfini et al. with 20%. Diluted pre-cultures have been used to inoculate tubes containing the synthetic must with different SO2 concentrations: 0, 50 and 100 mg/l. For each strain the test was set up in duplicate and the tubes, were kept at a temperature of 25 °C. The growth was observed after 24 hours recording the presence of turbidity in the liquid medium, by measuring the optical density of the solution at 600nm. The strains are considered resistant if the measure of spectrophotometric absorbance value is higher than 0.1 (OD value due to cell inoculation).

The SO_2 concentration tested correspond to the doses of SO_2 generally used in winery, although in synthetic must the antiseptic effect of the SO_2 is much more marked as several components able to sequester this molecule, such as tannins, the residue of the skin or lipids present in the natural must, are missing.

The Figure 2.3.7 shows the OD600 values of the cultures measured 24 hours after inoculation.

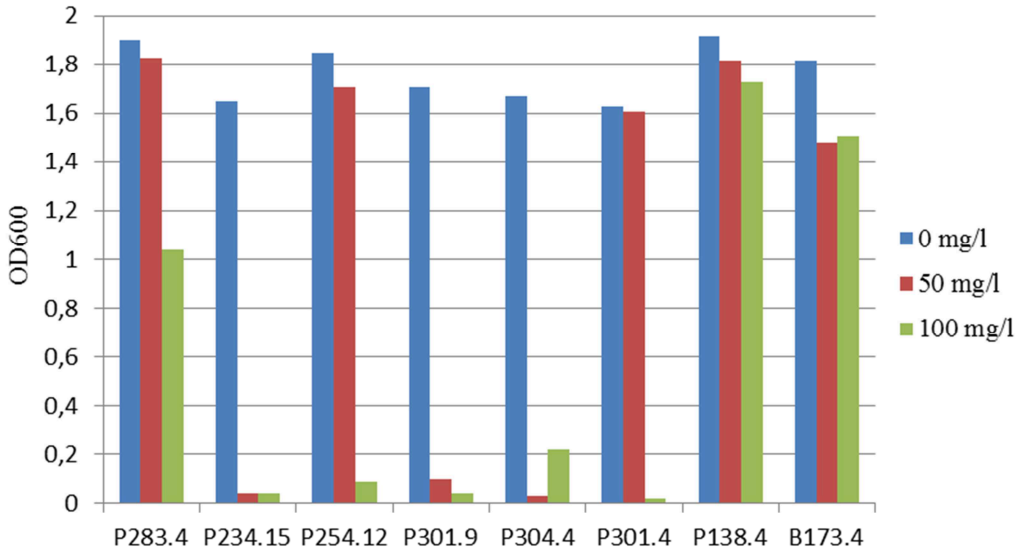


Figure 2.3.7 Yeast growth in the presence of different concentrations of sulphites.

Strain	SO_2 resistance (mg/l)
P283.4	100
P234.15	<50
P254.12	50
P301.9	<50
P304.4	50
P301.4	50
P138.4	100
B173.4	100

Table 2.3.6 Sulphites resistance levels.

As shown in Table 2.3.6 strains B173.4, P283.4 and P138.4 are less sensitive to sulfur dioxide and when sulphites are present at 50 mg/l, they show similar growth value of the control without sulphites, although the growth of the strain P283.4 at concentrations of 100 mg/l is slowed down. The growth of the strains P254.12 and P301.4 is inhibited when 100 mg/l of SO_2 is present in the medium, while P234.15, P301.9 and P304.4 are inhibited at both concentrations, revealing a low resistance level.

2.3.6 Copper resistance

The copper, besides being traditionally used in the vineyard for its antiseptic activity, is found in the composition of many pesticide used to inhibit growth of plant pathogens. The copper sprayed in vineyard may inhibit fermentation activity in cellar, if used at high concentrations. In addition, several commercial yeasts used in winemaking are able, by means of cell wall adsorption, to reduce the concentration of this metal in the wine (Vincenzini et al., 2005). With the aim to verify the copper resistance a growth inhibition assay was set up using the minimal medium YNB containing different concentrations of $CuSO_4$. The yeasts growth was determined by measuring the OD600 after 48 hours. Using

these conditions the strain sensitivity to the copper toxicity was determined by increasing the concentration of this metal (Table 2.3.7).

Strain	$CuSO_4$ 800µM	$CuSO_4$ 400µM	$CuSO_4$ 200µM	$CuSO_4$ 100µM	$CuSO_4$ 50µM	Control
P283.4	-	-	+	+	+	+
P234.15	-	+	+	+	+	+
P254.12	-	+	+	+	+	+
P301.9	-	+	+	+	+	+
P304.4	-	+	+	+	+	+
P301.4	-	+	+	+	+	+
P138.4	-	+	+	+	+	+
B173.4	-	-	+	+	+	+

Table 2.3.7 Copper resistance levels.

At concentrations equal or greater than 800 µM of $CuSO_4$ all strains tested evidence a complete growth inhibition. Strains B173.4 and P283.4 show the lower copper resistance. The other strains reveal to be quite resistance as they start growing within the first 24 hours in presence of 400 µM copper sulfate.

2.4 CONCLUSIONS

The technological characterization performed on the eight selected *S. cerevisiae* strains has shown a good degree of variability within each individual character, although always presenting suitable values from an enological point of view. All the strains showed good fermentation performance although some strains run fermentation more rapidly. A good level of variability was found when killer activity was assessed: three strains were found to produce toxins, two are sensitive and three neutral. The lowest level of variability was observed when the production SO_2 and H_2S was assessed. All strains produce intermediate level of SO_2 and H_2S. With respect to sulphate resistance three strains are rather sensitive, two show an intermediate resistance level and three are strongly resistant. The ethanol resistance level of the eight strains ranged from 13% up to 15% indicating a medium-to-high ethanol tolerance. Finally, the levels of copper resistance are quite high even if two strains seem to be more sensitive.

CHAPTER 3

Evaluation of the colonization ability of autochthonous *Saccharomyces cerevisiae* strains introduced in the vineyard and effects on must fermentation

3.1 INTRODUCTION

In the vineyard, yeasts may be transported from the soil to the grapes by various insects or by the wind. Surprisingly, fermentative species of *Saccharomyces* occur in very low numbers in grapes, the predominant microorganisms being apiculate yeasts and other oxidative species (Fleet & Heard, 1993). On the other hand, Mortimer & Polsinelli (1999) observed that damaged grape berries are rich depositories of *S. cerevisiae*, showing that the vineyard can be a natural store of *S. cerevisiae*. The importance of each yeast source, vineyard or winery, may vary greatly, depending on a large variety of factors, such as climatic conditions, including temperature and rainfall, the geographical location of the vineyard, the amount of SO_2, antifungal applications, the harvest technique, the grape variety, the age of the vineyard, and the soil type (Pretorius, 2000)

Since the beginning of the 1980s, the use of active dried *S. cerevisiae* yeast starters has been extensively generalised. Today, the majority of wine production is based on the use of active dried yeast, which ensures rapid and reliable fermentation, and reduces the risk of sluggish or stuck fermentation and of microbial contamination. Most commercial wine yeast strains available today have been selected in the vineyard for enological traits such as fermentation performance, ethanol tolerance, absence of off-flavors and production of desirable metabolites. These and other technological developments have contributed to an improvement in the quality of wine, and have enhanced the ability of winemakers to control the fermentation process and achieve specific outcomes.(Valero *et al*., 2005)

On the other hand, there is increasing interest in both indigenous strains of S. cerevisiae and wild yeast species that may contribute to the overall sensorial quality of

wine, (Pretorius *et al*., 1999); *Saccharomyces cerevisiae* strains seem to be widely distributed in a given viticultural region, and they can be found in consecutive years (Vezinhet *et al*., 1992; Torija *et al*., 2001); there are also strains predominant in fermenting microbial communities (Frezier & Dubourdieu, 1992; Sabate *et al*., 1998), suggesting the occurrence of specific native strains that can be associated with a *terroir*. High biodiversity level in vineyard is necessary to guarantee spontaneous fermentation that recently have been re-evaluated both for positively influencing sensorial characteristics of wine and for supporting a more "natural" management of winemaking process (Santamarìa et al., 2005). Preserving biodiversity is important in order to ensure the conservation of gene pools of technological importance. With regard to this, several studies have been performed with the aim of assessing the impact of winemaking practices – including the extensive use of active dried yeast – on the natural microbial community.

In order to evaluate the survival and the dynamics of commercial yeast over years, and the capacity to become members of the vineyard microbiota the INRA planned a large-scale study,in which the sampling plan was devised over a period of three years in six different vineyards (3 in France and 3 in Portugal). A total of 198 grape samples were collected at various distances from the wineries, before and after harvest, and yeast strains isolated after spontaneous fermentation were subsequently identified by molecular methods.

Among 3780 yeast strains identified, 296 isolates (7,8%) had a genetic profile identical to that of commercial yeast strains. Of this, 94% were recovered at very close proximity to the winery (10–200 m). and a large majority (78%) was found at sites very close (10–50 m) to the wineries. A major proportion (73%) was collected in post-harvest campaigns, indicating immediate dissemination. In most instances, the strains with a profile similar to that of a commercial strain were recovered from a vineyard in which the same commercial yeast was used in vinification, or had been used previously.

Analysis of population variations from year to year indicated that permanent implantation of commercial strains in the vineyard did not occur, but instead that these strains were subject to natural fluctuations of periodical appearance/disappearance like autochthonous strains.

Commercial yeasts are classically used in winemaking without any special

containment and are annually released in large quantities. Dispersal of commercial strains seems to be mainly mediated by water run-off and may also derive from macerated grape skin at dumping sites. Avoiding these behaviors it can significantly reduce the population size of commercial yeast strains around the winery.

Regarding autochthonous wine yeast biodiversity in vineyards around wineries where active dry yeasts have been used as fermentation starters, the results relating to the French winemaking region reveal that the large majority of different chromosomal patterns identified (91%) were found as unique patterns, indicating great biodiversity. There were differences in biodiversity according to the vineyard and year, showing that the biodiversity of *Saccharomyces* strains is influenced by climatic conditions and specific factors associated with the vineyards, such as age and size.

The yeast community of each year was characterized by the appearance of many new patterns, indicating the fact that the behaviour of the large majority of the strains was not perennial.

The biodiversity of *S. cerevisiae* strains after harvest was similar to that in the early campaign; moreover, a temporal succession of *S. cerevisiae* strains is shown. This fact, together with the differences in biodiversity levels verifies that other factors were more important than commercial yeast utilization in the biodiversity of the vineyard. (Valero *et a.l* 2007)

The same study carried out in the Vinho Verde region in the north of Portugal, published by Schuller et al. (2005), showed similar values to those found in Languedoc wine region of France. The vast majority of the patterns were unique, demonstrating an enormous biodiversity of *S. cerevisiae* strains in the Vinho Verde Region. The vast majority of the strains did not display a perennial behavior, being the flora of each year characterized by the appearance of many new patterns. Among all patterns only one showed a wide regional distribution with a perennial behavior providing preliminary evidence for a strain representing a ‘‘*terroir*’’ as described (Versavaud et al., 1995; Vezinhet et al., 1992). The appearance of this strain did not obey to a generalized pattern, but rather to sporadic presence, absence and reappearance, due to natural population fluctuations. The perennial appearance of this pattern is a consequence of its prevalence in the local microflora.

In contrast to the results from the French region, few of the grape samples collected before harvest initiated a spontaneous fermentation, compared to the samples collected after harvest, in a time frame of about 2 weeks. The associated strains were also much more diversified: 267 patterns among 1260 isolates compared to 30 patterns among 360 isolates in the post- and pre-harvest samples, respectively. With only one exception, autochthonous strain patterns from the early sampling stage did not appear in the late sampling stage, speaking in favor of a succession of S. cerevisiae strains.

To evaluate the industrial starter yeasts' ability to survive in nature and become part of the natural microbiota of musts, commercial yeast was disseminated voluntarily in an experimental vineyard in the Madrid region (Spain) (Cordero-Bueso G. *et al*.,2011) A large sampling plan was devised over 3 years, including samples of grapes, leaves, bark and soil. The disseminated yeast was well represented in the vineyard during the first 8 months. After 2 years, the commercial yeast strain had not survived in the sprayed plants, but a residual population was found in plants situated 50m east of the sprayed area. After 3 years, commercial yeast disseminated was not found in the sampled vineyard. Grapes and soil showed the highest number of yeasts isolated in the vegetative period, the bark being the main natural reservoir during the resting stages. The result of analysis of population variations from year to year indicated that permanent implantation of commercial strain (K1M) in the vineyard did not occur and its presence was limited in time.

In this work the effect of the release in vineyard of five strains among those previously characterized is reported. After the inoculum the presence of the strains were monitored by fermenting both grape bunches and bark portions. In this way the impact of the selected yeasts on native *S. cerevisiae* population was assessed. Finally, microvinifications were run to evaluate the contribution of the introduced strains on the onset of spontaneous fermentation.

3.2 MATERIALS AND METHODS

3.2.1 Yeast strains

For this study ecotypical strains were used. These yeasts were isolated from the vineyards in the winemaking area of Prosecco Superiore di Conegliano Valdobbiadene DOCG shown in Table 3.2.1

Strain	**Species**	**Origin**
P283.4	*Saccharomyces cerevisiae*	Vineyard
P234.15	*Saccharomyces cerevisiae*	Vineyard
P301.4	*Saccharomyces cerevisiae*	Vineyard
P138.4	*Saccharomyces cerevisiae*	Vineyard
B173.4	*Saccharomyces cerevisiae*	Vineyard

Table 3.2.1 Yeast strains used in this work

3.2.1.1 Culture media and growth condition

Media

Wallerstein Laboratory (WL medium) nutrient agar (Green & Gray, 1950).

Suspend 75 g WL nutrient agar (Oxoid) in a liter of distilled water.

Sterilize by autoclaving at 121 ° C for 15 minutes.

Plate Count Agar (PCA)

Suspend 20,5 g of PCA medium (Oxoid) in a liter of distilled water.

Sterilize by autoclaving at 121 ° C for 15 minutes.

YM solid agar medium

- 3 g L-1 yeast extract (Oxoid);
- 3 g L-1 malt extract (Oxoid);
- 5 g L-1 vegetatone peptone (DIFCO);

- 10 g L-1 glucose (PROLABO)
- 16 g L-1 Bacto Agar (DIFCO).

Adjust to volume with distilled water. Sterilize by autoclaving at 121 ° C for 15 minutes.

YPD (Yeast Extract/Peptone/Dextrose)

10 g L-1 yeast extract (OXOID)

20 g L-1 vegetatone peptone (DIFCO)

20 g L-1 glucose (PROLABO)

Adjust to volume with distilled water. Sterilize by autoclaving at 121 ° C for 15 minutes.

Synthetic nutrient medium (MSN) (Delfini, 1995)

Macronutrients

0,1 g L-1 $CaCl_2$

0,1 g L-1 NaCl

1 g L-1 KH_2PO_4

0,5 g L-1 $MgSO_4 \cdot 7H_2O$

3 g L-1 tartaric acid 123

Micronutrients

0,2 mg L-1 $NaMoO_4 \cdot 2H_2O$

0,4 mg L-1 $ZnSO_4 \cdot 7H_2O$

0,5 g L-1 H_3BO_3

0,04 mg L-1 $CuSO_4 \cdot 5H_2O$

0,1 mg L-1 KJ

0,4 mg L-1 $FeCl_3 \cdot 6H_2O$

0,4 mg L-1 $MnSO_4 \cdot H_2O$

Vitamins

400 µg L-1 pyridoxine hydrochloride

400 µg L-1 thiamine hydrochloride

2000 µg L-1 Inosite

20 μg L-1 Biotin
400 μg L-1 Calcium pantothenate
400 μg L-1 Nicotinic acid amide
200 μg L-1 P-amino-benzoic acid

Variable components
0,3 g L-1 (NH4)2SO4
0,3 g L-1 (NH4)2HPO4
200 g L-1 Glucose
0,2 g L-1 Hydrolyzed Casein

Prepare the micronutrients and vitamins in a 100 times concentrated aqueous solution and use the 1%. Dissolve all components in distilled water, adjust the pH with KOH of the resulting solution to pH 3.2. Sterilize by autoclaving at 100 ° C for 5 min.

Solution
Ringer Solution for dilutions (1/4 *strenght*; Dept. of Health & Social Security, 1937). Dissolve one tablet preparation (LAB M, International Diagnostics Group) in 500 ml of deionised water. Sterilize by autoclaving at 121 ° C for 15 minutes.

Growth conditions

The yeast strains were grown at 25°C. The liquid cultures to be used for fermentation trials were subjected to orbital shaking (130 rpm).

3.2.2 Yeast sampling and isolation from vineyards

Grape bunches isolation
The collection has been made, at each stage, avoiding touching the grapes with hands and sterilizing scissors periodically in order to minimize contamination.

Stomaker sterile bags were used, filled with about 500 g of grapes (corresponding to one or two bunches, depending on size) and closed for the laboratory transport.
Samples collected in the vineyard were transferred to the laboratory where 10 g of sugars (5g of fructose and 5g of glucose) and 500µl of sulphur dioxide at 5% v/v, to facilitate the development of *Saccharomyces* strains, were added.
Bags were closed with a foam rubber cap previously sterilized in order to avoid the increase of pressure inside the bag, while maintaining the internal environment isolated from the outside. Each sample was then manually pressed and left to ferment spontaneously (at room temperature) for 2 to 3 weeks with skins, stalks and pips.
The fermentation process was monitored by measuring, for each bag, the daily weight loss.

Bark portion isolation

The sampling was carried out by collecting bark portions from the main vines row. Bark portions were collected scratching vine stock with a spatula sterilized each time with denatured alcohol to avoid any kind of contamination.
Samples collected in the vineyard (in quantity to fill a 50 mL Falcon) were transferred to the laboratory where were put into 100 ml-Erlenmeyer flasks sealed with silicon cap and supplied with bowed glass pipettes and filled with 100 ml of synthetic must (Delfini, 1995). 100µl of sulphur dioxide at 5% v/v and 10mL of vaseline oil, to prevent apiculate yeasts and moulds development, were added.
Fermentation process was followed monitoring the weight loss daily. The fermentations were considered completed when weight loss was lower than 0,1 g within 24 hours.

Yeasts isolation

During the fermentation, when the amount of CO_2 produced reached 3 - 4 g/100 ml of must or g of grape, 3 ml of product were taken, the appropriate serial dilutions (1:10) were made using Ringer solution. Hundred µl of the last three dilutions were plated on WL medium. After 5 days at 25°C, colonies count was performed and 11 colonies with *Saccharomyces*-like morphology were randomly collected.

3.2.3 Yeast storage and purification

All the colonies belonging to the *Saccharomyces* genus were grown on YPD liquid medium for 24hours at 25°C, then centrifuged and resuspended in 2 ml of a sterile solution composed of YPD medium and 40% of glycerol (1:1). The vials were stored at -80°C.

3.2.4 DNA amplification

3.2.4 1 Sample preparation for DNA amplification

Yeast colonies (1–2mm diameter), grown for 1–3 days, were picked up with a sterile toothpick from YM plates and resuspended in 20 µL of sterile deionized water in 0.5mL tubes. Two microlitres of the suspension were used for PCR amplification.

3.2.4.2 SAC26-SAC18 multiplex PCR

The identification of the colonies belonging to the genus *Saccharomyces* took place adopting the method developed by Nardi *et al.* (2006). The various components of the reaction mixture were used in the following final concentrations:

Table 3.2.2 PCR master mix composition

Primer SAC26F	0,2 µM
Primer SAC26R	0,2 µM
Primer SAC18F	2 µM
Primer SAC18R	2 µM
dNTPs (Amersham)	200 µM
Taq polimerasi (Promega)	0,02 U/µ
Buffer	1X
DNA	2 µl cellular suspension

Primers utilized are reported below (table 3.2.3).

Table 3.2.3 Primers for SAC26-SAC18 amplification

Name	Length	Sequence (5'-3')
SAC26F	22 nt	GAGAGGGCAACTTTGGGRCCGT
SAC26R	27 nt	ACCATTATGCCAGCATCCTTGACTTAC
SAC18F	23 nt	CTGCGAATGGCTCATTAAATCAG
SAC18R	25 nt	CCCTAACTTTCGTTCTTGATTAATG

The thermal protocol was the following: initial incubation at 95°C for 5 min to allow cell lysis and DNA denaturation, followed by 35 cycles composed of denaturation at 95°C for 30 s, annealing at 54°C for 45 s and extension at 72°C for 90 s. A final extension step was added at 72°C for 5 min.

Amplified samples were run on 1,2% agarose gel with 0,1 μg/ml of ethidium bromide. The running was performed with TBE 0,5X (44,5 mM Tris, 44,5 mM boric acid, 1 mM EDTA) on a potential difference of 50-110 V.

Digital images were acquired with an EDAS290 image capturing system (Kodak, Rochester, NY).

3.2.5 Yeasts species identification by ITS1-5,8S-ITS2 region amplification and RFLP analysis

3.2.5.1 Sample preparation for DNA amplification

Yeast colonies (1–2mm diameter), grown for 1–3 days, were picked up with a sterile toothpick from YM plates and resuspended in 20 μL of sterile deionised water in 0.5mL tubes. Two microlitres of the suspension were used for PCR amplification.

3.2.5.2 ITS1-5,8S-ITS2 region amplification

The various components of the reaction mixture were used in the following final concentrations:

Table 3.2.4. PCR master mix composition

ITS1	2 µM
ITS4	2 µM
dNTPs (Amersham)	200 µM
Taq polimerasi (Promega)	0,02 U/µ
Buffer	1X
DNA	2 µl cellular suspension

Primers utilized are reported below (table 3.2.5).

Table 3.2.5 Primers for ITS1-ITS4 amplification

Name	Length	Sequence (5'-3')	Source
ITS1	19 nt	TCCGTAGGTGAACCTGCGG	White *et al*.,1990
ITS4	20 nt	TCCTCCGCTTATTGATATGC	White *et al*.,1990

The thermal protocol was the following: initial incubation at 95°C for 5 min to allow cell lysis and DNA denaturation, followed by 35 cycles composed of denaturation at 95°C for 30 s, annealing at 54°C for 30 s and extension at 72°C for 90 s. A final extension step was added at 72°C for 5 min.

Amplified samples were run on 1,2% agarose gel with 0,1 µg/ml of ethidium bromide. The running was performed with TBE 0,5X (44,5 mM Tris, 44,5 mM boric acid, 1 mM EDTA) on a potential difference of 50-110 V.

Digital images were acquired with an EDAS290 image capturing system (Kodak, Rochester, NY).

3.2.5.3 ITS1-4 RFLP analysis

The amplification products of the region ITS1-5,8S-ITS2 of rDNA were digested with enzymes HaeIII and MaeI (Amersham). The digestions were performed in 20 µl volumes reaction containing 10 U of enzymes and 10 µl of the amplified. The reactions were conducted at 37°C for 16 h.

3.2.6 Amplification of inter-δ region.

3.2.6.1 Sample preparation for DNA amplification

Yeast colonies (1–2mm diameter), grown for 1–3 days, were picked up with a sterile toothpick from YM plates and resuspended in 20 µL of sterile deionized water in 0.5mL tubes. Two microlitres of the suspension were used for PCR amplification.

3.2.6.2 PCR-amplification of delta sequences

The various components of the reaction mixture were used in the following final concentrations:

Table 3.2.6 PCR master mix composition

Delta 12	1 µM
Delta 21	1 µM
dNTPs (Amersham)	200 µM
Taq polimerasi (Promega)	0,02 U/µ
Buffer	1X
DNA	2 µl cellular suspension

Primers utilized are reported below (table 3.2.7).

Table 3.2.7 Primers for ITS1-ITS4 amplification

Name	Length	Sequence (5'-3')	Source
Delta 12	19 nt	TCAACAATGGAATCCCAAC	Legras *et al*.,2003
Delta 21	20 nt	CATCTTAACACCGTATATGA	Legras *et al*.,2003

The thermal protocol was the following: initial incubation at 95°C for 4 min to allow cell lysis and DNA denaturation, followed by 35 cycles composed of denaturation at 95°C for 30 s, annealing at 46°C for 30 s and extension at 72°C for 90 s. A final extension step was added at 72°C for 10 min.

Amplified samples were run on 1% agarose gel with 0,1 µg/ml of ethidium bromide. The running was performed with TBE 0,5X (44,5 mM Tris, 44,5 mM boric acid, 1 mM EDTA) on a potential difference of 100 V.

Digital images were acquired with an EDAS290 image capturing system (Kodak, Rochester, NY).

3.2.7 Mitochondrial DNA analysis

3.2.7.1 Yeasts total DNA extraction

All the yeast cells present on YM agar plate after 48-hours incubation at 25 °C, were collected, resuspended in 1 ml of sterile water and then centrifuged at 14000 rpm for 3 minutes in an Eppendorf microcentrifuge. After the supernatant have been poured off, the cells were resuspended in 500 µl of a solution containing 50 mM Tris-HCl, 20 mM EDTA, pH 7.4 and transferred to a 2 ml Eppendorf tube containing 0.3 g of glass beads of 425-600 µm (Sigma) and vortex for 3 minutes. 50 µl of 10% SDS were then added to the samples that were incubated in a thermostatic bath at 65°C for 30 minutes. At the end 200 µl of potassium acetate 5M were added and the samples were left on ice for 30 minutes. The tubes were centrifuged at 14000 rpm for 10 minutes. 600 µl of supernatant was transferred to an Eppendorf tube and 600 µl of cold isopropanol were added. The samples were kept at room temperature for 5 minutes, stirring by inversion and then centrifuged at 14000 rpm for 10 minutes. The supernatant was removed and 500 µl of 70% ethanol were added. After centrifugation at 14000 rpm for 10 minutes and the supernatant discarding, the pellet was dried for 1 h at 37 ° C. The samples were resuspended in 50 µl of sterile water, to which 1.5

µl (10 mg / ml) of RNase (Amersham Bioscience E70194Z) were added. The samples were left at room temperature for 15-20 minutes and finally stored at -20 °C.

3.2.7.2 Total DNA enzyme restriction

The total DNA digestions were performed in 15 µl of volumes reaction containing 10 U of *HinfI* enzyme (Fermentas) and 10 µl of extracted DNA. The reactions were performed at 37 °C for 2 h.

3.2.7.3 Genetic profile analysis

Restriction profiles were compared using BioNumerics V.6.6 (Applied Maths) software that allows, by a matrix construction, to calculate the similarity level between profiles and to convert it into a dendrogram. For the matrix construction was used the Dice similarity coefficient which considers the electrophoretic bands position, but not their intensity. Moreover, for the dendrogram construction, determined by the UPMGA method, the "optimization" and "tolerance" values, which determine the minimum variability degree of a profile than other more similar, were those recommended by the program.

3.2.8 Inoculum preparation and yeasts release in vineyard

The yeasts were grown on plates containing YPD medium at 25 °C for 3 days. A loopful of yeast cells was transferred from the plate into 5 ml of YPD liquid medium. The tubes were incubated for 24 hours at 28°C (120 rpm) until stationary phase (about 10^7 -10^8 cell/ml) was reached. Erlenmeyer flasks containing 100 ml of YPD liquid medium were inoculated with 1ml of the overnight culture and incubated 17 hours at 25°C under orbital shaking (120 rpm). The cells concentration present in the culture medium was measured by flow cytometry (Partec).

<u>First yeasts release (2013)</u>

The suitable volume of pre-colture was transferred in 3 L of sterile deionised water to a final concentration of 7,14 x 10^4 cells/ml (corresponding to 1 x 10^6 cells/ml in 14 ml of cell sospension, volume used to inoculate one meter of grape vine shoot).

A cells suspension containing the five selected *Saccharomyces cerevisiae* strains was transfered into a nebulizer and sprayed on the main shoot of the plant. For each vine row $2x10^6$ cells per linear meter have been sprayed.

Second yeasts release (2014)

The suitable volume of pre-colture was transferred in 3 L of sterile deionised water to a final concentration of 7,14 x 10^6 cells/ml (corresponding to 1 x 10^8 cells/ml in 14 ml of cell sospension, volume used to inoculate one meter of grape vine shoot).

A cells suspension containing the five selected *Saccharomyces cerevisiae* strains was transfered into a nebulizer and sprayed on the main shoot of the plant. For each vine row $2x10^8$ cells per linear meter have been sprayed.

3.2.9 Quantification of yeasts and bacteria present on the shoot surface

A quantity of 0.5 g of bark sample was resuspended in 50 ml of physiological solution and appropriate dilutions were plated on WL medium containing 100 µg/ml of chloramphenicol (for yeasts determination) and in PCA medium containing 200 µg/ml of cycloheximide (for bacteria determination). The plates were incubated at 25°C aerobically. Plates count were performed after 5 days of incubation in the case of yeasts, while for bacteria after 10 days of incubation.

3.2.10 Microvinifications

Harvest 2013

Microvinifications were carried out at the experimental winery (Veneto Agricultura) in Conegliano. When Glera grapes had reached complete maturation the manual harvesting was performed. Two quintals of bunches for each of the two vine rows used in the study were collected, in both vineyards. Two spontaneous fermentations for each of the rows considered in the trial were performed. After crushing, for each fermentation, the must and the skins were placed in steel fermenters of 1 hl capacity, about 70 liters of must were obtained. The following maceration at 20°C lasted 24 hours, afterwards the must was pressed to separate the grape pomace. After 24 hours of decantation, the must was transferred to a new fermenter to separate the lees. The musts were fermented at 20 °C, without addition of sulfur dioxide. A fermentation activator (59.88% ammonium

phosphate, thiamine hydrochloride 0.6%, cellulose 39.52%) was added at a concentration of 10 g/hl.

Must samples were collected to determine the sugars amount by the enzymatic method (EC ™ Wine Glucose + Fructose Total HL / ML Diffchamb). The fermentation was considered completed when the sugars concentration was lower than 1 g/l. Yeast quantification was performed by plate count on WL agar plates when alcohol content was about 6,0% v/v and at the end of fermentation. Form the isolation plates 25 randomly chosen colonies with *Saccharomyces*-like morphology, were purified and stored in glycerol.

Harvest 2014

In order to avoid contamination of commercial yeast used in cellar the microvinifications were performed in a warehouse located far from the wineries, provided by the consortium of Prosecco di Conegliano Valdobbiadene DOCG and the pressing was performed manually. Two quintals of bunches for each of the two vine rows used in the study were collected, in both vineyards. Two spontaneous fermentations for each of the rows considered in the trial were performed.

After crushing, the must and the skins were placed in steel fermenters of 1 hl capacity about 70 liters of must were obtained and were fermented at 20 °C. Grapes obtained from each row was used to conduct two spontaneous fermentations, whose one in the presence of 50 mg/l of SO_2. Must samples were collected to determine the sugars amount by the enzymatic method (EC ™ Wine Glucose + Fructose Total HL / ML Diffchamb). The fermentation was considered completed when the sugars concentration was lower than 1 g/l. Yeast quantification was performed by plate count on WL agar plates when alcohol content was about 6,0% v/v.

Form the isolation plates 30 randomly chosen colonies with *Saccharomyces*-like morphology, were purified and stored in glycerol.

3.2.11 Chemical analyses

At the end of each fermentation alcohol content, pH and total acidity were determined following the official methods of analysis proposed by the Office International de la Vigne et du Vin (OIV).

3.3 RESULTS AND DISCUSSION

3.3.1 The vineyards

In this study two vineyards in the area of Prosecco Superiore di Conegliano Valdobbiadene DOCG were chosen: one managed using conventional farming and the other organic.

The two vineyards are within 20km of each other. The organic vineyard is located in the countryside of Follina (Figure 3.3.1 a and c) in a flat area delimited on two sides by hills and distant from roads and cellars. It is a young vineyard (six years old) and for this reason the grapevine shoots have an average diameter of 1.5-2 cm with a thin bark; the vineyard cultivated with conventional farming techniques, is located near Conegliano (Figure 3.3.1 b and d) and unlike the organic vineyard, is closed to roads and cellars. It is 10 years old showing grape vine shoots more robust with an average diameter of 10 cm.

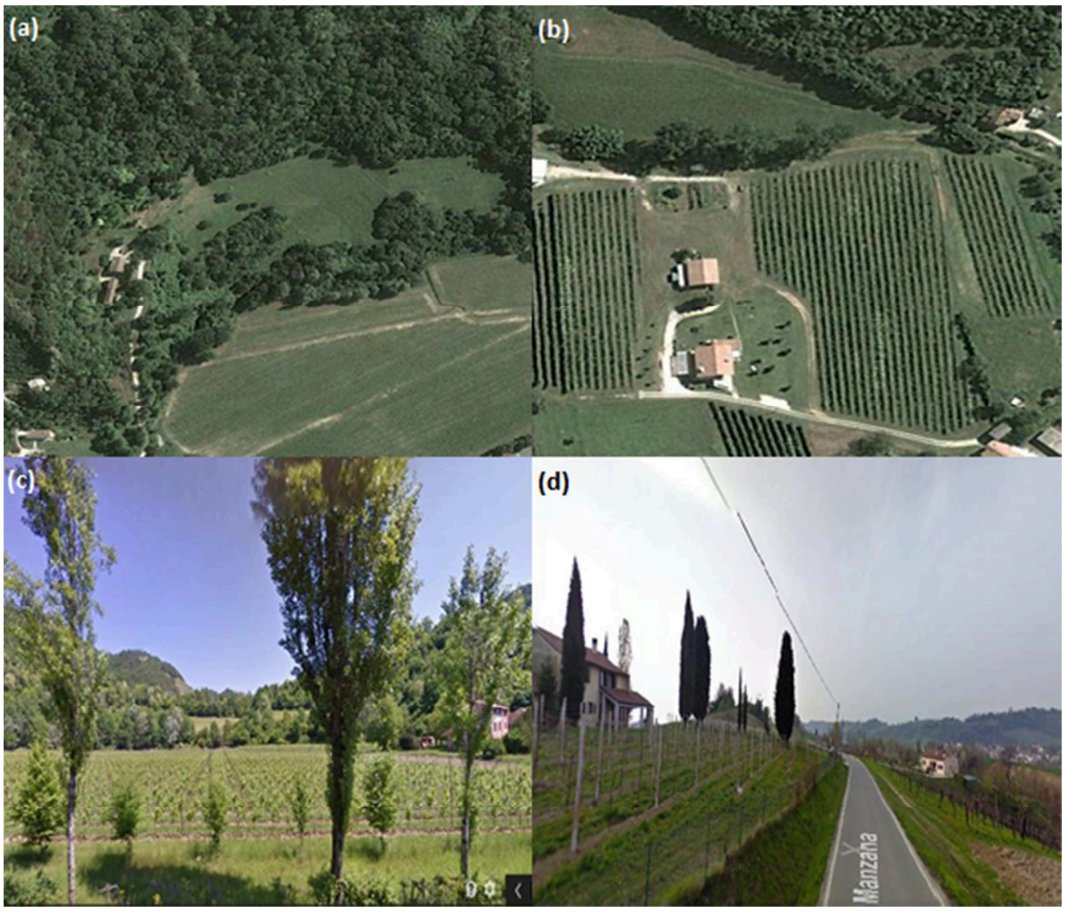


Figure 3.3.1 Organic vineyard located in Follina (a and c) and the conventional one in Conegliano (b and d).

3.3.2 Genetic variability of *S. cerevisiae* strains in the selected vineyards

In order to evaluate the genetic variability of *S. cerevisiae* strains associated to the vineyards a sampling was conducted during pre-harvest period (in September 2013). Ten bunches and ten bark (cortex) portions were collected. In each vineyards, two rows (L and C) were selected and 10 bunches, one from each randomly chosen plant, were individually transferred in sterile bags and named as follows: 1CGL1-10 first bunch sampling from the conventional vineyard row L, and 1CGC1-10 first sampling of bunches from the conventional vineyard row C. Moreover, 10 bark portions were collected from the same plants. The 10 samples of bark were named as followed: 1CTL1-10 first bark sampling of from conventional vineyard row L and 1CTC1-10 first bark sampling from conventional vineyard row C. With the same procedure bunches and bark samples were collected from organic vineyard (1BGL1-10, 1BGC1-10, 1BTL1-10, 1BTC1-10).

In each sterile bag the grape bunch was manually crushed (Figure 3.3.2), while the fragments of bark were placed in Erlenmeyer flask containing 100ml of MSN (Figure 3.3.3).

All samples were fermented as described in "Materials and Methods" section. During fermentation the decrease in weight due to the production of CO_2 was monitored while ethanol percentage was obtained using the following formula: weight loss% * 1,285 (Delfini 1995).

Figure 3.3.2 Bunch sampling

a **b**

Figure 3.3.3 a) Removal of bark portion from the vine shoot; b) bark-portion fermentation.

The Figure 3.3.4 fermentation kinetics were reported. Yeasts present in grape bunches 1CGL3, 1CGL6, 1CGL7, 1CGL9, 1CGL10 (5/10), collected from row L in the conventional vineyard, fermented rather vigorously with a good amount of CO_2 produced, which could indicate the presence of *S. cerevisiae*. From these sample, when 3-4 g/l of CO_2 produced was achieved, yeast isolation was performed. For each sample, 12 colonies were purified and subjected to the Multiplex PCR for identification of the genus *Saccharomyces*. The method allows the identification of yeasts belonging to this genus on the basis of nucleotide differences within the DNA region coding for ribosomal RNA (rDNA). In *S. cerevisiae*, this sequence is present on chromosome XII, repeated in a hundred copies placed in tandem (Johnston et al, 1997) and is transcribed into a single stretch of size 35S. It is divided into 3 main units encoding the RNA molecules that constitute the subunits of the ribosome:

• 26S (large subunit, LS) with 3392-bp size,

• 18S (small subunit, SS) with 1799-bp size,

• 5,8S with 155-bp size.

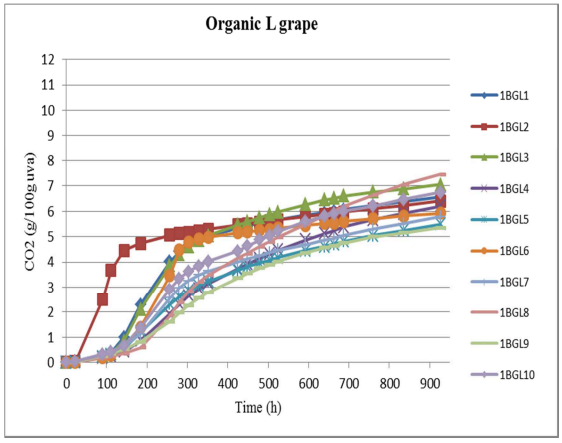
There is another DNA region coding for ribosomal RNA whose size is 120 bp and is encoded by an independent region on the complementary strand.

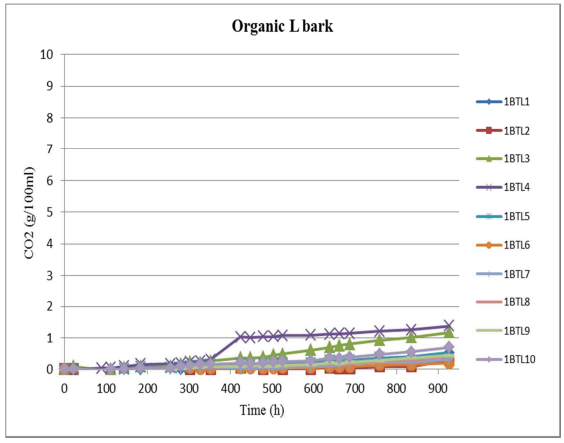
The DNA region coding for ribosomal RNA is a powerful tool for microorganism identification, since the nucleotide sequence variations observed among the different species are related to phylogenetic distance and taxonomic position. Within rDNA sequence the fragment that carries most of the genetic variability is the D1/D2 region of 26S DNA, whose sequences are available in GenBank. By means of multiple alignment (ClustalW) of D1/D2 sequences of all the species belonging to *Saccharomyces* genus, it was possible to identify two short DNA fragments that are highly conserved specifically in this genus. The two short sequences were used to design amplification primers (Sac26), obtaining a *Saccharomyces* specific amplicon. A second pair of primers (Sac18) was designed to have an internal amplification control: in this case a sequence belonging to the region of the 18s rDNA, highly conserved among all oenological yeasts was chosen. Although the colonies morphology was attributable to that of the specie *S. cerevisiae* none of the colonies tested gave positive results, when analyzed by Multiplex PCR (Table 3.3.1).

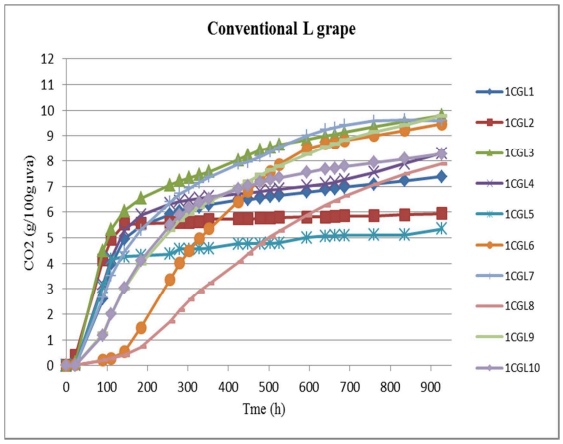
Yeasts present in the bark portions 1CTL1, 1CTL3, 1CTL4 1CTL6 (4/10) collected from row L in the conventional vineyard, fermented rather vigorously with a good amount of CO_2 produced, which could indicate the presence of *S. cerevisiae*. The colonies with *Saccharomyces* –like morphology isolated from these fermentations were subjected to Multiplex PCR, as well. All sample but 1CTL6 showed the presence of *S. cerevisiae*. Regarding the row C in conventional vineyard the grape bunches that showed *Saccharomyces*-like kinetics were 1CGC1, 1CGC2, 1CGC4, 1CGC5, 1CGC6, 1CGC8, 1CGC10 (7/10). Multiplex PCR revealed that only samples 1CGC2 and 1CGC5 contained *S. cerevisiae*.

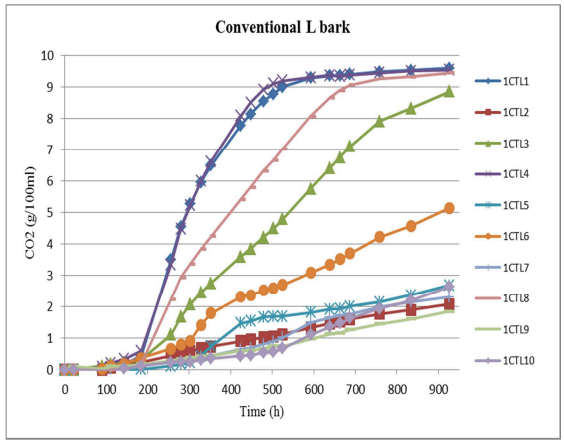
In the case of the bark portions collected from plant in the row C 1CTC6 and 1CTC10 (2/10) showed the presence of *S. cerevisiae*.

Surprisingly the bark and bunch samples collected from the organic vineyard, in both row L and C, did not evidence any fermentation indicating the absence of fermenting microorganism.









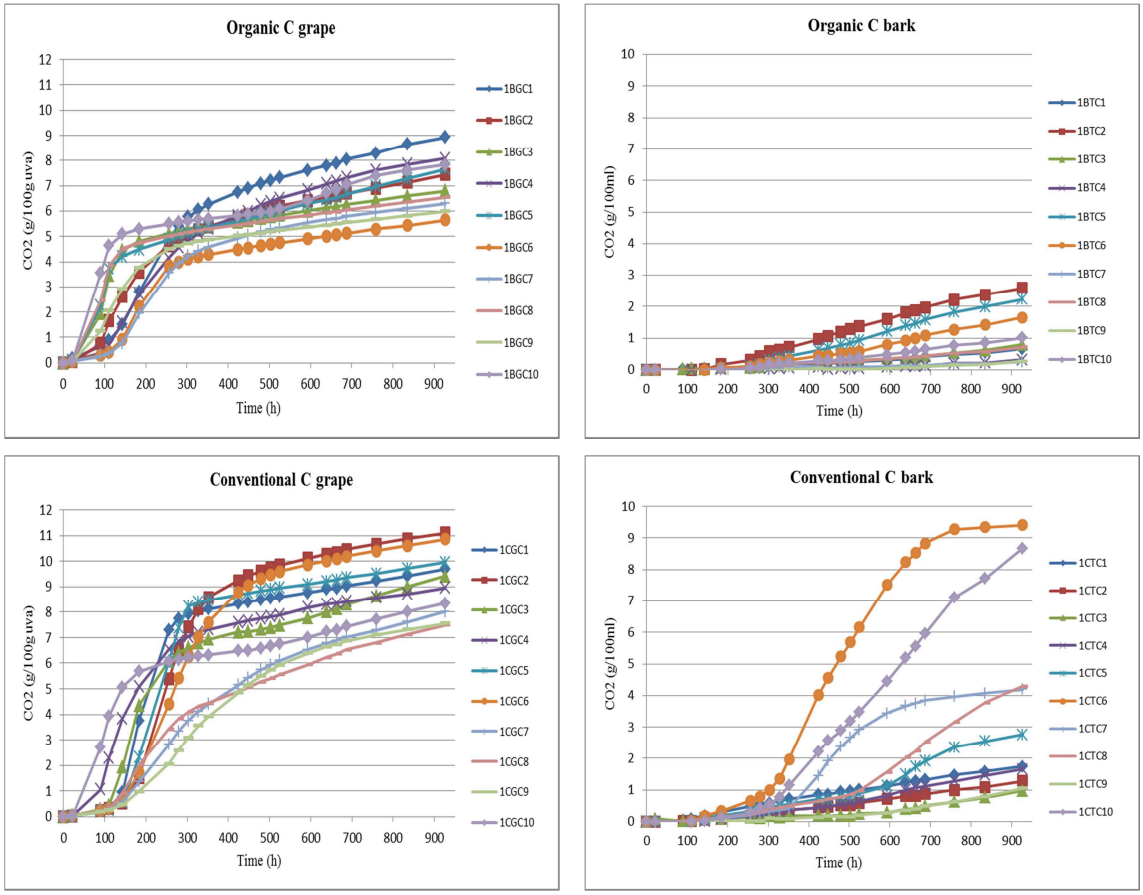


Figure 3.3.4 Fermentation kinetics of grapes bunches and bark samples obtained from the vine rows in the organic and conventional vineyards. The sampling was conducted before the inoculum of the selected strains.

Conventional Vineyard	Grape	Bark	Fermentation similar to *S. cerev*isiae	*% S. cerevisiae*	Genetic profile	Presence
Row L	1CGL1		no	–	–	–
	1CGL2		no	–	–	–
	1CGL3		si	0%	–	–
	1CGL4		no	–	–	–
	1CGL5		no	–	–	–
	1CGL6		si	0%	–	–
	1CGL7		si	0%	–	–
	1CGL8		no	–	–	–
	1CGL9		si	0%	–	–
	1CGL10		si	0%	–	–
		1CTL1	si	100%	Y1.1 Y1.2	10% 90%
		1CTL2	no	–	–	–
		1CTL3	si	100%	Y3.1	100%
		1CTL4	si	100%	Y4.1 Y4.2	20% 80%
		1CTL5	no	–	–	–
		1CTL6	si	0%	–	–
		1CTL7	no	–	–	–
		1CTL8	no	–	–	–
		1CTL9	no	–	–	–
		1CTL10	no	–	–	–
Row C	1CGC1		si	0%	–	–
	1CGC2		si	31% (13 out of 42 colonies)	G2.2	100%
	1CGC3		no	–	–	–
	1CGC4		si	0%	–	–
	1CGC5		si	100%	DV10	100%
	1CGC6		si	0%	–	–
	1CGC7		no	–	–	–
	1CGC8		si	0%	–	–
	1CGC9		no	–	–	–
	1CGC10		si	0%	–	–
		1CTC1	no	–	–	–
		1CTC2	no	–	–	–
		1CTC3	no	–	–	–
		1CTC4	no	–	–	–
		1CTC5	no	–	–	–
		1CTC6	si	100%	C6.1	100%
		1CTC7	no	–	–	–
		1CTC8	no	–	–	–
		1CTC9	no	–	–	–
		1CTC10	si	100%	C10.1	100%

Table 3.3.1 Presence of *S. cerevisiae* in grape bunches and bark samples obtained from the conventional vineyard. The sampling was conducted before the inoculum of the selected strains.

All the isolates belonging to the genus *Saccharomyces* were subjected to further genetic analysis for species attribution. For this reason a protocol present in literature was used. This is based on the analysis of the sequence variability of the DNA region called ITS (Internal Transcribed Spacer), that flanks the rDNA fragments. The polymorphism of this sequence is much higher than that associated with the genes encoding 18S and 26S rRNA (Cai et al., 1996; James et al., 1996) (Figure 3.3.5). The sequence variability is extremely high, when the microorganisms belong to different species, while drops dramatically when strains of the same specie are considered. This intra-specific polymorphism can be highlighted by amplification of the ITS region and subsequent analysis of the restriction profile using appropriate enzymes (Esteve-Zarzoso et al., 1999). In this way you get a quick method, easy to perform, for the yeasts identification, which has the advantage of providing not only information relevant to the strain to be recognized but also to the rest of the microbial population. All isolates tested were found to belong to the specie *S. cerevisiae*.

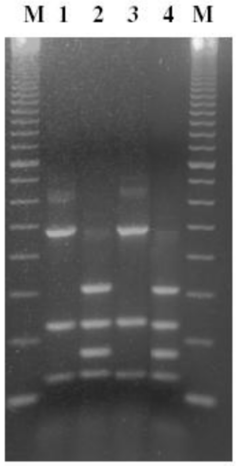


Figure 3.3.5 Separation of some *Saccharomyces* species by ITS analysis using *Hae*III enzymatic digestion. Lane: M, marker 100bp (Amersham Bioscience); 1, *S. mikatae*; 2, *S. paradoxus*; 3 *S. kudriavzwevii*; 4, *S. cerevisiae*.

The intraspecific genetic variability of *S. cerevisiae* isolates was investigated amplifying the interdelta DNA regions (interdelta analysis). The analysis was performed using the primer pair delta12 and delta21 (Legras and Karst, 2003) able to amplify these hypervariable regions of the genome of this species.

In Figure 3.3.6, by way of example, the interdelta profile of the strains inoculated in the vineyard is shown.

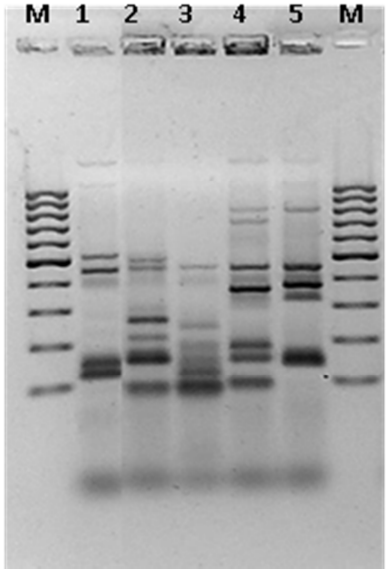


Figure 3.3.6 Example of interdelta profiles. M marker "100-bp DNA ladder" Amersham. Wells: from (1) to (5) strains introduced in the vineyard: P301.4, P283.4, P234.15, P138.4, B173.4.

The profiles obtained were compared visually and those identical were grouped. To further investigate the genetic variability of yeast isolates a method present in literature has been chosen (Querol et al., 1996). By this procedure the restriction profiles of the mitochondrial DNA is obtained by enzymatic digestion of the total DNA. This is the genetic characterization system used to identify wine strains belonging to the genus *Saccharomyces*, in particular using *Hinf*I as restriction enzyme (Lopez et al., 2001; Schuller et al., 2004).

It is a technique that offers many advantages, such as the reproducibility and speed of execution. Moreover It was also demonstrated that the level of resolution of this technique is comparable with that obtained by interdelta analysis (Shuller, Valero et al., 2004). For each interdelta profile one representative isolate has been chosen and grown on YPD agar medium. After DNA extraction, the enzymatic digestion with the enzyme *Hinf*I followed by electrophoretic run was performed. An example of mitochondrial DNA (mitDNA) profiles obtained is shown in Figure 3.3.7. The comparison of mitDNA profiles with those of 80 commercial strains, to verify the possible presence of commercial strains in the vineyard, was performed using the software BioNumerics that calculates the level of similarity

between profiles through the construction of a matrix. The output of the analysis is a dendrogram. In the construction of the matrix the Dice similarity coefficient that considers the position of the electrophoretic band, but not its intensity was used. Moreover in the dendrogram construction, based on the UPMGA association method, the value of the parameters "optimization" and "tolerance", which determine the minimum degree of variability of a profile compared to those most similar, were those calculated by the program.

Overall, in the analysis a total of nine genetic profiles have been identified. Only one was identical to the profile of the commercial strain DV10, widely used in the past in the wineries of this region (Table 3.3.1). The strain was isolated in the sample 1CGC5. Generally from each fermented sample (grape bunch or bark portion) a unique strain (mitDNA profile) was isolated except for samples 1CTL1 and 1CTL4 where two different strains in each fermentation were present.

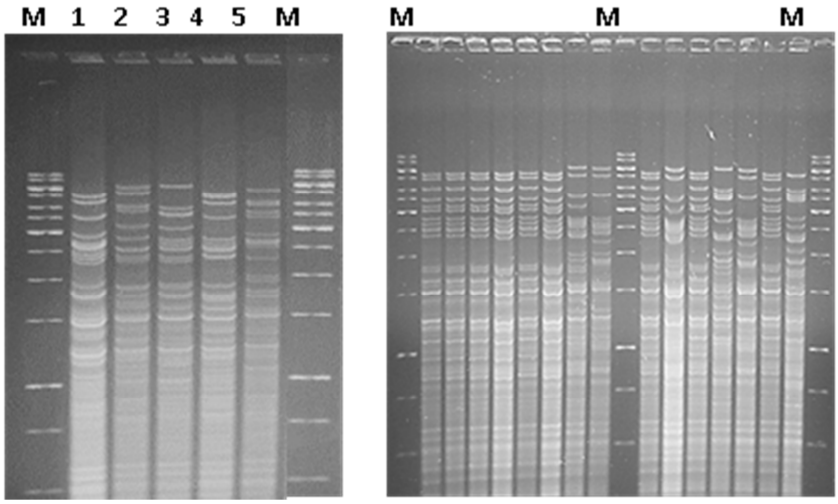


Figure 3.3.7 Genetic profiles obtained by mitochondrial DNA analysis (a) of the five strains used in the vineyard: M marker "1-kb DNA ladder" (Amersham), P138.4 1, P283.4 2, P301.4 3, P234.15 4, B173.4 5; (b) example of profiles obtained by analyzing isolates from vineyard.

3.3.3 Introduction of selected strains in the vineyard (first release)

With the aim to evaluate the extent of the microbial population that introduced yeasts would have been faced, ten days before strains inoculation (March 2013) total yeasts and bacteria quantification by means of standard plate counts was performed by collecting bark portion from the two vineyards. For each vineyard two samples from the row L (11LB,

31LB, 10LC, 30LC) and a sample from the row C (20NC, 21NB) were collected (Table 3.3.2). The samples were resuspended in physiological solution and the appropriate dilutions were plated respectively on WL plates containing 100 µg/ml of chloramphenicol (for the yeasts determination) and on PCA plates containing 200 µg/ml of cycloheximide (for the bacteria determination). Both media were incubated at 25°C aerobically.

Vineyard	Sample	Yeasts		Bacteria	
		(CFU/g d.w.)	Dev. St.	(CFU/g d.w.)	Dev. st.
Organic	11LB	6,75E+06	5,14E+05	5,73E+08	1,77E+08
	31LB	9,17E+06	1,82E+06	5,04E+08	8,05E+07
	20NB	1,21E+07	3,25E+06	4,44E+08	7,06E+07
Conventional	10LC	3,26E+04	7,87E+03	1,82E+07	5,93E+06
	30LC	9,29E+04	1,21E+04	1,96E+07	7,14E+06
	21NC	1,84E+05	1,01E+04	1,19E+07	5,16E+06

Table 3.3.2 Quantification of the microbiota present in the bark samples collected 10 days before inoculation. Values are expressed per gram of bark, dry weight (d.w.)

The results reveal that the microbial concentration is significantly higher (1 log) in organic vineyard. Moreover bark samples transferred in MSN did not show fermentation activity indicating that neither *S. cerevisiae* strains nor fermenting yeasts were present. Thus, we can suppose that the yeasts population present on the vine shoot of the organic vineyard is predominantly oxidative, while the higher yeast concentration than that in conventional vineyard could be due to the vineyard age: younger vines would be able to release a larger amount of exudates and thus better support the microbial growth.

Afterwards, the inoculum of the five selected yeasts was performed by spraying the microbial culture directly on the main vine shoot of the row L of each vineyard.

Each row has been sprayed with 2 x 10^6 cells (each strain) per meter of shoot using a gardening nebulizer (figure 3.3.8).

a **b**

Figure 3.3.8 Strains inoculum on the vine shoot of (a) the conventional vineyard and (b) the organic one.

Three days after the inoculation total yeasts quantification by means of standard plate counts was performed by collecting bark portion from the two vineyards to assess any changes in the microbiota. From row L of each vineyards three samples (11lb, 21LB, 31LB, 10LC, 20LC, 30LC) were collected. The samples were resuspended in physiological solution and the appropriate dilutions were plated in WL plates containing 100 μg/ml of chloramphenicol. The plates were incubated at 25°C aerobically. The results (Table 3.3.3), show in both cases an increase in the population size, although different concentration was found in the two vineyards. This excludes the hypothesis that the increase is due to the introduction of selected yeasts, and suggests fluctuations due to environmental changes. The result was confirmed by the Multiplex PCR analysis of 50 colonies with *Saccharomyces*-like morphology, collected from the isolation plates obtained from the investigation of each vineyard. No yeast belonging to the genus *Saccharomyces* was found.

Vineyard	Sample	Yeasts (CFU/g d.w.)	Dev. St.
Organic	11LB	3,84E+08	3,04E+07
	21LB	1,82E+08	2,02E+07
	31LB	8,88E+08	9,80E+07
Conventional	10LC	2,19E+05	5,05E+04
	20LC	4,40E+05	1,48E+05
	30LC	5,24E+05	1,48E+05

Table 3.3.3 Quantification of the microbiota present in the bark samples collected 3 days after inoculation. Data are expressed per gram of bark, dry weight d.w.

3.3.4 Evaluation of the colonization ability of the selected yeasts introduced in vineyard

To evaluate the colonization ability of the five yeast strains introduced in the vineyard, barks sampling were collected two, six and twelve months after the inoculum. At each sampling 10 bark portions were collected from the sprayed vine row (L) and 10 from the control (C), in both vineyards. Finally grapes were harvested and spontaneous fermentations were run. During the fermentation process the must was sampled when ethanol reached 6% and at the end of fermentation to evaluate strain composition.

3.3.4.1 Colonization after two months

After two months (May 2013) a second bark sampling was performed. A total of 20 samples were collected from both organic and conventional vineyards: 10 samples from each sprayed row L (conventional vineyard 2CTL1-10, organic vineyard 2BTL1-10) and 10 from each control row C (conventional vineyard 2CTC1-10, organic vineyard 2BTC1-10). All the bark portion were treated as previously described.

Yeasts present in bark samples 2CTL2, 2CTL3, 2CTL6, 2CTL7, 2CTL8, 2CTL9 (6/10), collected from the sprayed row L o conventional vineyard, fermented rather vigorously with a good amount of CO_2 produced, which could indicate the presence of *S. cerevisiae* (Figure 3.3.9). The following genetic investigations (Table 3.3.4) revealed that in the sample 2CTL7, the twelve colonies analyzed had the same mitDNA profile identical to that of the strain B173.4, one of the five selected strains introduced in vineyard.

The commercial strain DV10 (2CTL2) and the strain Y1.1 (2CTL9), already identified in the first sampling, were still present. The bark samples 2CTL3, 2CTL6, 2CTL8 carried in each case one new genetic profile. Seven out of ten bark samples collected in the control row (C) showed vigorous fermentations, which could indicate the presence of *S. cerevisiae*. During the fermentation of the bark samples 2CTC1, 2CTC5, 2CTC8 and 2CTC10 isolates carried a mitDNA profile identical to that of the strain profile Y3.1 already found in this vineyard; in the bark sample 2CTC2 the commercial strain DV10 was isolated, while in the samples 2CTC3 and 2CTC9 two strains with a new genetic profile, T10 and T14, were identified.

In organic vineyard only one bark sample (1/10) showed vigorous fermentations, which could indicate the presence of *S. cerevisiae*. The mitDNA profile of the isolates from the sample 2BTL9 fermentation was identical to that of the strain P301.4, one of the five selected strains introduced in vineyard.

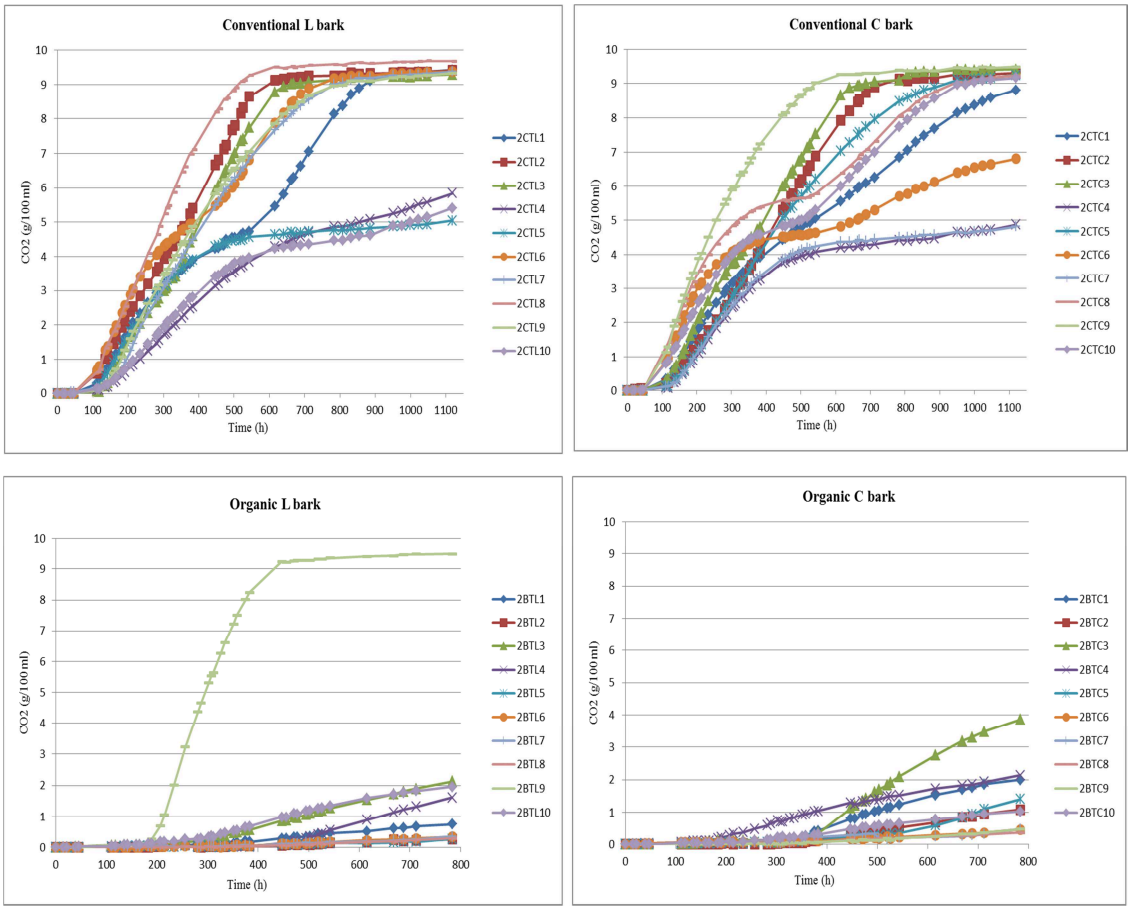


Figure 3.3.9 Fermentation kinetics obtained by inoculating bark samples collected from sprayed (row L) and untreated vineyards (row C). The sampling was performed two months after the spray.

Vineyard	Bark	Fermentation similar to *S. cerev*isiae	*% S. cerevisiae*	Introduced strains	Genetic profile	Presence
Conventional	2CTL1	si	25%	no	C6.1	100%
Row L	2CTL2	si	90%	no	DV10	100%
	2CTL3	si	63%	no	T1	100%
	2CTL4	no	–	–	–	–
	2CTL5	no	–	–	–	–
	2CTL6	si	100%	no	T2	100%
	2CTL7	si	100%	B173.4	B173.4	100%
	2CTL8	si	100%	no	T3	100%
	2CTL9	si	81%	no	Y1.1	100%
	2CTL10	no	–	–	–	–
Row C	2CTC1	si	90%	no	Y3.1	100%
	2CTC2	si	100%	no	DV10	100%
	2CTC3	si	100%	no	T10	100%
	2CTC4	no	–	–	–	–
	2CTC5	si	100%	no	Y3.1	100%
	2CTC6	no	–	–	–	–
	2CTC7	no	–	–	–	–
	2CTC8	si	64%	no	Y3.1	100%
	2CTC9	si	100%	no	T14	100%
	2CTC10	si	90%	no	Y3.1	100%
Organic	2BTL1	no	–	–	–	–
Row L	2BTL2	no	–	–	–	–
	2BTL3	no	–	–	–	–
	2BTL4	no	–	–	–	–
	2BTL5	no	–	–	–	–
	2BTL6	no	–	–	–	–
	2BTL7	no	–	–	–	–
	2BTL8	no	–	–	–	–
	2BTL9	si	81%	si	P301.4	100%
	2BTL10	no	–	–	–	–
Row C	2BTC1	no	–	–	–	–
	2BTC2	no	–	–	–	–
	2BTC3	no	–	–	–	–
	2BTC4	no	–	–	–	–
	2BTC5	no	–	–	–	–
	2BTC6	no	–	–	–	–
	2BTC7	no	–	–	–	–
	2BTC8	no	–	–	–	–
	2BTC9	no	–	–	–	–
	2BTC10	no	–	–	–	–

Table 3.3.4 Presence of *S. cerevisiae* in bark samples collected from the conventional and organic vineyard (sprayed vine row L and untreated vine row C) two months after spraying.

3.3.4.2 Colonization six months after inoculation

After six months from strain inoculum (September 2013), 20 bark samples were collected from the organic and the conventional vineyards. Samples collected were named as follows: conventional vineyard row L 3CTL1-10, row C 3CTC1-10; organic vineyard row L 3BTL1-10, row C 3BTC1-10. Bark samples were treated as described above. Yeasts present in bark samples 3CTL1, 3CTL2, 3CTL3, 3CTL4, 3CTL5, 3CTL6,3CTL7, 3CTL8,3CTL10 (8/10), collected from the sprayed row L o conventional vineyard, fermented rather vigorously with a good amount of CO_2 produced, which could indicate the presence of *S. cerevisiae* (figure 3.3.10). The following genetic investigations (Table 3.3.5) revealed the presence in this vine row of the inoculated strain P173.4, which constituted the 33% of the yeast present in the sample 3CTL1 and 90% in 3CTL2. In this sample 10% of the colonies had a genetic profile identical to that of the strain C6.1, already identified in the pre-spray sampling, but in the row C. In samples 3CTL3 and 3CTL4 100% of the colonies showed the genetic profile that identified the strain Y3.1, which was present also in the first sampling. In samples 3CTL1, 3CTL5, 3CTL7, 3CTL8 new strains, present in different percentages, were identified. In the sample 3CTL6, no isolate belongs to the genus *Saccharomyces*. In this sampling the commercial strain DV10 was present in the sample 3CTL.10. The analysis of isolates coming from the control row C has led to the identification of the strain Y3.1 in 3 bark samples (3CTC1, 3CTC4 and 3CTC9). The bark sample 3CTC10 contained 63% of isolates with the mitDNA profile identical to the commercial strain VIN13, while 37% has a new genetic profile (T15). In samples 3CTC3 and 3CTC8 the fermentation was run by the strain T10 already isolated in this row. The strain T13, never isolated before, was found in the sample 3CTC5.

Among the bark samples collected in the organic vineyard, only 3BTL6 fermented rather vigorously, which could indicate the presence of *S. cerevisiae*. All the colonies isolated and subjected to genetic analysis, are identical to the strain P301.4 that is one of the inoculated strains.

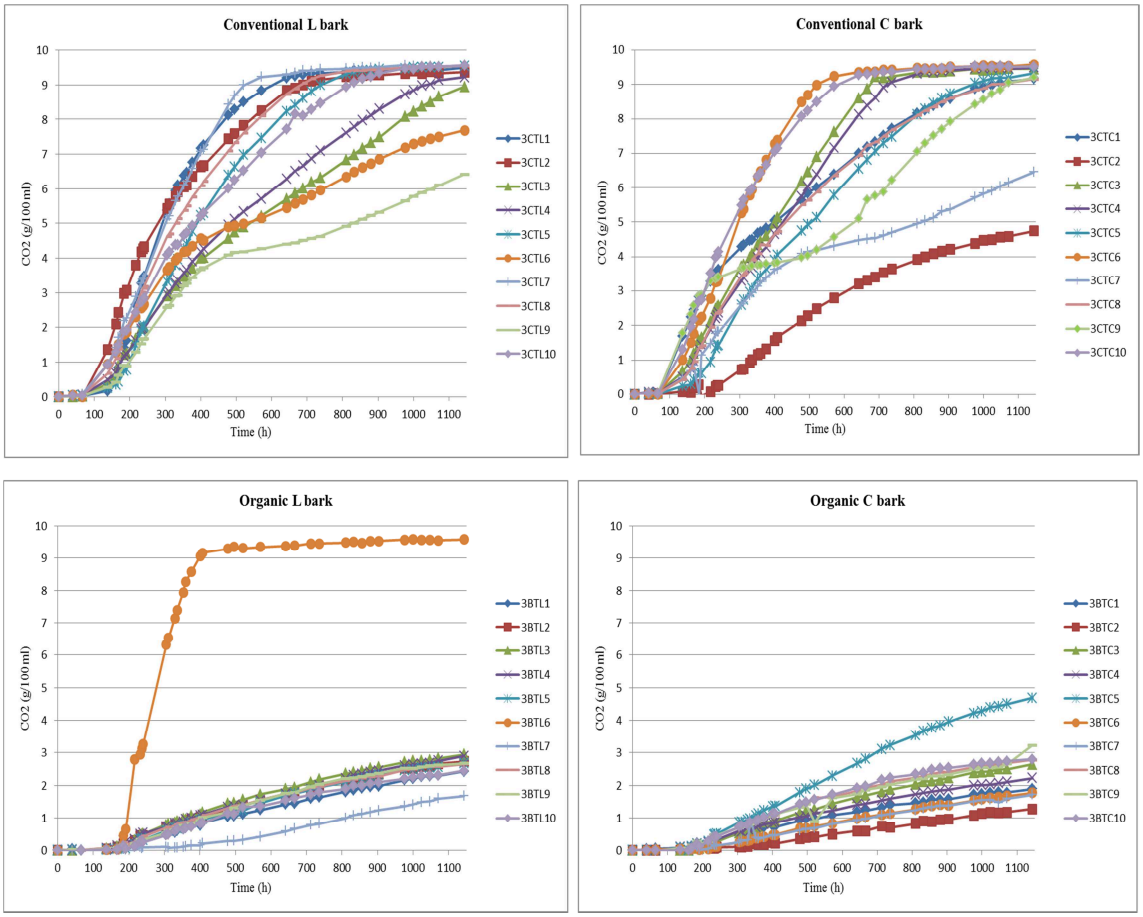


Figure 3.3.10 Fermentation kinetics obtained by inoculating bark samples collected from sprayed (row L) and untreated vineyards (row C). The sampling was performed six months after the spray.

Vineyard	Bark	Fermentation similar to *S. cerevisiae*	*% S. cerevisiae*	Introduced strains	Genetic profile	Presence
Conventional	3CTL1	si	54%	B173.4	B173.4	33%
Row L				no	T4	67%
	3CTL2	si	83%	B173.4	B173.4	90%
				no	C6.1	10%
	3CTL3	si	80%	no	Y3.1	100%
	3CTL4	si	66%	no	Y3.1	100%
	3CTL5	si	100%	no	T5	100%
	3CTL6	si	0%	–	–	–
	3CTL7	si	72%	no	T6	75%
				no	T7	25%
	3CTL8	si	70%	no	T6	15%
				no	T8	85%
	3CTL9	no	–	–	–	–
	3CTL10	si	83%	no	DV10	100%
Row C	3CTC1	si	100%	no	Y3.1	100%
	3CTC2	no	–	–	–	–
	3CTC3	si	78%	no	T10	100%
	3CTC4	si	90%	–	Y3.1	100%
	3CTC5	si	100%	no	T13	100%
	3CTC6	si	0%	–	–	–
	3CTC7	no	–	–	–	–
	3CTC8	si	100%	no	T10	100%
	3CTC9	si	100%	no	Y3.1	100%
	3CTC10	si	72%	no	VIN13	63%
				no	T15	37%
Organic	3BTL1	no	–	–	–	–
Row L	3BTL2	no	–	–	–	–
	3BTL3	no	–	–	–	–
	3BTL4	no	–	–	–	–
	3BTL5	no	–	–	–	–
	3BTL6	si	90%	P301.4	P301.4	100%
	3BTL7	no	–	–	–	–
	3BTL8	no	–	–	–	–
	3BTL9	no	–	–	–	–
	3BTL10	no	–	–	–	–
Row C	3BTC1	no	–	–	–	–
	3BTC2	no	–	–	–	–
	3BTC3	no	–	–	–	–
	3BTC4	no	–	–	–	–
	3BTC5	no	–	–	–	–
	3BTC6	no	–	–	–	–
	3BTC7	no	–	–	–	–
	3BTC8	no	–	–	–	–
	3BTC9	no	–	–	–	–
	3BTC10	no	–	–	–	–

Table 3.3.5 Yeasts presence in bark samples collected from the conventional and organic vineyard (rows L and C) six months after spraying.

3.3.4.3 Colonization one year after inoculation

In April 2014, 20 bark samples were collected from the organic and the conventional vineyards. The samples were collected from the sprayed rows L (conventional vineyard 4CTL1-10, organic vineyard 4BTL1-10) and from the untreated rows C (conventional vineyard 4CTC1-10, organic vineyard 4BTC1-10). The bark samples were treated as described above.

Yeasts present in bark samples 4CTL3, 4CTL5, 4CTL6, 4CTL7, 4CTL8, 4CTL9, 4CTL10 (6/10), collected from the sprayed row L of conventional vineyard, fermented rather vigorously, which could indicate the presence of *S. cerevisiae* (Figure 3.3.11).

The strain Y3.1, already identified in the previous sampling, was again present. The samples where this strain was found were the following: 4CTL3, 4CTL7, 4CTL9, 4CTL10. One year after spraying in the sample 4CTL5 was present the strain C6.1, which is, as discussed above, a strain isolated in the pre-spraying sampling. In the bark samples 4CTL6 and 4CTL9, two new strains were found (Table 3.3.6). Regarding samples collected in the control row C of the conventional vineyard 4/10 (4CTC3, 4CTC6, 4CTC8 and 4CTC10) showed vigorous fermentations, which could indicate the presence of *S. cerevisiae*. In the samples 4CTC3 and 4CTC10 all the isolates showed the mitDNA profile of the strain T10, already identified in previous sampling. The strain Y3.1 was present in the fermentations of the bark samples 4CTC6 and 4CTC8 where it represented 91% of the isolates while 9% was identified as T10.

Regarding the samples collected from the organic vineyard, no samples showed vigorous fermentations, which could indicate the presence of *S. cerevisiae*.

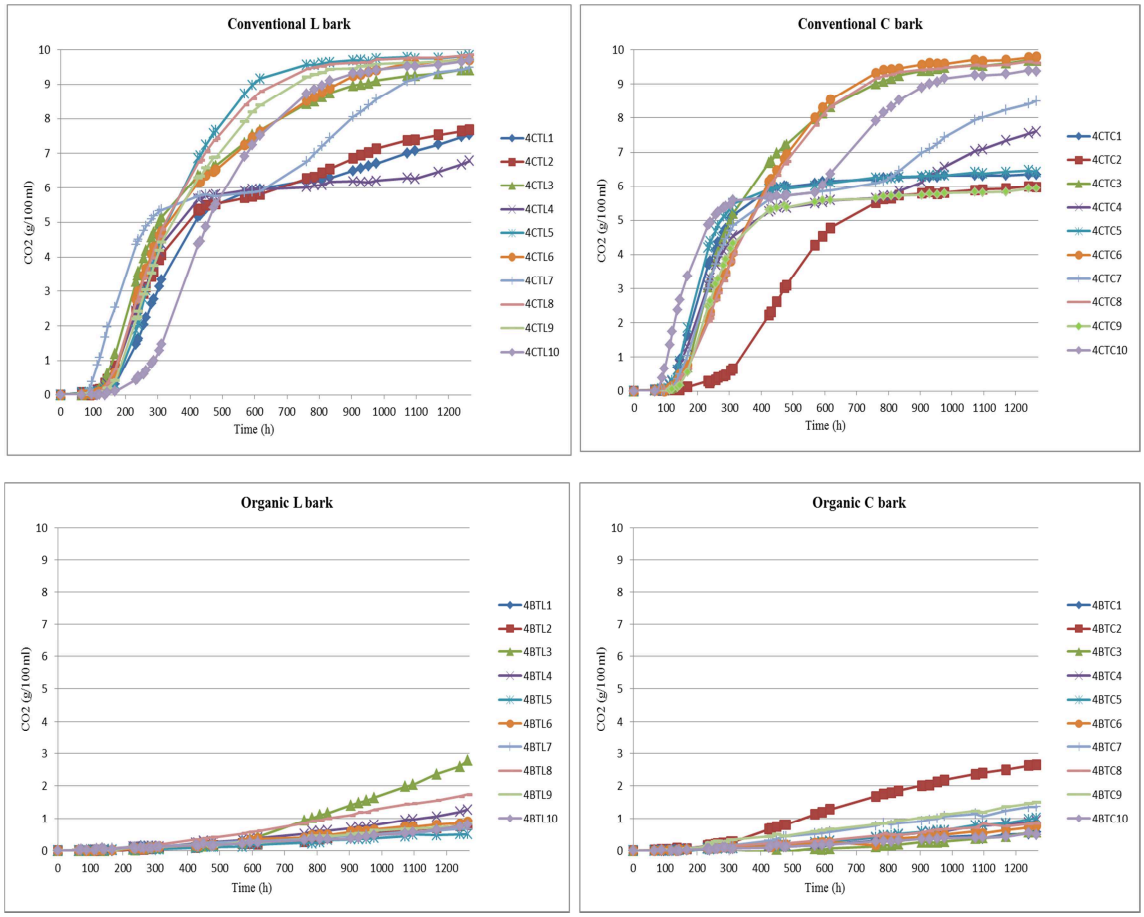


Figure 3.3.11 Fermentation kinetics obtained by inoculating bark samples collected from sprayed (row L) and untreated (row C) vineyards. The sampling was performed one year after the spray.

Vineyard	Bark	Fermentation similar to *S. cerev*isiae	*% S. cerevisiae*	Introduced strains	Genetic profile	Presence
Conventional	4CTL1	no	–	–	–	–
Row L	4CTL2	no	–	–	–	–
	4CTL3	si	82%	no	Y3.1	100%
	4CTL4	no	–	–	–	–
	4CTL5	si	100%	no	C6.1	100%
	4CTL6	si	100%	no	T9	100%
	4CTL7	si	100%	no	Y3.1	100%
	4CTL8	si	100%	no	T10	100%
	4CTL9	si	100%	no	Y3.1	100%
	4CTL10	si	100%	no	Y3.1	100%
Row C	4CTC1	no	–	–	–	–
	4CTC2	no	–	–	–	–
	4CTC3	si	100%	no	T10	100%
	4CTC4	no	–	–	–	–
	4CTC5	no	–	–	–	–
	4CTC6	si	100%	no	Y3.1	100%
	4CTC7	no	–	–	–	–
	4CTC8	si	100%	no no	Y3.1 T10	91% 9%
	4CTC9	no	–	–	–	–
	4CTC10	si	100%	no	T10	100%
Organic	4BTL1	no	–	–	–	–
Row L	4BTL2	no	–	–	–	–
	4BTL3	no	–	–	–	–
	4BTL4	no	–	–	–	–
	4BTL5	no	–	–	–	–
	4BTL6	no	–	–	–	–
	4BTL7	no	–	–	–	–
	4BTL8	no	–	–	–	–
	4BTL9	no	–	–	–	–
	4BTL10	no	–	–	–	–
Row C	4BTC1	no	–	–	–	–
	4BTC2	no	–	–	–	–
	4BTC3	no	–	–	–	–
	4BTC4	no	–	–	–	–
	4BTC5	no	–	–	–	–
	4BTC6	no	–	–	–	–
	4BTC7	no	–	–	–	–
	4BTC8	no	–	–	–	–
	4BTC9	no	–	–	–	–
	4BTC10	no	–	–	–	–

Table 3.3.6 Presence of *S. cerevisiae* in bark samples collected from the conventional and organic vineyard (sprayed vine row L and untreated vine row C) one year after spraying.

The results indicate that in the conventional vineyard there is a greater genetic variability with respect to *S. cerevisiae* strains, including some of the selected strains introduced with the inoculum. In particular we find the presence of one of five inoculated strains, B173.4, which was isolated in two sampling (two and six months after spraying).

In conventional vineyard we observed the presence of new genetic profiles, not identified in the pre-spraying sampling, and the commercial strain DV10.

From organic vineyard, no indigenous strains have been isolated. The only strain belonging to the specie *S. cerevisiae,* isolated two and six months after treatment, was P301.4, one of the five selected strains introduced with the inoculum.

The results suggest that the inoculated strains colonized the vine shoot, but only for short periods. Regarding the numerical difference of fermenting yeasts that was detected in the two vineyards, it could be linked to different ages of the vineyard.

The conventional vineyard is older than the organic one, and has a different shoot structure, which is thicker and more robust. Due to the more complex structure, the old conventional vineyard constitutes a better habitat for yeast than the young organic one.

3.3.5 Vinifications

At harvest about two quintals of grapes were collected, for each row. First, the bunches were removed from the conventional vineyard and after ten days from the organic one. Two spontaneous fermentations, for each vine row, were run at the experimental winery (Veneto Agricultura) in Conegliano. Must chemical analyses was reported in Table 3.3.7.

Regarding conventional vineyard, the grapes coming from the vine row C showed a sugars accumulation higher than those of the row L, but this did not affect the total acidity concentration that was quite similar. The grape harvest from the organic vineyard, showed an average sugar concentration lower than that of organic vineyard and higher values of total acidity. This result indicates a slightly lower degree of ripening of the grapes from the organic vineyard than that of the conventional one.(Table 3.3.7).

Must	Sugars (g/l)	pH	Total acidity (g/l)
Conventional			
L	163	3,32	5,60
C	185	3,52	4,56
Organic			
L	168	3,24	7,7
C	150	3,22	7,8

Table 3.3.7 Chemical analysis of the musts

After crushing, for each fermentation, the must and the skins were placed in steel fermenters of 1 hl capacity, about 70 liters of must were obtained. The following maceration at 20°C lasted 24 hours, afterwards the must was pressed to separate the grape pomace. After 24 hours of decantation, the must was transferred to a new fermenter to separate the lees. The musts were fermented at 20°C, without addition of sulfur dioxide.
Only in the case of the must obtained from grapes harvested from the conventional vineyard, row C, containing 185 g/l of sugar, the fermentation was very slow and lasted 26 days with a sugar residue of 26 g/l. Despite that, ethanol concentration was 9.3% (Table 3.3.8). Even if in the other cases the fermentation times were shorter, the fermentation process was hard suggesting the poor presence of *S. cerevisiae* strain.

Yeast quantification was performed by plate count on WL agar plates when alcohol content was about 6,0% v/v and at the end of fermentation. For each sampling, from the isolation plates 26 randomly chosen colonies with *Saccharomyces*-like morphology, for a total of about 400 colonies, were collected and the mitDNA profile of each isolates was determined.

Must	Sugars (g/l)	Alcohol (%V/V)	pH	Total acidity (g/l)	Days of fermentation
Conventional					
L	1	9,39	3,50	5,5	17
C	26	9,35	3,64	4,6	26
Organic					
L	3	9,35	3,24	7,7	13
C	1	8,96	3,22	7,8	13

Table 3.3.8 Chemical analysis of the wines produced with grapes harvested in the conventional and organic vineyard. The fermentation time is also reported.

Each mitDNA profile was compared, using the software BioNumerics, with those of commercial strains present in the profile collection, with those of the strains previously isolated and with those of the five selected yeasts inoculated in vineyard.

Regarding the conventional vineyard, the genetic analysis of the isolates collected in fermented grapes of the sprayed vine row L evidences the presence of eight strains in total both at 6% ethanol and at the end of fermentation (Figure 3.3.12).

Four strains are commercial yeasts and are present both at 6% ethanol and at the end of fermentation.

The commercial strain mainly present in both fermentations is Fruity Flavour, which represent 61% of the total isolates collected in the first sampling and 42% at the end of fermentation. The percentage of all commercial strains reaches 77% and 69% of the isolates yeasts collected respectively at 6% ethanol and at the end of fermentation.

The remaining strains (4 to 6% ethanol and 4 at the end of fermentation), were isolated only in one of the two sampling times indicating a notable succession of yeasts during fermentation, although the total number is very low. Indeed, researches have detected during spontaneous fermentations the simultaneous presence of up to ten strains (Fleet, 2003).

It is interesting to note that one of the four strains present in the sample collected at 6% ethanol is the selected strain B173.4, also found in vineyard two and six months after inoculation, which accounts for 4% of the present population.

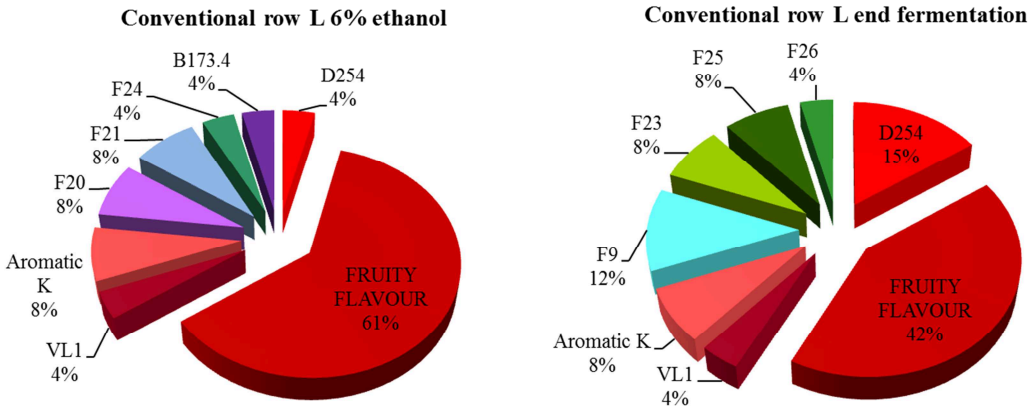


Figure 3.3.12 *S. cerevisiae* strains isolated at 6% ethanol and at the end of fermentation during spontaneous fermentation of grapes harvested in the sprayed vine row L of the conventional vineyard.

The genetic analysis of the isolates collected in fermented grapes of the untreated vine row C evidences a higher genetic variability: 14 and 13 different strains were isolated at 6% ethanol and at the end of fermentation, respectively. A High percentage of commercial strains (43% and 39%, respectively) is found, as in the case of sprayed vine row L. Four of the 5 strains present at 6% ethanol and 6 strains at the end of fermentation shows MitDNA profiles identical to the commercial strains, Fruity Flavour, Aromatik, VL1 and D254, isolated from sprayed grapes, as well.

Nine autochthonous strains were isolated at 6% ethanol and 7 at the end of fermentation, one of which F23 in a higher percentage, also found in the vinification with sprayed grapes. In addition, two strains (F21 and F28), are present both at 6% of ethanol and at the end of fermentation in small percentage. Among these, only the strain F21 was found during the vinification of sprayed grapes.

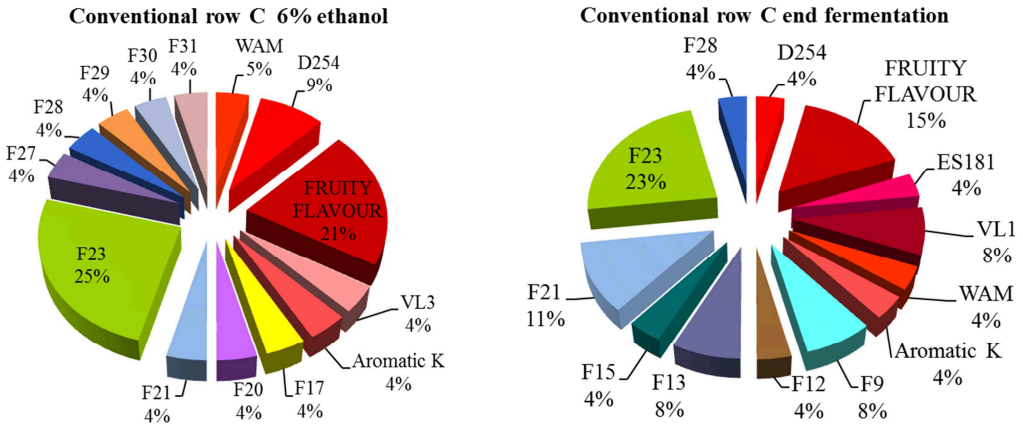


Figure 3.3.13 *S. cerevisiae* strains isolated at 6% ethanol and at the end of fermentation during spontaneous fermentation of grapes harvested in the untreated row C of the conventional vineyard

Surprisingly in inoculated organic vineyard, the level of genetic variability is very similar to what found for the conventional vineyard. the presence of 8 strains in the sample collected at 6% ethanol and 9 strains at the end of fermentation was assessed (Figure 3.3.14). The same commercial strains found in the vinification of conventional grape were found. They constitute 76% and 65% of the total of yeast collected. Also in this case the strain Fruity Flavour was the most present. In samples collected at 6% ethanol and at the end of fermentation were identified three and five indigenous strains, respectively. The strain F18 shows a good colonizing ability as it was isolated both in samples collected at 6% ethanol and at the end of fermentation (16% and 19%).

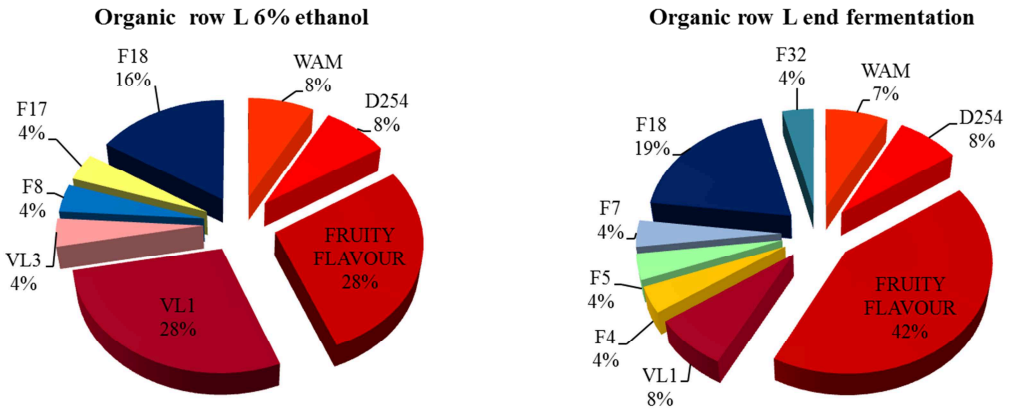


Figure 3.3.14 *S. cerevisiae* strains isolated at 6% ethanol and at the end of fermentation during spontaneous fermentation of grapes harvested in the sprayed row L of the organic vineyard.

The genetic analysis of the isolates collected in fermented grapes of the untreated vine row C evidences an extremely low level of genetic variability. Four strains are commercial yeasts and are present both at 6% of ethanol that at the end of fermentation, accounting for 92% and 73% of the total isolates (Figure 3.3.15). Also in this case the strain mostly present is Fruity Favour followed by ES181, both present in the sample collected at 6% ethanol and at the end of fermentation. This two strains constitute 80% and 54% of the total isolates collected in the sample at 6% ethanol and at the end of fermentation, respectively.

Only one autochthonous strain was isolated at 6% ethanol, constituting 8% of the total isolates, and 2 autochthonous strains at the end of fermentation, consituting 27% of the total isolates. Among them the strain F9 is present in both samples.

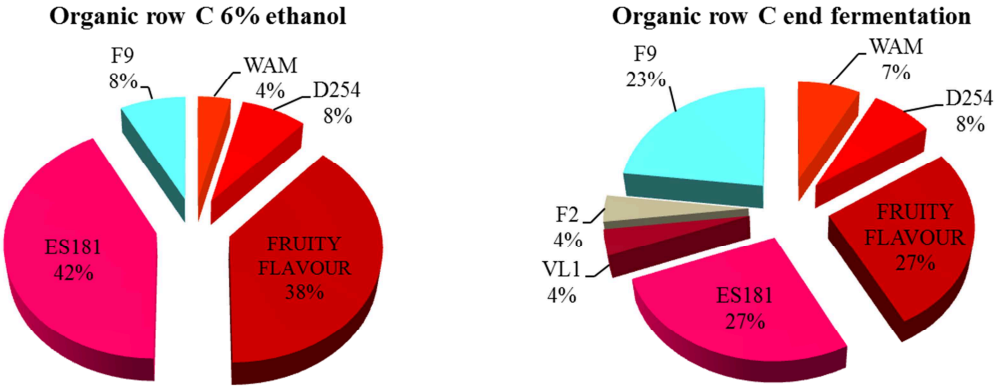


Figure 3.3.15 *S. cerevisiae* strains isolated at 6% ethanol and at the end of fermentation during spontaneous fermentation of grapes harvested in the untreated row C of the organic vineyard.

By comparing all the genetic profiles, two strains F9 and F17 were found to be present in the grape coming from both vineyards. F9 was isolated at the end of fermentation from grapes coming from the conventional vineyard (both sprayed and untreated). F17 instead was found in conventional untreated vineyard and isolated at 6% ethanol, and in biological treated vineyard, at 6% ethanol.

3.3.6 Genetic variability of *S. cerevisiae* strains in the vineyards selected for the second release

The following year, the strains inoculation was repeated using higher cell concentrations (April 2014). In this new trial, to avoid interference from the previous inoculation, a new vine row, named L2, was chosen for the strain inoculation, both in the conventional and organic vineyards. In order to assess the genetic variability of the *S. cerevisiae* population associated with the new rows before strain release, bark sampling in the new vine rows was performed (April 2014).

Therefore ten bark samples were collected from both the organic and conventional vineyards. Samples from conventional vineyard were named 4C2TL1-10, from organic vineyard 4B2TL1-10) and processed as previously described.

Yeasts present in bark samples 4C2TL4, 4C2TL5, 4C2TL6, 4C2TL8 (4/10), collected in the conventional vineyard, fermented rather vigorously, which could indicate the presence of *S. cerevisiae* (Figure 3.3.16).

The following genetic investigations (Table 3.3.9) revealed, among the identified strains, the Y3.1 already isolated in previous sampling. Samples containing showing this mitDNA profile are 4C2TL5 and 4C2TL8. In samples 4C2TL4 and 4C2TL6, two new strains T11 and T12 were found. Regarding the samples collected from the organic vineyard, no samples showed vigorous fermentations, which could indicate the presence of *S. cerevisiae.* The absence of fermenting yeast in the samples collected from the organic vineyard may be due to the vineyard age (the vines are only six year old). Moreover this vineyard was planted in an area where vine growing was not practiced previously, neither wineries are present. The plants in the conventional vineyard are older than those of the organic vineyard. Therefore different structure vine shoots is present. They are greatly thickened and robust. These conditions could be a better *habitat* for the establishment of the yeasts.

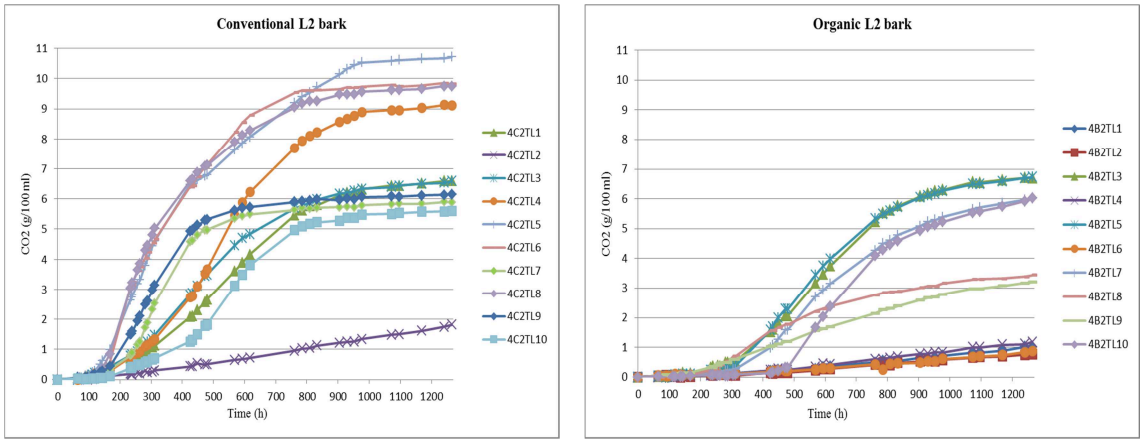


Figure 3.3.16 Fermentation kinetics of bark samples collected in the two vine rows (L2) selected for the second strain inoculum in conventional and organic vineyards. The sampling was conducted before the inoculum of the selected strains.

Vineyard	Bark	Fermentation similar to *S. cerevisiae*	*% S. cerevisiae*	Introduced strains	Genetic profile	Presence
Conventional row L2	4C2TL1	NO	–	–	–	–
	4C2TL2	NO	–	–	–	–
	4C2TL3	NO	–	–	–	–
	4C2TL4	SI	9%	NO	T11	100%
	4C2TL5	SI	100%	NO	Y3.1	100%
	4C2TL6	SI	100%	NO	T12	100%
	4C2TL7	NO	–	–	–	–
	4C2TL8	SI	100%	NO	Y3.1	100%
	4C2TL9	NO	–	–	–	–
	4C2TL10	NO	–	–	–	–
Organic row L2	4B2TL1	NO	–	–	–	–
	4B2TL2	NO	–	–	–	–
	4B2TL3	NO	–	–	–	–
	4B2TL4	NO	–	–	–	–
	4B2TL5	NO	–	–	–	
	4B2TL6	NO	–	–	–	–
	4B2TL7	NO	–	–	–	–
	4B2TL8	NO	–	–	–	–
	4B2TL9	NO	–	–	–	–
	4B2TL10	NO	–	–	–	–

Table 3.3.9 Presence of *S. cerevisiae* in bark samples collected in the two vine rows (L2) selected for the second strain inoculum in conventional and organic vineyards. The sampling was conducted before the inoculum of the selected strains.

3.3.7 Introduction of selected strains in the vineyard (second release)

In April 2014, the inoculum of the five selected yeasts was performed by spraying a 100 fold concentrated microbial culture directly on the main vine shoot of the row L2 of each vineyard.

Each row has been sprayed with 2 x 10^8 cells (each strain) per meter of shoot using a gardening nebulizer (figure 3.3.8).

3.3.8 Evaluation of the colonization ability of the selected yeasts introduced in vineyard

To evaluate the colonization ability of the five yeast strains introduced in the vineyard, barks sampling were collected two and four months after the inoculum. At each sampling 10 bark portions were collected from the sprayed vine row (L) treated the previous year, from the sprayed vine row (L2) just treated and 10 from the control row (C), in both vineyards. Finally grapes were harvested and spontaneous fermentations were run. During the fermentation process the must was sampled when ethanol reached 6% to evaluate strain composition.

3.3.8.1 Colonization two months after inoculation

In June 2014 bark samples were collected and named as follows: those from conventional vineyard, row L2, 5C2TL1-10, row L, 5CTL1-10, row C, 5CTC1-10; those from organic vineyard, row L2, 5B2TL1-10, row L 5, BTL1-10, row C, 5BTC1-10. The bark samples were processed as previously described.

Yeasts present in bark samples 5C2TL6, 5C2TL1, 5C2TL3, 5C2TL2 (4/10), collected in the conventional vineyard from vine row L2, fermented rather vigorously, which could indicate the presence of *S. cerevisiae* (Figure 3.3.17). The following genetic analysis (Table 3.3.10) revealed that all these samples containe the same yeast strain, never isolated before (T18).

Regarding the vine row sprayed the year before L, the samples 5CTL7, 5CTL10, 5CTL2, 5CTL8, 5CTL3, 5CTL4 and 5CTL1 (7/10) fermented rather vigorously, which could indicate the presence of *S. cerevisiae* (Figure 3.3.17). The following genetic investigations (Table 3.3.10) revealed that in three samples (5CTL1, 5CTL8 and 5CTL10) the strain Y3.1 is present; in the sample 5CTL4 the strain T10 was isolated. This yeast, as Y3.1, was previously isolated; in the sample 5CTL7 a new strain (T16) was found. In the remaining two samples no colonies are belonging to the genus *Saccharomyces*.

In the control vine row (C), only one sample (5CTC7) fermented vigorously and allowed the isolation of a new strain (T17).

Regarding the samples collected from the organic vineyard, no samples showed vigorous fermentations, which could indicate the presence of *S. cerevisiae.*

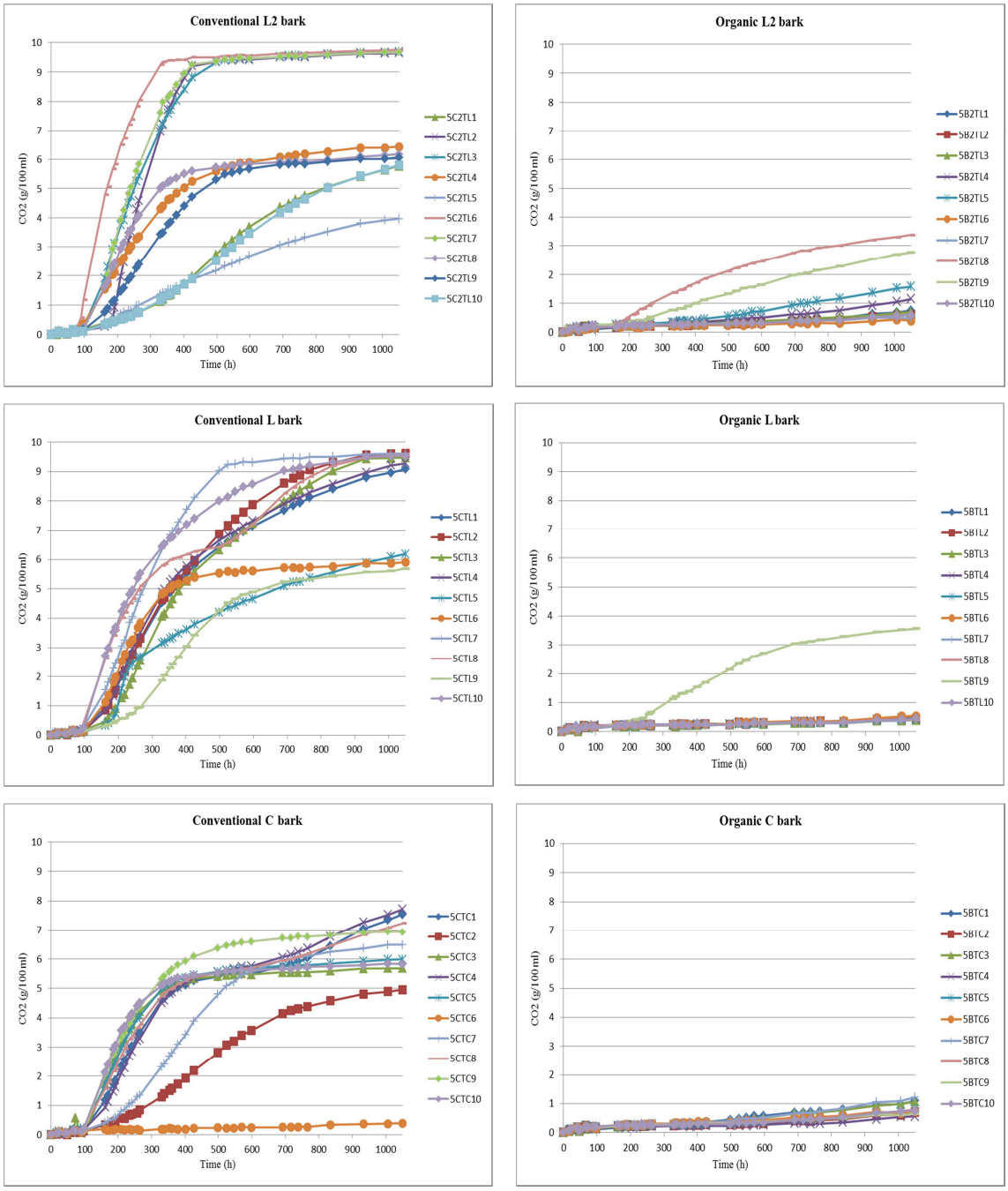


Figure 3.3.17 Fermentation kinetics obtained by inoculating bark samples collected from sprayed (row L2 and L) and untreated (row C) vineyards. The sampling was performed two months after spraying.

Vineyard	Bark	Fermentation similar to *S. cerevisiae*	*% S. cerevisiae*	Introduced strains	Genetic profile	Presence
Conventional row L	5CTL1	si	91%	no	Y3.1	100%
	5CTL2	si	–	–	–	–
	5CTL3	si	–	–	–	–
	5CTL4	si	100%	no	T10	
	5CTL5	no	–	–	–	–
	5CTL6	no	–	–	–	–
	5CTL7	si	90%	no	T16	100%
	5CTL8	si	91%	no	Y3.1	100%
	5CTL9	no	–	–	–	–
	5CTL10	si	100%	no	Y3.1	100%
Conventional row C	5CTC1	no	–	–	–	–
	5CTC2	no	–	–	–	–
	5CTC3	no	–	–	–	–
	5CTC4	no	–	–	–	–
	5CTC5	no	–	–	–	–
	5CTC6	no	–	–	–	–
	5CTC7	si	9%		T17	100%
	5CTC8	no	–	–	–	–
	5CTC9	no	–	–	–	–
	5CTC10	no	–	–	–	–
Conventional row L2	5C2TL1	no	–	–	–	–
	5C2TL2	si	100%	no	T18	100%
	5C2TL3	si	100%	no	T18	100%
	5C2TL4	no	–	–	–	–
	5C2TL5	no	–	–	–	–
	5C2TL6	si	100%	no	T18	100%
	5C2TL7	si	100%	no	T18	100%
	5C2TL8	no	–	–	–	–
	5C2TL9	no	–	–	–	–
	5C2TL10	no	–	–	–	–

Table 3.3.10 Yeasts presence in bark samples collected from the conventional and organic vineyard (rows L2, L and C) two months after spraying.

3.3.8.2 Colonization four months after inoculation

In August 2014, a further bark sampling was performed. Bark samples were named as follows: those from conventional vineyard, row L2 5C2TL1-10, row L 6CTL1-10, row C 6CTC1-10; those from organic vineyard, row L2, 6B2TL1-10, row L, 65BTL1-10, row C 6BTC1-10. In the conventional vineyard yeasts present in bark samples collected from row L2, 6C2TL8, from row L, 6CTL2 and 6CTL9, and from row C 6CTC1, 6CTC3, 6CTC8 and 6CTC10, fermented rather vigorously, which could indicate the presence of *S. cerevisiae* (Figure 3.3.18).

The following genetic investigations (Table 3.3.11) have revealed the presence of the strain T10 in all the colonies isolated from the sample 6CTC3, and of the strain Y3.1 in the samples 6CTL9, 6CTC8, 6CTC10 and 6C2TL8. Both strains have been already identified in previous samplings.

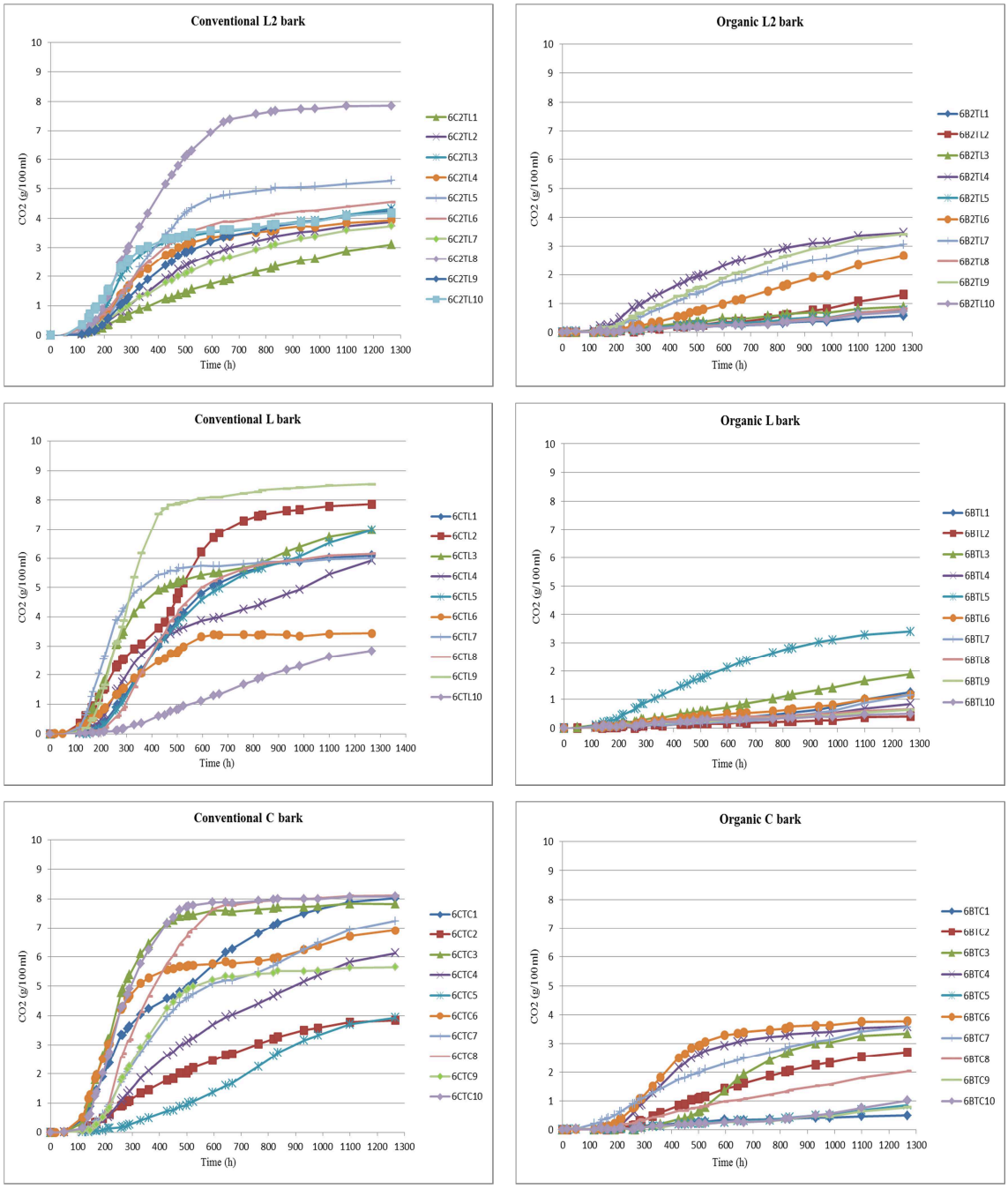


Figure 3.3.18 Fermentation kinetics obtained by inoculating bark samples collected from sprayed (row L2 and L) and untreated (row C) vineyards. The sampling was performed four months after spraying.

Vineyard	Bark	Fermentation similar to *S. cerevisiae*	*% S. cerevisiae*	Introduced strains	Genetic profile	Presence
Conventional row L	6CTL1	no	–	–	–	–
	6CTL2	si	0%	–	–	–
	6CTL3	no	–	–	–	–
	6CTL4	no	–	–	–	–
	6CTL5	no	–	–	–	–
	6CTL6	no	–	–	–	–
	6CTL7	no	–	–	–	–
	6CTL8	no	–	–	–	–
	6CTL9	si	100%	no	Y3.1	100%
	6CTL10	no	–	–	–	–
Conventional row C	6CTC1	si	0%	–	–	–
	6CTC2	no	–	–	–	–
	6CTC3	si	100%	no	T10	100%
	6CTC4	no	–	–	–	–
	6CTC5	no	–	–	–	–
	6CTC6	no	–	–	–	–
	6CTC7	no	–	–	–	–
	6CTC8	si	100%	no	Y3.1	100%
	6CTC9	no	–	–	–	–
	6CTC10	si	100%	no	Y3.1	100%
Conventional row L2	6C2TL1	no	–	–	–	–
	6C2TL2	no	–	–	–	–
	6C2TL3	no	–	–	–	–
	6C2TL4	no	–	–	–	–
	6C2TL5	no	–	–	–	–
	6C2TL6	no	–	–	–	–
	6C2TL7	no	–	–	–	–
	6C2TL8	SI	100%	NO	Y3.1	100%
	6C2TL9	no	–	–	–	–
	6C2TL10	no	–	–	–	–

Table 3.3.11 Yeasts presence in bark samples collected from the conventional and organic vineyard (rows L2, L and C) two months after spraying.

Regarding the samples collected from the organic vineyard, no samples showed vigorous fermentations, which could indicate the presence of *S. cerevisiae.*

The results confirm that in conventional vineyard a higher number of strains, therefore higher genetic variability, is present. Moreover, new genetic profiles, not identified in pre-spraying sampling, were found.

In organic vineyard the presence of *Saccharomyces* strains is confirmed to be very poor.

The results obtained indicate that a higher concentration of the inoculum did not improve the strains ability to colonize the vine rows of the two vineyards. However it is important to emphasize that *Saccharomyces cerevisiae* is not the microorganism usually found in vineyard, although is known to be present. In this environment very different oxidative yeasts are largely present and they seem to impede significantly the establishment of the inoculated strains, despite their vineyard origin.

3.3.9 Vinifications

At harvest about two quintals of grapes were collected, for each row. First, the bunches were removed from the conventional vineyard and after ten thirteen days from the organic one. No vinivication was run using grapes from vine row L, inoculated the previous year. In order to avoid contamination of commercial yeast used in cellar, the vinifications were performed in a warehouse located far from the wineries, provided by the consortium of Prosecco di Conegliano Valdobbiadene DOCG and the pressing was made manually. After crushing must and skins were placed in one hectoliter-capacity steel fermenters containing about 70 liters of must.

Grapes collected from each row were used to conduct two spontaneous fermentations, whose one in the presence of 50 mg/l of SO_2. Must chemical analyses was reported in Table 3.3.12.

In relation to conventional vineyard, the grapes collected from vine row C show an higher sugar accumulation than those of the row L2. In organic vineyard the two rows present similar sugar concentration. In both cases, grapes did not reached full maturity, due

to the unfavorable weather conditions, as confirmed by the high values of total acidity observed.

Must	Sugars (g/l)	pH	Total acidity (g/l)
Conventional			
L2	134	3,15	8,60
C	159	3,14	7,80
Organic			
L2	148	3,27	9,48
C	151	3,24	9,43

Table 3.3.12 Chemical analysis of the musts obtained with grapes harvested in the conventional and organic vineyard.

In all fermentations, yeasts were able to consume all the sugars present in the must (Table 3.3.13), producing 8.40 to 9.11% ethanol. During fermentation the decrease of reducing sugars was monitored. As expected, in musts added with sulfur dioxide the fermentation started late compared to that without sulphite. When grapes from conventional vineyard were used, in must without SO_2 the fermentation was closed within 9 days, while in must added with sulfur dioxide the fermentation was completed in 12 days. Due to lower sugar concentration, in the musts obtained with grapes from organic vineyard the fermentation closed within 8 days.

Must	Sugars (g/l)	Alcohol (%V/V)	pH	Total acidity (g/l)	Days of fermentation
Conventional					
L2	1,58	8,40	3,16	8,55	9
L2 + SO_2	1,00	8,50	3,17	8,60	12
C	4,56	9,03	3,15	7,80	9
C + SO_2	3,73	9,11	3,16	7,75	12
Organic					
L2	1,57	8,50	3,27	9,50	8
L2 + SO_2	1,49	8,70	3,26	9,47	8
C	1,59	8,90	3,24	9,45	8
C + SO_2	1,58	9,10	3,25	9,42	8

Table 3.3.13 Chemical analysis of the wines produced with grapes harvested in the conventional and organic vineyard. The fermentation time is also reported.

Yeast quantification was performed by plate count on WL agar plates when alcohol content was about 6,0%. For each sampling, from the isolation plates 30 randomly chosen colonies with *Saccharomyces*-like morphology, for a total of about 240 colonies, were collected and the mitDNA profile of each isolates was determined.

Each mitDNA profile was compared, using the software BioNumerics, with those of commercial strains present in the profile collection, with those of the strains previously isolated and with those of the five selected yeasts inoculated in vineyard.

Regarding conventional vineyard, in the wine made with grapes coming from the inoculated vine row L2, the genetic analysis show the presence of eight strains when no sulphite was added, and five in presence of 50 mg/l of SO_2. Four strains are commercial yeasts and are present both at 6% ethanol and at the end of fermentation.

As previously found, commercial strain are abundantly present (Figure 3.3.19). In the fermenting must without sulphite five commercial yeasts were isolated, which constitute 77% of the analyzed isolates. They are ES181, VL1, Fruity Flavour and Cru 211. The most present commercial strain accounts for 40% of the isolates. In the presence of sulfur dioxide the commercial strains ES181, VL1 and Wam were identified constituting up to 94% of the total isolates. Among them the yeast Wam reached 77% of the total.

Furthermore, in the fermentation without sulphite addition, three autochthonous strains (23% of the total) were isolated, including the strain P14 that has been isolated also in presence of SO_2. In this condition an additional autochthonous strain, which constitutes the 6% of the total, was isolated. All the autochthonous strains isolated from must fermentations, although their number is very limited, have never been found previously in vineyard on vine shoots. Data in the literature indicate, during spontaneous fermentation, the simultaneous presence of dozens of different strains (Fleet, 2003). The results, apparently in contrast with that reported in the literature, may be due to the large presence of commercial yeasts, very competitive in grape must and therefore able to dominate the fermentation process. In fact, their excellent technological features could have allowed these yeasts to impose on the autochthonous *S. cerevisiae* strains less performant in fermentation condition. The presence of commercial strains isolated in wine is certainly due to vineyard contamination as fermentations were carried out in an environment, the warehouse, where vinifications have never been conducted previously.

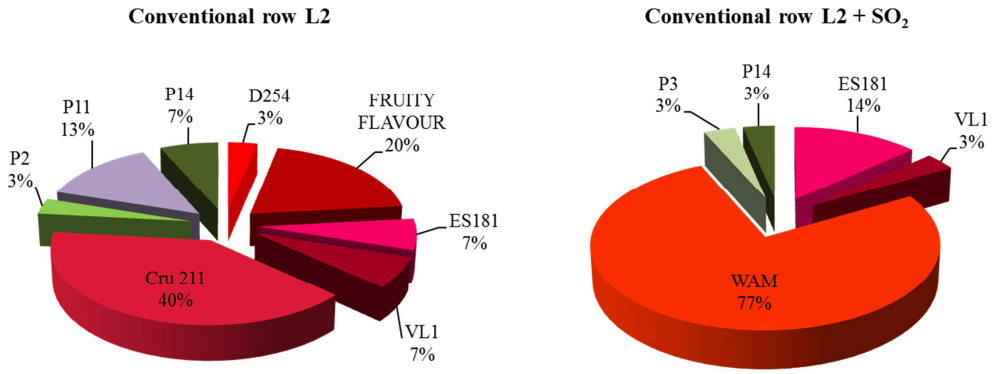


Figure 3.3.19 *S. cerevisiae* strain frequency. Yeasts were isolated at 6% ethanol during spontaneous fermentation of grapes harvested in inoculated row L2 of the conventional vineyard. Vinifications were run with and without SO_2 addition.

Regarding conventional vineyard, in wine made with grapes coming from the control vine row C, where no SO_2 was added, the genetic variability was extremely poor. Only four different strains were isolated. Moreover 87% of the isolates showed a profile identical to that of commercial strains ES181, DV10 and Cru 211. As occurred in wine obtained from the inoculated rowL2, Cru211 is the prevalent (47%). The only autochthonous strain, P11 (13%), was also isolated in fermenting must obtained from inoculated grapes (L2). In the wine with sulphite 9 strains were identified. A larger number of commercial strains were found (6 corresponding to 70% of the total isolates) compared to that in wine without sulphite. Together with the commercial yeasts, three autochthonous strains (30%) were found. Among them P4 was present at a higher percentage then the others and P14 was also found in vinifications with inoculated grapes (L2). The cause of higher genetic variability level, especially as regards the commercial yeasts, observed in the presence of sulphites could be attributed to a lower resistance to sulfur dioxide of the strains that have dominated the wine without sulphite. It is important to note that SO_2 resistance is a technological trait that has been largely used in the past to select wine commercial strains.

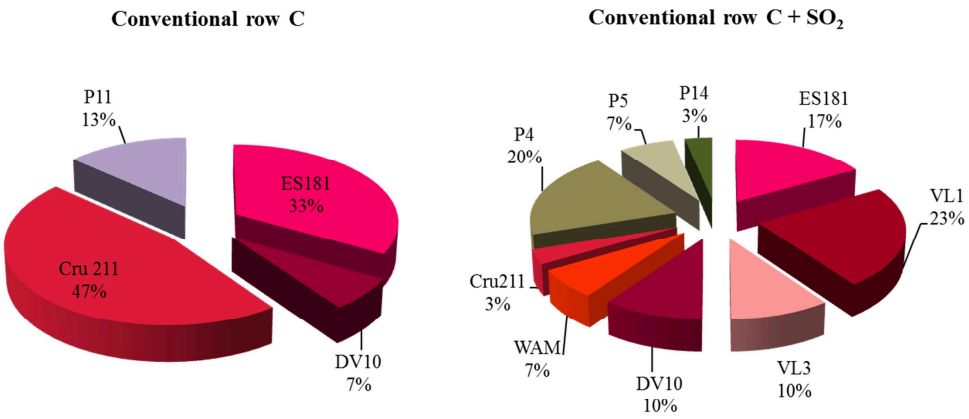


Figure 3.3.20 *S. cerevisiae* strain frequency. Yeasts were isolated at 6% ethanol during spontaneous fermentation of grapes harvested in the control row C of the conventional vineyard. Vinifications were run with and without SO_2 addition.

Surprisingly, in inoculated organic vineyard, the level of genetic variability is very similar to that found in conventional vineyards. The presence of 7 strains was identified in the sample collected from fermenting must without sulfites and 5 strains in sample where SO_2 was not present (Figure 3.3.21). As previously observed, a remarkable presence of commercial strains, most of them already isolated in fermenting must with grapes from conventional vineyard, was found. They constitute 57% and 60% of the total isolate collected from the samples, without and with sulfites, respectively. In this case the strain VL1 was the most present. In the samples collected from the fermentation without sulfites four autochthonous strains were also identified. One of them, B173.4, belongs to the group of the five selected strains introduced in vineyard and constitutes 3% of the total of the isolates. Surprisingly, this strain has never been isolated from bark samples in vineyard. In samples collected from must fermentation in presence of sulphites three autochthonous strains, were identified. Two of them, P14 and P7, also present in the fermentation without sulfites.

In particular, the strain P14 showed good colonizing ability as irrespective of SO_2 presence it was isolated at high percentage (34%).

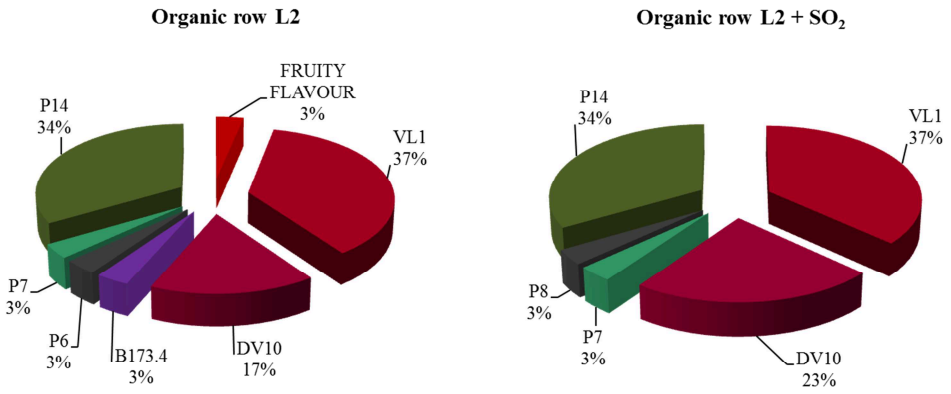


Figure 3.3.21 *S. cerevisiae* strain frequency. Yeasts were isolated at 6% ethanol during spontaneous fermentation of grapes harvested in the sprayed row L2 of the organic vineyard. Vinifications were run with and without SO_2 addition.

In must fermentation with uninoculated grapes (vine row C) 7 and 8 different strains were isolated in with and without sulphites condition, respectively. In the latter condition 6 out of 7 strains are commercial yeast and represent 93% of the total isolates (Figure 3.3.22). They are ES181, VL1, Fruity Flavour, DV10, Wam and Cru 211. The most present strain is VL1 followed by Cru 211. In fermenting must with sulphite added 4 strains (84% of the total) were identified as commercial yeast, ES181, VL3, VL1 and Wam. The latters were present most frequently (37%). The autochthonous isolates constitute only 7% of the total in the fermentation without sulfites. The genetic analysis evidenced the presence of a single strain. When sulphite was added the autochthonous isolates constitute 16% of the total and 4 strains are identified.

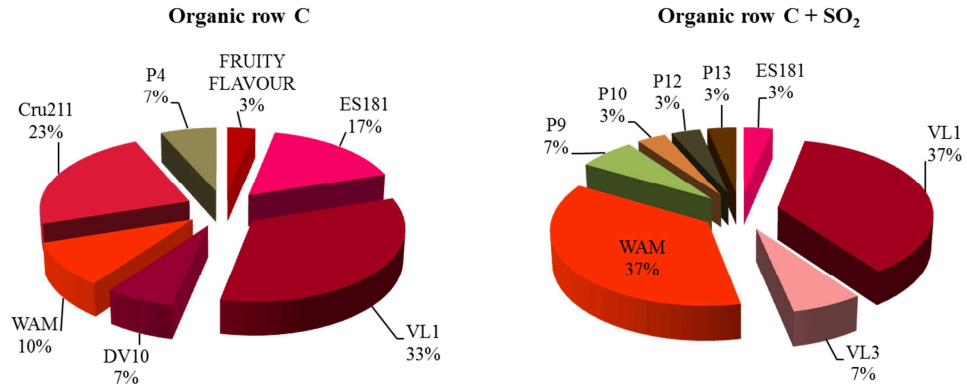


Figure 3.3.22 *S. cerevisiae* strain frequency. Yeasts were isolated at 6% ethanol during spontaneous fermentation of grapes harvested in the uninoculated row C of the organic vineyard. Vinifications were run with and without SO_2 addition.

Comparing all mitDNA profiles obtained two strains, P4 and P14, were identified in vinification of both conventional and organic grapes. Moreover only P4 strain was isolated both on vine shoot (conventional vineyards, row C) and in fermenting must added with SO_2 (organic vineyard, row C).

P14 was isolated in fermenting must obtained from grapes coming both from inoculated organic vineyard and conventional vineyard (sprayed and untreated).

The presence of these two "transversal" strains could suggest that they are commercial yeasts but unfortunately their genetic profiles are not present in the mitDNA database. So their identification is not possible, yet.

3.3.10 Genetic variability associated with two vineyards

The total genetic variability obtained during this experiment is well represented by the dendrogram reported in Figure 3.3.23. By means of analyzing more than 910 isolates collected from vineyard and fermenting must, 60 different genetic profiles, corresponding to as many strains, were found. None of the strains isolated during vinification process was found previously in the vineyard. This result indicates that such environments are driven by diverse selective forces allowing different strains to colonize the vine shoot or the fermenting must. Furthermore no native strain isolated during the first vinification (previous experimentation) was detected in the second. This indicates a significant alternation of strains probably due to seasonal and contingent factors. Finally, looking at the distribution of strains in the dendrogram does not seem to be a relationship between genetic profiles and origin of the strain (vineyard or wine). It is important to emphasize, however, that the analysis method chosen is not relevant from a phylogenetic point of view.

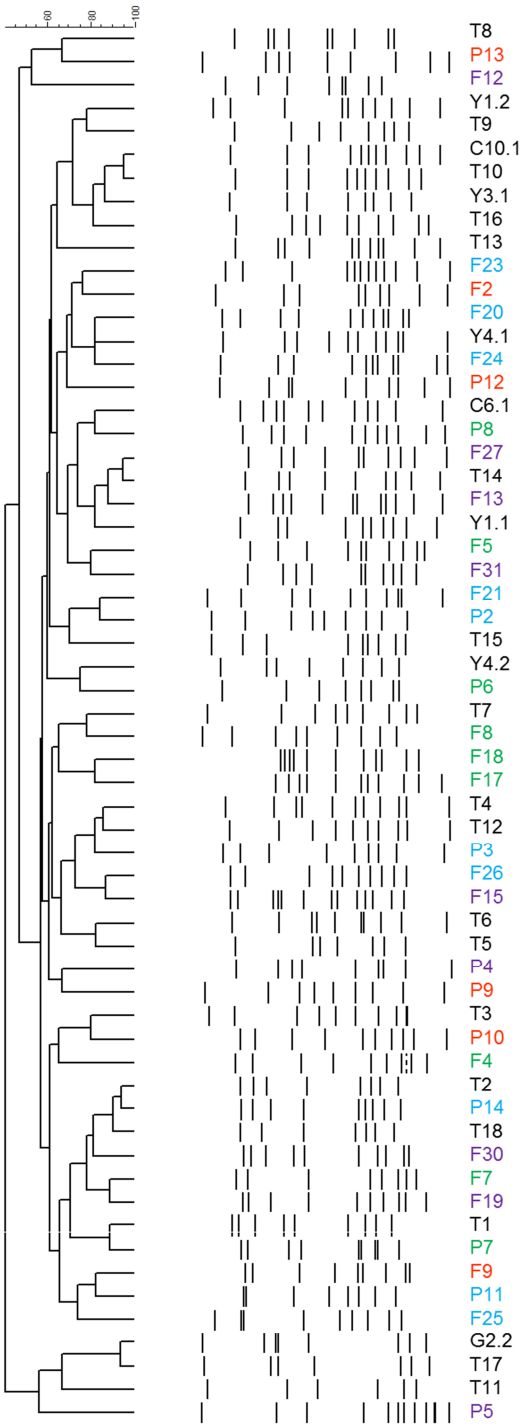


Figura 3.3.23 Dendrogram representing the genetic relationships among the autochthonous strains. Y, C and T indicate isolates from vine shoots of the conventional vineyard; G isolates from grape of conventional vineyard; F isolates from first vinification and P isolates from second vinifications. In addition, the blue color indicates isolates from the inoculated conventional vineyard, violet isolates from the untreated conventional vineyard, green from inoculated organic vineyard and orange from untreated organic vineyard.

3.4 CONCLUSIONS

Regardless of the inoculum concentration the ability to colonize the vineyard environment by selected yeasts introduced was very poor. After the first inoculation, regarding conventional vineyard, only the strain B173.4, was found, although on vine shoot of different plants, in the samples collected both at two and six months after spraying. In organic vineyard the selected strain P301.4, found both after two and six months on vine shoot of different plants, was the only strain of *S. cerevisiae* isolated in vineyard. During the second year in conventional vineyard the inoculated yeasts were not found either on the vine shoots of the row inoculated at low concentration or from the row inoculated 100 times higher concentration. The presence of the introduced strains in the vinification was svery poor, as well. Only the strain B173.4 was isolated during the fermentation of the inoculated grapes of the conventional vineyard during the first year. The same strain was isolated the following year during must fermentations of inoculated grapes of the organic vineyard. The poor colonizing ability shown by strains could be attributed to several causes. Surely the harsh climatic conditions encountered in the last two years (cold and rainy spring, cold summers) may have influenced yeast persistence. However it is important to emphasize that *Saccharomyces cerevisiae* However it is important to emphasize that *Saccharomyces cerevisiae* is not the microorganism usually found in vineyard, although is known to be present. In this environment oxidative, but also fermentative, yeasts are largely present and they seem to impede significantly the establishment of the inoculated strains, despite their vineyard origin.

This interpretation is supported by the data obtained by the plate count of microbial population on vine shoots performed during the first year. In both conventional and organic vineyard a strong presence of yeasts has been detected. In particular in organic vineyard these yeasts are unable to ferment the synthetic must indicating a predominantly oxidative metabolism.

Among the results obtained by this experiment, the absence of S. cerevisiae in the samples coming from the barks and bunches of organic vineyard, was perhaps the most unexpected observation.

In particular fermentation kinetics obtained by inoculating bark fragments in synthetic must indicate that in those samples are absent not only *S. cerevisiae* strains but also other genera

of fermenting yeasts. This could be due to the age of the organic vineyard that could host better oxidative than fermenting species. On the contrary, in the conventional 10-years old vineyard numerous autochthonous strains were collected. In particular Y3.1 and T10 were isolated in both years in different bark samples isolated from inoculated and untreated vine shoots. We can therefore conclude that there are specific strains permanently associated with the conventional vineyard. All the other 23 autochthonous strains isolated are sporadically present with large fluctuations during the two years.

It is also interesting to note the presence of commercial yeasts, in the bark samples collected in vineyard. DV10 and VIN13 are two commercial strains widely used in the vinification of Prosecco. In particular DV10 has a genetic profile shred by a group of yeasts with a long wine history, including EC1118 and QA23.

During vinifications were isolated several commercial strains, as well. They always dominated the fermentations. Among them DV10 was the only the strain that was previously found in vineyard. Many of these have been identified both in the fermentation of the grapes coming from the organic and conventional vineyard indicating the same contamination in vineyard, despite the two vine parcels are within 20km of each other.

Finally, the autochthonous strains and the majority of commercial strains (all but one, DV10) isolated during vinification, have not been found previously in the vineyard. This result indicates that such environments are driven by diverse selective forces allowing different strains to colonize the vine shoot or the fermenting must.

CHAPTER 4

Effect of pairwise strain inoculation on the fermentation activity and the production of secondary compounds

4.1 INTRODUCTION

One of the aims of the modern oenology is to propose new starter cultures based on multiple yeast species or strains in order to increase wine flavour complexity that nowadays is limited, due to the wide use of pure yeast monocultures (Barrajón et al., 2011; Fleet, 2003). Several studies have already described fermentation with mixtures of non-*Saccharomyces* yeasts together with *S. cerevisiae* strains, and mixtures of different strains of *S. cerevisiae*, finding chemical differences in wines (Capece et al., 2013). When using particular yeast cultures to obtain a special character or style in the final product a dominant growth of the inoculate strain(s) is required. The ability of inoculated starter to compete with native yeasts present in the grape must is a fundamental aspect. Only if this condition is guaranteed, the inoculated starter can produce the desired effect into the final product (Capece et al., 2011).

During fermentation a strict yeast-to-yeast interaction occurs, involving an exchange of metabolites, which might affect the succeeding strain metabolic behaviour allowed the production of different profile of volatile aroma substances (Cheraiti et al., 2005). Howell et al. (2006) confirmed these interactions, noting that the profiles of wines made by mixed culture fermentation were different from those produced in monoculture fermentation. It must be highlighted that these differences could not be produced by blending individually-fermented wines.

In this work, the 8 selected strains, previously described, were used to perform pair strain fermentations in order to evaluate the colonization ability of each strain. Changing in the fermentation kinetics and metabolite production were also investigated.

4.2 MATERIALS AND METHODS

4.2.1 Yeast strains

For this study ecotypical strains were used. These yeasts were isolated from the vineyards in the winemaking area of Prosecco Superiore di Conegliano Valdobbiadene DOCG shown in Table 4.2.1

Strain	**Species**	**Origin**
P283.4	*Saccharomyces cerevisiae*	Vineyard
P234.15	*Saccharomyces cerevisiae*	Vineyard
P254.12	*Saccharomyces cerevisiae*	Vineyard
P301.9	*Saccharomyces cerevisiae*	Vineyard
P304.4	*Saccharomyces cerevisiae*	Vineyard
P301.4	*Saccharomyces cerevisiae*	Vineyard
P138.4	*Saccharomyces cerevisiae*	Vineyard
B173.4	*Saccharomyces cerevisiae*	Vineyard

Table 4.2.1 Yeast strains used in this work

4.2.2 Culture media and growth condition

Media

YM solid agar medium

- 3 g L-1 yeast extract (Oxoid);
- 3 g L-1 malt extract (Oxoid);
- 5 g L-1 vegetatone peptone (DIFCO);
- 10 g L-1 glucose (PROLABO)
- 16 g L-1 Bacto Agar (DIFCO).

Adjust to volume with distilled water. Sterilize by autoclaving at 121 ° C for 15 minutes.

YPD (Yeast Extract/Peptone/Dextrose)

10 g L-1 yeast extract (OXOID)

20 g L-1 vegetatone peptone (DIFCO)

20 g L-1 glucose (PROLABO)

Adjust to volume with distilled water. Sterilize by autoclaving at 121 ° C for 15 minutes.

Synthetic nutrient medium (MSN) MS300

Macroelements

200 g glucose

0,155 g $CaCl_2 \cdot 2H_2O$

0,2 g NaCl

0,75 g KH_2PO_4

0,25 g $MgSO_4 \cdot 7H_2O$

0,5 g K_2SO_4

0,46 g $(NH_4)Cl$

6 g malic acid

6 g citric acid

Microelements

4 mg $MnSO_4 \cdot H_2O$

4 mg $ZnSO_4 \cdot 7\ H_2O$

1 mg $CuSO_4 \cdot 5H_2O$

1 mg KI

0,4 mg $CoCl_2$

1 mg H_3BO_3

1 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$

Vitamins

20 mg Myo-inositol

2 mg Nicotinic acid

1,5 mg Calcium Panthotenate
0,25 mg Thiamine hydrochloride
0,25 mg Pyridoxine hydrochloride
0,003 mg Biotin

Amino acids
3,70 g leucine
5,80 g threonine
1,40 g glycine
38,60 g glutamine
11,10 g alanine
3,40 g valine
2,40 g methionine
2,90 g phenylalanine
6,00 g serine
2,50 g histidine
1,30 g lysine
1,00 g cysteine
46,80 g proline
1,40 g tyrosine
13,70 g tryptophan
2,50 g isoleucine
3,40 g aspartic acid
9,20 g glutamic acid
28,60 g arginine

Final pH 3.2
Prepare the amino acids in a 1 litre aqueous solution and use 13,09 ml per litre of must. Dissolve all components in distilled water, adjust the pH with KOH of the resulting solution to pH 3.2.

Solution

Ringer Solution for dilutions (1/4 *strenght*; Dept. of Health & Social Security, 1937). Dissolve one tablet preparation (LAB M, International Diagnostics Group) in 500 ml of deionised water. Sterilize by autoclaving at 121 ° C for 15 minutes.

Growth conditions

The yeast strains were grown at 25°C. The liquid cultures, to be used for fermentation trials, were subjected to orbital shaking (130rpm).

4.2.3 Fermentation trials on synthetic must

4.2.3.1 Inoculum preparation and fermentation setting

The yeasts were grown on YPD agar medium at 25 °C for 3 days. A loopful was collected from the plate and used to inoculate 5 ml of YPD liquid medium. The tubes were incubated for 24 hours at 25°C under orbital shacking until stationary phase (about 10^7 cells/ml) was reached. One milliliter of overnight culture was transferred into 100 ml of YPD liquid medium and incubated 17 hours at 25 °C under orbital shaking (130 rpm).

Cell concentration was measured by flow cytometry. The cultures were diluted in Ringer solution to 1-5 x 10^5 cells/ml. Flow cytometric measurements were carried out using a CyFlwo flow cytometer (Partec) equipped with a DPSS laser (diode-pumped solid-state). Excitation of 20 mW at 488 nm was used to analyze forward scatter (FSC) and side scatter (SSC). Amplification was carried out at logarithmic scales and measurement of the events was triggered by the FSC signal. The data were processed with the software Flowmax (Partec). Cell contentration was measured by microscopy direct count using Thoma counting chamber.

Suitable volumes of culture were inoculated in 100 ml of MSN in order to obtain a concentration of 1 x 10^6 cells/ml in single-strain fermentations and 5 x10^5 cells/ml, for each strain, in co-fermentations. After the inoculum the Erlenmeyer flask were sealed with silicon cap and supplied with a bowed glass pipette.

Samples were collected when alcohol content was about 6,5% and colony isolation was performed on YPD agar plates. Twelve colonies, randomly chose, were submitted to amplification of inter-δ region. At the end of fermentation supernatant was stored at -20 °C

to be used for determination of reducing sugar, acetic acid, succinic acid and glycerol concetrations.

4.2.3.2 Monitoration of the fermentation process

Fermentation process was monitored daily by measuring the weight loss due to CO_2 production. Fermentation process was considered completed when weight loss was lower than 0,1 g within 24 hours.

4.2.4 Amplification of inter-δ region

4.2.4.1 Sample preparation for DNA amplification

Yeast colonies (1–2mm diameter), grown for 1–3 days, were picked up with a sterile toothpick from YPD plates and resuspended in 20 µL of sterile deionized water in 0.5mL tubes. Two microlitres of the suspension were used for PCR amplification.

4.2.4.2 PCR-amplification of delta sequences

The various components of the reaction mixture were used in the following final concentrations:

Table 4.2.2 PCR master mix composition

Delta 12	1 µM
Delta 21	1 µM
dNTPs (Amersham)	200 µM
Taq polimerasi (Promega)	0,02 U/µ
Buffer	1X
DNA	2 µl cellular suspension

Primers utilized are reported below (table 4.2.3).

Table 4.2.3 Primers for interdelta amplification

Name	Length	Sequence (5'-3')	Source
Delta 12	19 nt	TCAACAATGGAATCCCAAC	Legras *et al*.,2003
Delta 21	20 nt	CATCTTAACACCGTATATGA	Legras *et al*.,2003

The thermal protocol was as follows:
initial incubation at 95°C for 4 min to allow cell lysis and DNA denaturation, followed by 35 cycles composed of denaturation at 95°C for 30 s, annealing at 46°C for 30 s and extension at 72°C for 90 s. A final extension step was added at 72°C for 10 min.
Amplified samples were run on 1% agarose gel with 0,1 μg/ml of ethidium bromide. The running was performed with TBE 0,5X (44,5 mM Tris, 44,5 mM boric acid, 1 mM EDTA) at 100 V.
Digital images were acquired with an EDAS290 image capturing system (Kodak, Rochester, NY).

4.2.5 Chemical analysis of fermented must

At the end of each fermentation, the amount of residual glucose, glycerol, succinic acid and acetic was determined by means of HPLC analysis. Components separation was carried out using a Waters 1525 binary HPLC pump with an Aminex ion exclusion column to HPX_87H 300 mm x 7.8 mm. A Waters 2414 Refractive Index Detector was set at 600nm wavelength for the determination of ethanol, glycerol and glucose, while for the detection of the peaks related to organic acids we used a Waters 2487 Dual Absorbance detector set at 210nm wavelength. A calibration has been done for each individual compound and it was used to calculate the corresponding g/L in each sample.

4.2.6 Statistical analysis

The software XLSTAT, vers.7.5.2, was used to performed the analysis of variance (one-way ANOVA), followed by the Tukey "post-hoc" test. The analysis was conducted by comparing the averages of three independent replications and differences were considered statistically significant for p-value less than 0.05. The same software was used to performed Principal Component Analysis (PCA).

4.3 RESULTS AND DISCUSSION

4.3.1 Evaluation of the fermentative performance

In this work the eight vineyard strains of *Saccharomyces cerevisiae* were used to run, in synthetic must MS300, single-strain fermentation and co-fermentation performing, in these cases, pairwise strain inoculates. A total of 8 single-strain fermentation and 28 co-fermentations were run. Each co-fermentation was indicated with a letter as shown in table 4.3.1.

Strains inoculated	**P283.4** Killer						
P234.15 Neutral	S	**P234.15** Neutral					
P254.12 Killer	D	G	**P254.12** Killer				
P301.9 Sensitive	O	L	B	**P301.9** Sensitive			
P304.4 Neutral	BB	T	E	P	**P304.4** Neutral		
P301.4 Sensitive	X	V	A	AA	W	**P301.4** Sensitive	
P138.4 Neutral	H	I	F	M	K	Z	**P138.4** Neutral
B173.4 Killer	Q	U	C	N	R	Y	J

Figure 4.3.1 Co-fermentation plan formulated in this work

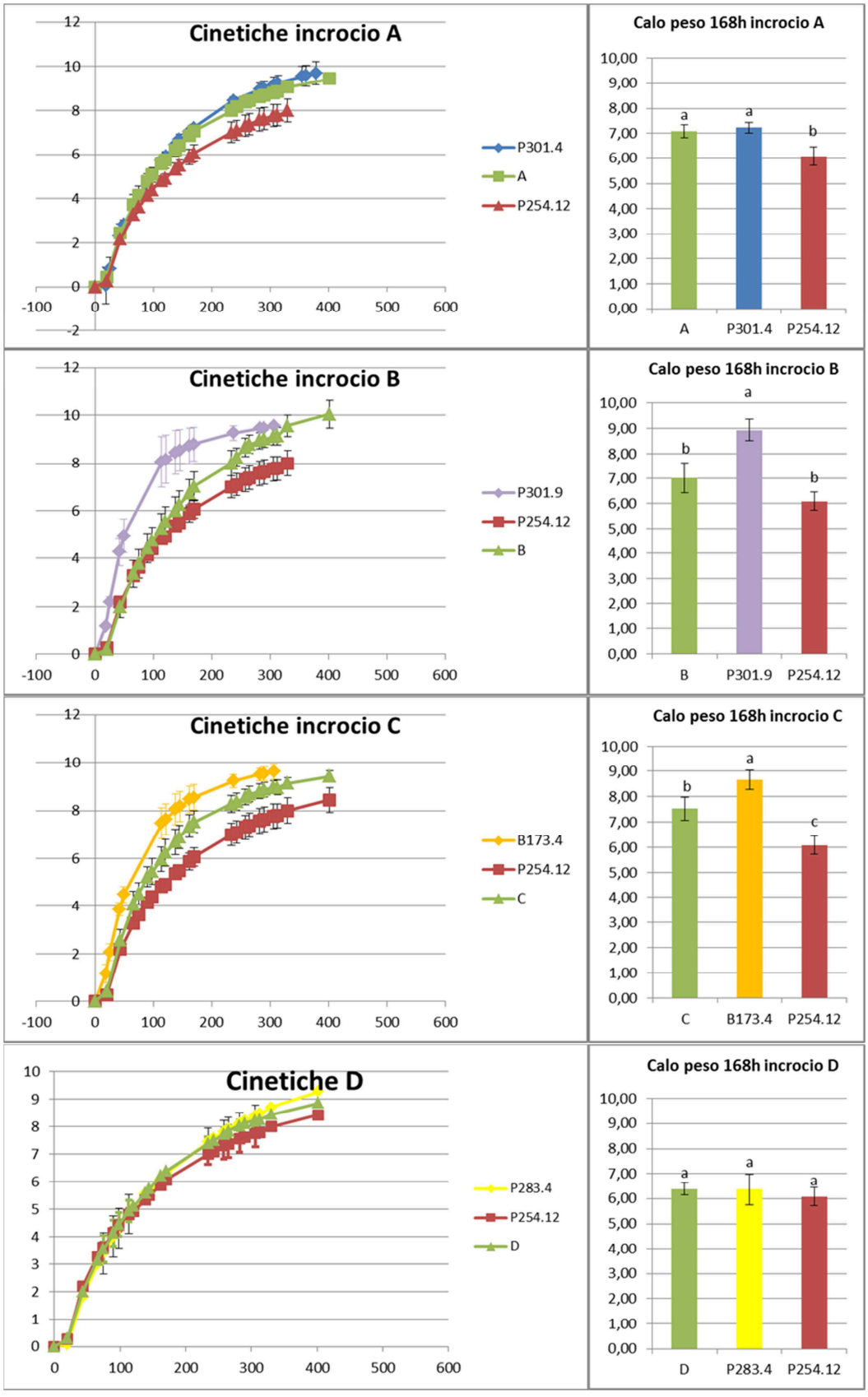
Single or pairwise strain was inoculated in synthetic must MS300 mimicking the enological environment. Each strain was inoculated at a concentration of about 10^6 cells/ml in single-strain fermentations and $5x10^5$ cells/ml in co-fermentation. The advantage to use synthetic must with respect to natural juice for preliminary physiological assessments, is to standardize growth conditions and to facilitate significantly daily growth monitoring operations.

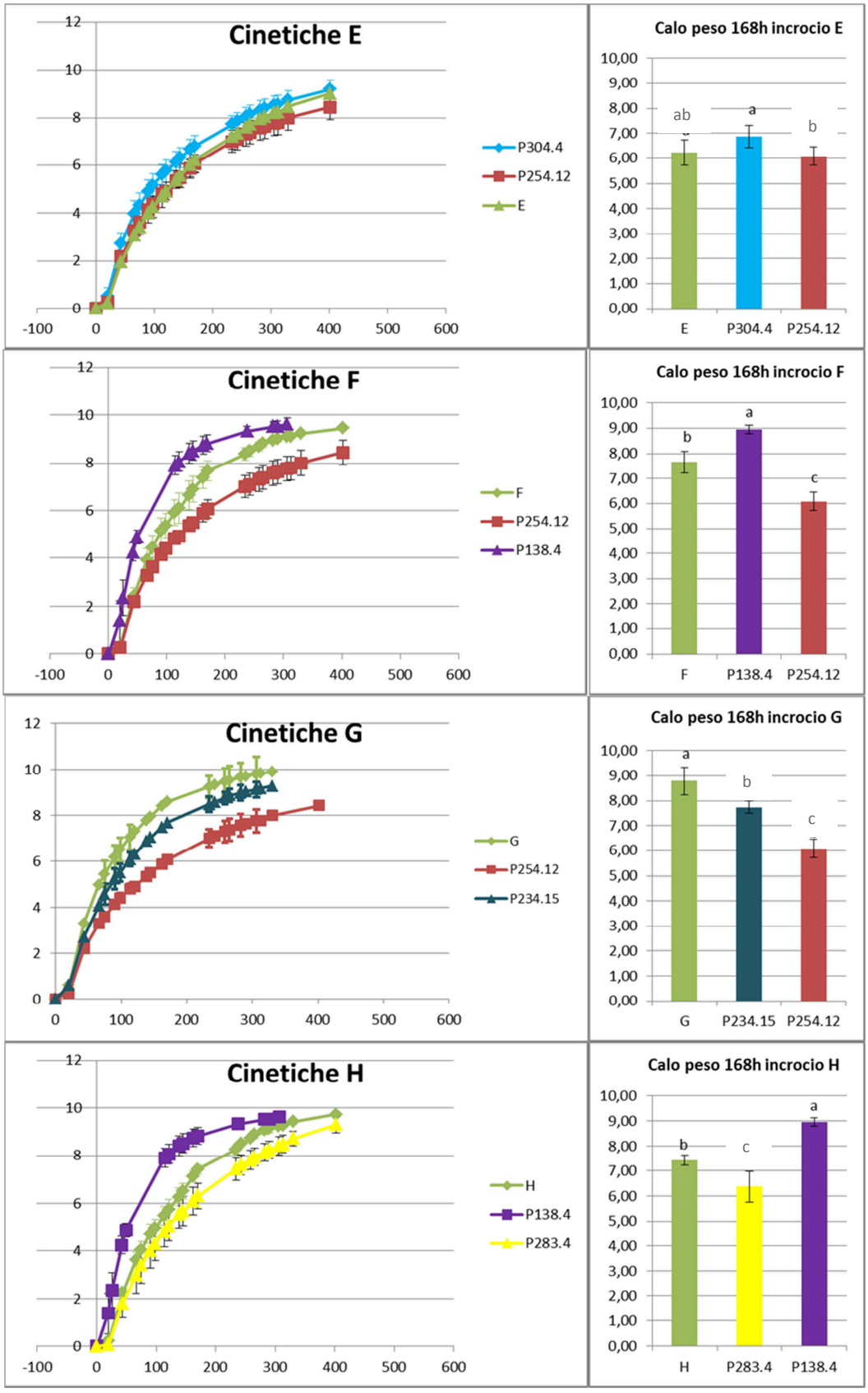
The fermentation trend was followed by daily monitoring of the decrease in weight of the flasks, due to the loss of CO_2 produced in fermentation. Each time, the dynamics of co-fermentations was compared with those of single-strain fermentations, in order to evaluate how the simultaneous presence of both strains can influence the single-strain fermentation performance.

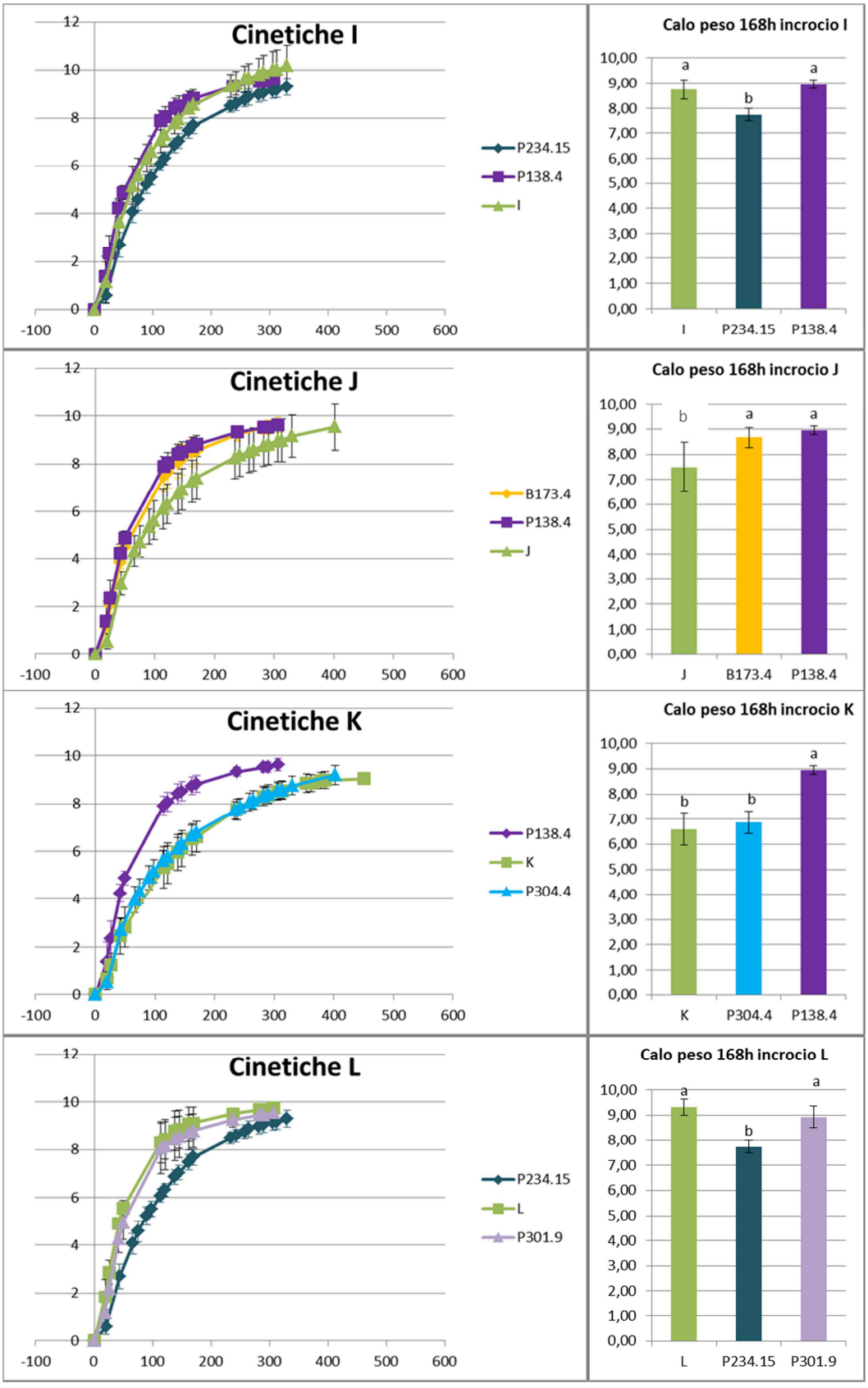
The fermentation kinetics and the decrease in weight (as CO_2 produced) after 168 are reported in Figure 4.3.2. Each trial was performed in triplicate.

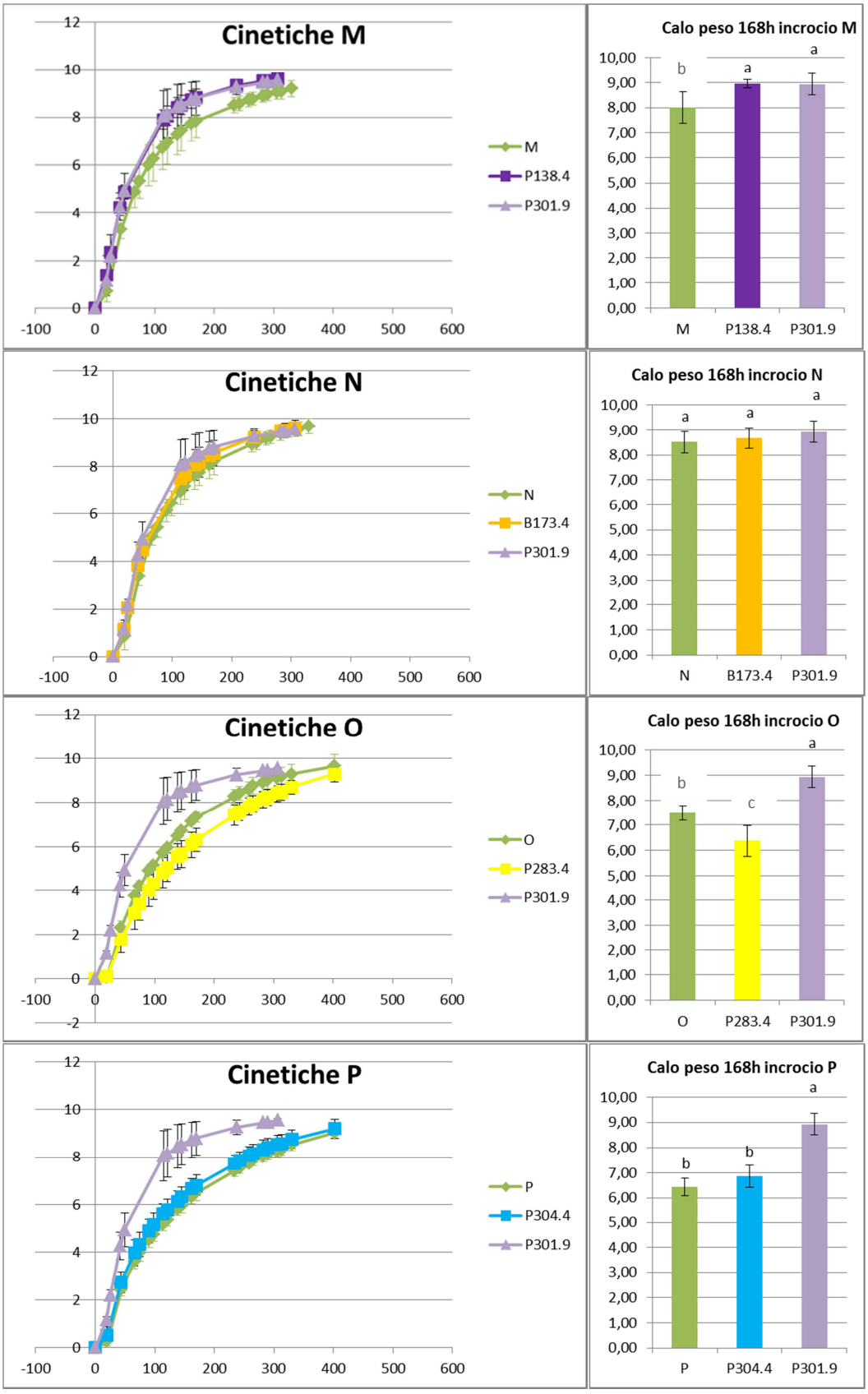
The co-fermentations lasted 15-16 days as the single-strain fermentations showing no differences in fermentation time On the basis of the data collected, the dynamics of the 28 co-fermentation can be divided into five group: thirteen co-fermentation (46,4%) showed similar trend to one of the two single strain-fermentations, 6 (21,4%) showed trend similar to both single-strain fermentations, 6 (21,4%) showed intermediate trend between the two single-strain fermentation; in only one case (P254.12 co-inoculated with P234.15) co-fermentation (3,6% of the total) showed better trend than the two single-strain fermentations and finally only two co-fermentation (7,1%) showed a slower fermentation trend than the two single-strain fermentations: in both co-fermentation strain P304.4 was involved, in one case coupled with P283.4 and in the other with P301.4.

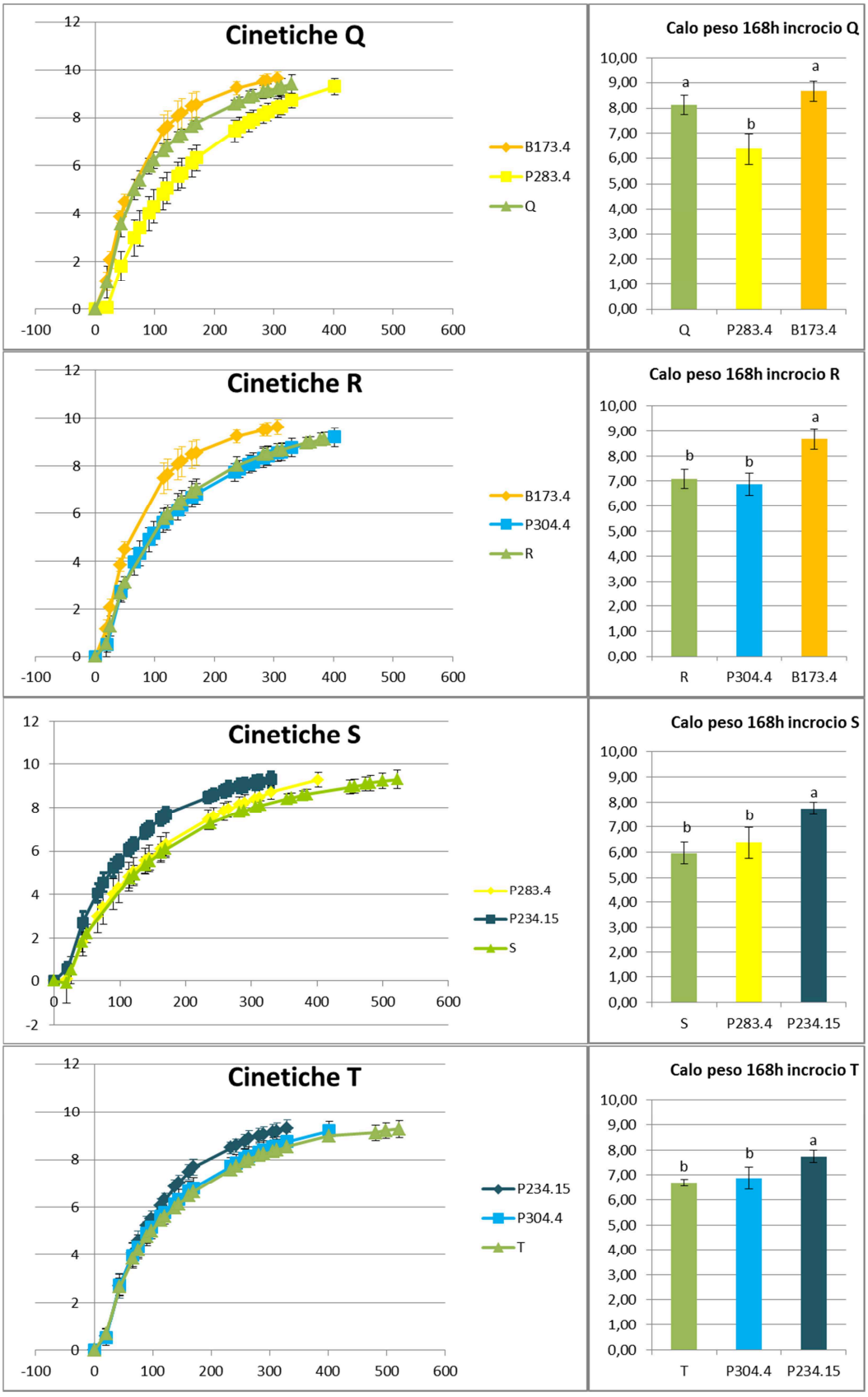
Statistical analysis, performed on the weight loss measurements (CO_2 production) after 168 hours, confirmed, in most of the cases, the previous results. Notwithstanding, co-fermentation B after 7 days shows a weight loss statistically similar to one of the two strains, but the trend is intermediate between the single-strain fermentations. The co-fermentations J and M show trends similar to both the single-strain fermentations, but the weight loss after 168 hours is statistically lower to those of the single-strain fermentations.
The behavior of P304.4 is particularly interesting. This strain is present in both co-fermentations, W and BB, which show a fermentation trend worse than the two single-strain fermentations. P304.4 when inoculated with another strain tend to modify the fermentation trend that become more similar to that of P304.4 single-strain fermentation, except in the case of co-fermentation E where it is inoculated with the strain P254.12. In fact, the strain P254.12 in co-fermentation always improves its fermentation trend, particularly when it is co-inoculated with P234.15 (co-fermentation G).

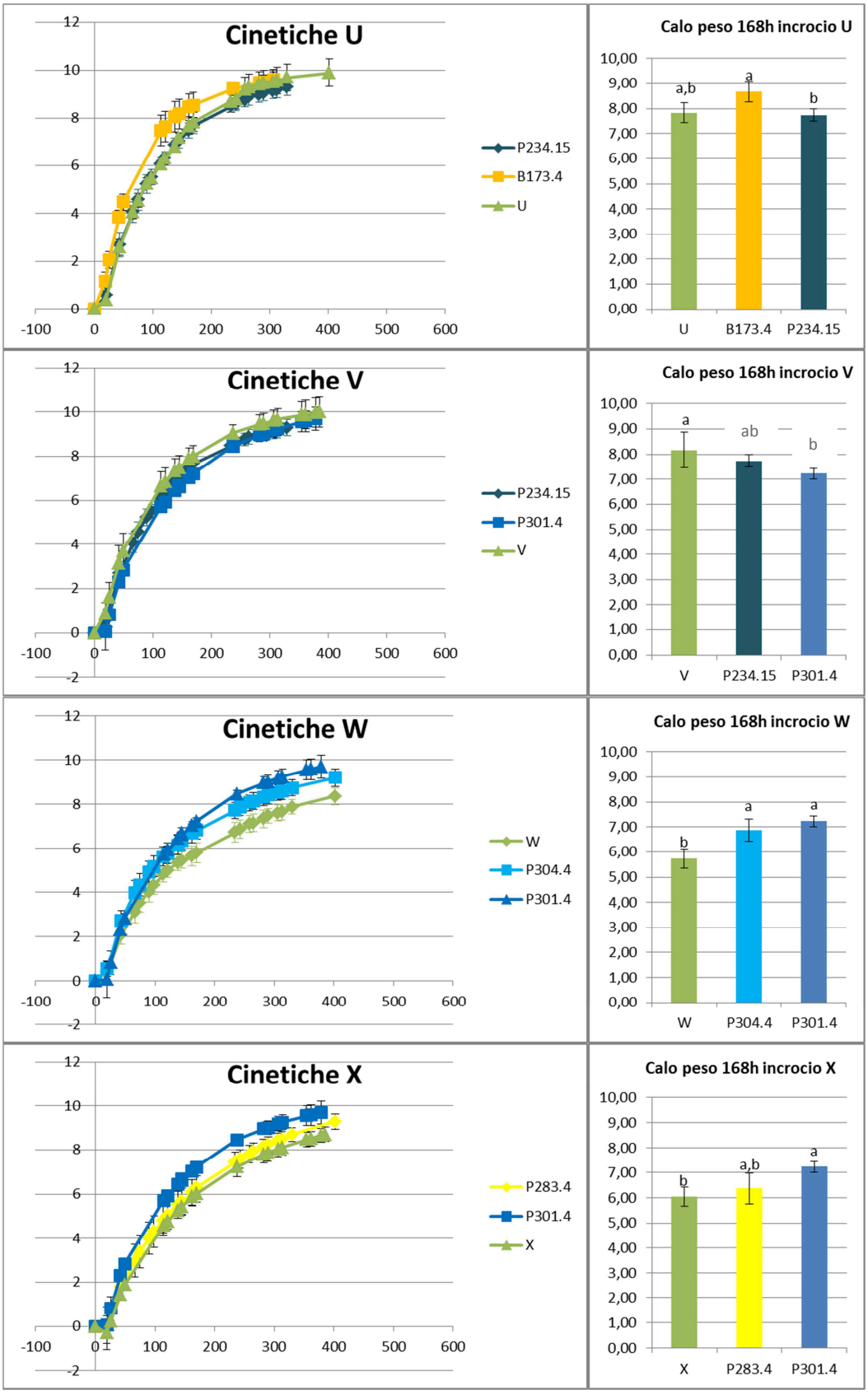

Cinetiche incrocio A
P301.4
A
P254.12
Calo peso 168h incrocio A
a
a
b
A
P301.4
P254.12
Cinetiche incrocio B
P301.9
P254.12
B
Calo peso 168h incrocio B
b
a
b
B
P301.9
P254.12
Cinetiche incrocio C
B173.4
P254.12
C
Calo peso 168h incrocio C
b
a
c
C
B173.4
P254.12
Cinetiche D
P283.4
P254.12
D
Calo peso 168h incrocio D
a
a
a
D
P283.4
P254.12


Cinetiche E
P304.4
P254.12
E
Calo peso 168h incrocio E
ab
a
b
E
P304.4
P254.12
Cinetiche F
F
P254.12
P138.4
Calo peso 168h incrocio F
b
a
c
F
P138.4
P254.12
Cinetiche G
G
P254.12
P234.15
Calo peso 168h incrocio G
a
b
c
G
P234.15
P254.12
Cinetiche H
H
P138.4
P283.4
Calo peso 168h incrocio H
b
c
a
H
P283.4
P138.4


Cinetiche I
P234.15
P138.4
I
Calo peso 168h incrocio I
a
b
a
I
P234.15
P138.4
Cinetiche J
B173.4
P138.4
J
Calo peso 168h incrocio J
b
a
a
J
B173.4
P138.4
Cinetiche K
P138.4
K
P304.4
Calo peso 168h incrocio K
b
b
a
K
P304.4
P138.4
Cinetiche L
P234.15
L
P301.9
Calo peso 168h incrocio L
a
b
a
L
P234.15
P301.9


Cinetiche M
M
P138.4
P301.9
Calo peso 168h incrocio M
b
a
a
M
P138.4
P301.9
Cinetiche N
N
B173.4
P301.9
Calo peso 168h incrocio N
a
a
a
N
B173.4
P301.9
Cinetiche O
O
P283.4
P301.9
Calo peso 168h incrocio O
b
c
a
O
P283.4
P301.9
Cinetiche P
P
P304.4
P301.9
Calo peso 168h incrocio P
b
b
a
P
P304.4
P301.9


Cinetiche Q
B173.4
P283.4
Q
Calo peso 168h incrocio Q
Q
P283.4
B173.4
Cinetiche R
B173.4
P304.4
R
Calo peso 168h incrocio R
R
P304.4
B173.4
Cinetiche S
P283.4
P234.15
S
Calo peso 168h incrocio S
S
P283.4
P234.15
Cinetiche T
P234.15
P304.4
T
Calo peso 168h incrocio T
T
P304.4
P234.15


Cinetiche U
P234.15
B173.4
U
Calo peso 168h incrocio U
a,b
a
b
U
B173.4
P234.15
Cinetiche V
P234.15
P301.4
V
Calo peso 168h incrocio V
a
ab
b
V
P234.15
P301.4
Cinetiche W
W
P304.4
P301.4
Calo peso 168h incrocio W
b
a
a
W
P304.4
P301.4
Cinetiche X
P283.4
P301.4
X
Calo peso 168h incrocio X
b
a,b
a
X
P283.4
P301.4

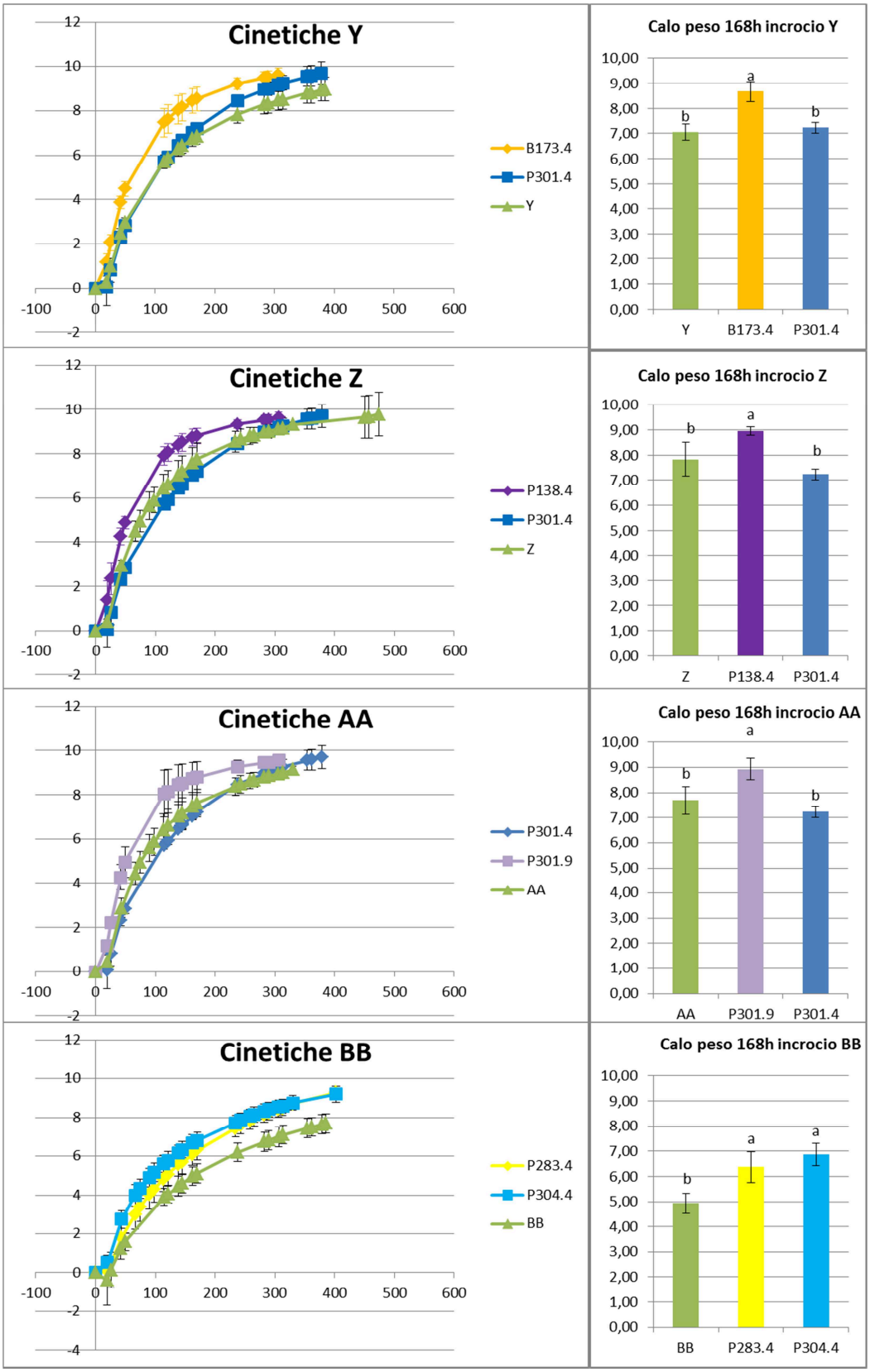


Figure 4.3.2 Fermentation kinetics and CO_2 production (g/100ml of synthetic must) after168 h. Statistical analysis: one-factor ANOVA (p value < 0.05). Different letters indicate statistically significant differences.

4.3.2 Residual glucose and secondary compounds production

4.3.2.1 Residual glucose

The yeast finds in synthetic must the glucose (200 g/l) that they need as carbon and energy source. Survival ability of the yeast during the fermentation process depends on its adaptation ability to higher ethanol concentrations (Ribereau-Gayon *et al.*, 2006). The potential of ethanol production increases with the sugar amount in the must.

For this reason the residual glucose concentration is an index of yeast ability to close the fermentation and to resist to increasing ethanol concentrations.

Regarding single-strain fermentation, residual glucose ranges from 0.38 to 11.72 g/l. As shown in Figure 4.3.3, the fermentations containing the lowest residual glucose were those relating the strains P301.9, P138.4, B173.4 and P301.4, therefore with remarkable ability to survive in increasing ethanol concentrations, while a larger residue was observed for the strain P283.4 and P304.4.

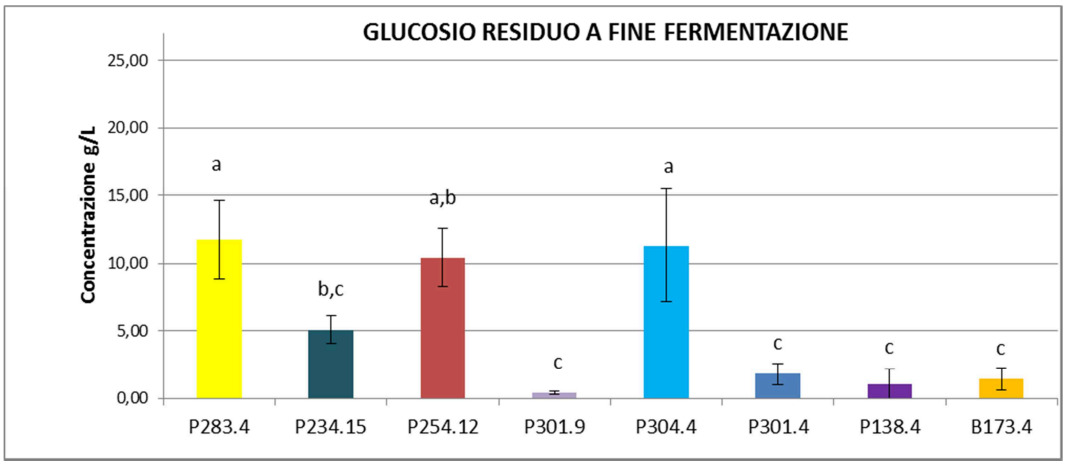


Figure 4.3.3 Residual glucose in single-strain fermentations. Statistical analysis: one-factor ANOVA (p value < 0.05). Different letters indicate statistically significant differences.

The residual glucose concentration found for co-fermentations were compared with those produced by the single-strain fermentations (Figure 4.3.4).

Among the co-fermentations ten and the respective single-strain fermentations show a glucose residual concentration less than 5 g/l, therefore it is possible to affirm that both

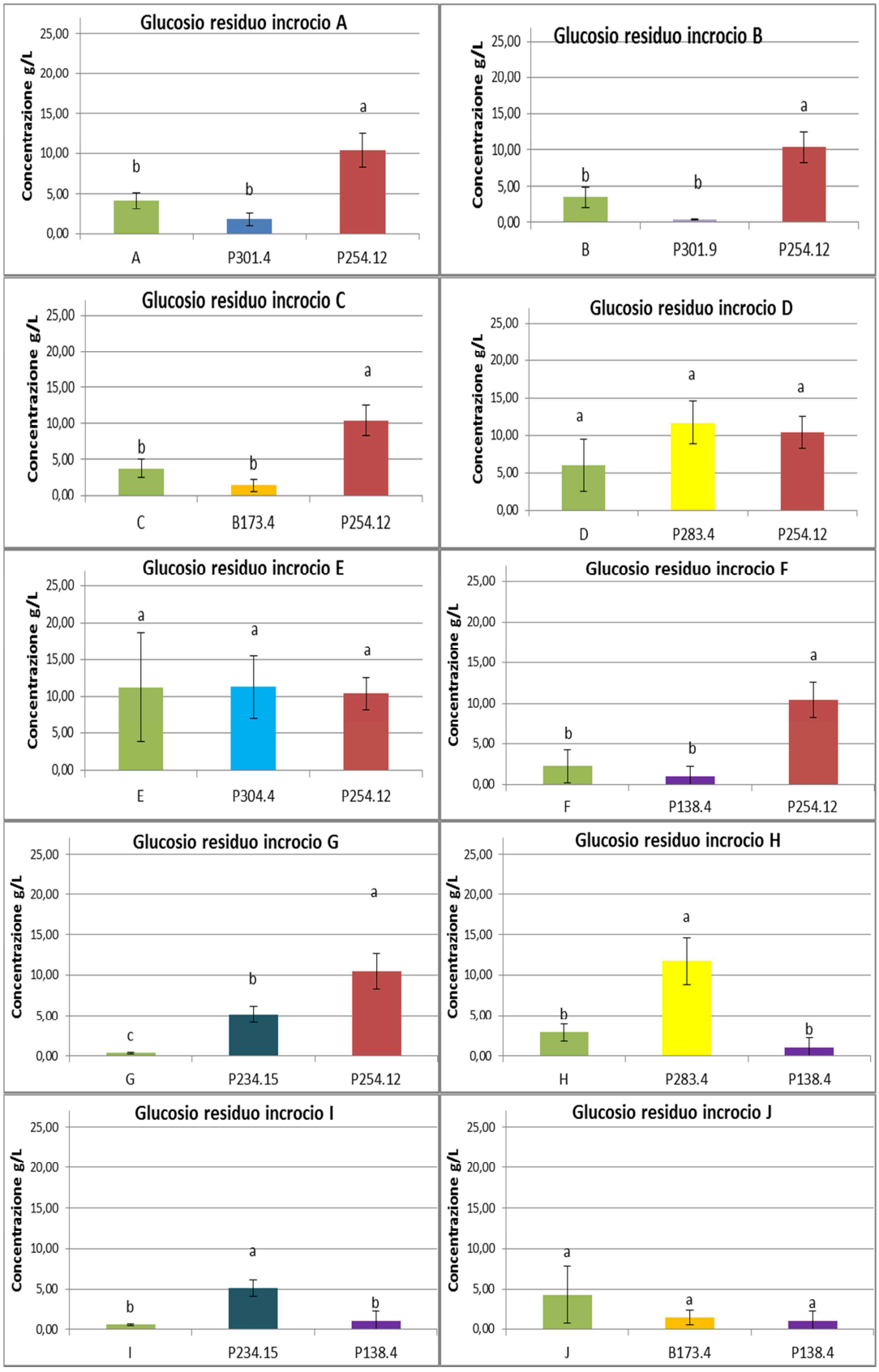
the single strain and the co-fermentation have successfully completed the fermentation process.

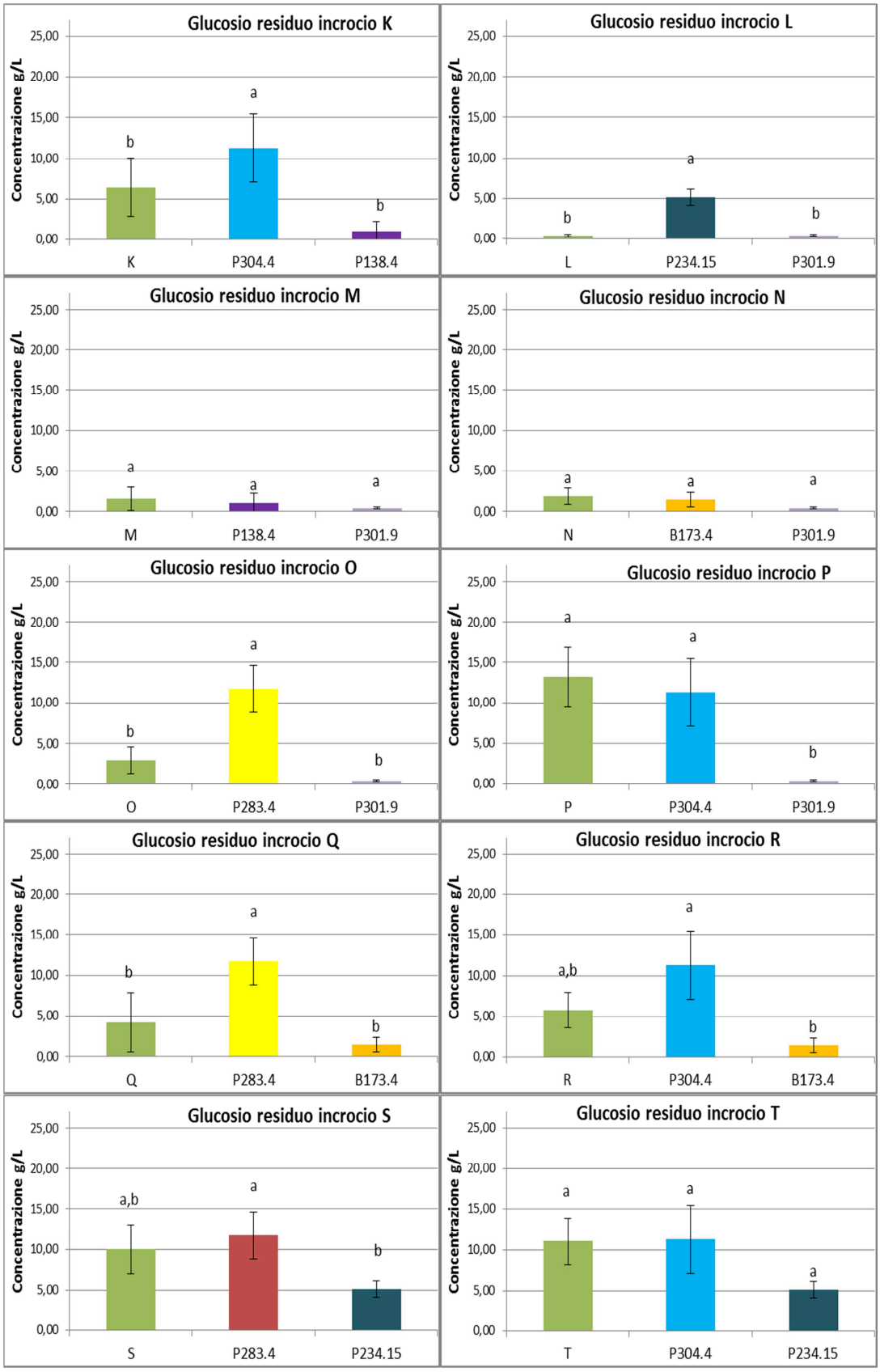
Eleven co-fermentations exhibit a glucose residue similar to one of the two single-strain and, of these, five confirmed the fermentation trend previously discussed, five show an intermediate kinetics, while the co-fermentation W shows worse fermentation kinetics than the strains P304.4 and P301.4.

Five co-fermentations do not have a glucose residue statistically different to that obtained with the corresponding single-strain fermentations. Of these, three show a fermentation kinetics similar of both single-strain fermentation, two show a kinetics similar to one of two the single-strain fermentations.

Interestingly the co-culture G has a residual sugar statistically lower than the P234.15 and P254.12 strains, this data confirms a better fermentation trend than the single-strain fermentations.

Finally, the high residual sugar present in the co-fermentation BB confirms the bad fermentation performance, in fact its kinetics is worse than those obtained with the single-strain fermentations.


Glucosio residuo incrocio A
Concentrazione g/L
25,00
20,00
15,00
10,00
5,00
0,00
b
b
a
A
P301.4
P254.12
Glucosio residuo incrocio B
Concentrazione g/L
b
b
a
B
P301.9
P254.12
Glucosio residuo incrocio C
Concentrazione g/L
b
b
a
C
B173.4
P254.12
Glucosio residuo incrocio D
Concentrazione g/L
a
a
a
D
P283.4
P254.12
Glucosio residuo incrocio E
Concentrazione g/L
a
a
a
E
P304.4
P254.12
Glucosio residuo incrocio F
Concentrazione g/L
b
b
a
F
P138.4
P254.12
Glucosio residuo incrocio G
Concentrazione g/L
c
b
a
G
P234.15
P254.12
Glucosio residuo incrocio H
Concentrazione g/L
b
a
b
H
P283.4
P138.4
Glucosio residuo incrocio I
Concentrazione g/L
b
a
b
I
P234.15
P138.4
Glucosio residuo incrocio J
Concentrazione g/L
a
a
a
J
B173.4
P138.4


Glucosio residuo incrocio K
Concentrazione g/L
25,00
20,00
15,00
10,00
5,00
0,00
b
a
b
K
P304.4
P138.4
Glucosio residuo incrocio L
Concentrazione g/L
b
a
b
L
P234.15
P301.9
Glucosio residuo incrocio M
Concentrazione g/L
a
a
a
M
P138.4
P301.9
Glucosio residuo incrocio N
Concentrazione g/L
a
a
a
N
B173.4
P301.9
Glucosio residuo incrocio O
Concentrazione g/L
b
a
b
O
P283.4
P301.9
Glucosio residuo incrocio P
Concentrazione g/L
a
a
b
P
P304.4
P301.9
Glucosio residuo incrocio Q
Concentrazione g/L
b
a
b
Q
P283.4
B173.4
Glucosio residuo incrocio R
Concentrazione g/L
a,b
a
b
R
P304.4
B173.4
Glucosio residuo incrocio S
Concentrazione g/L
a,b
a
b
S
P283.4
P234.15
Glucosio residuo incrocio T
Concentrazione g/L
a
a
a
T
P304.4
P234.15

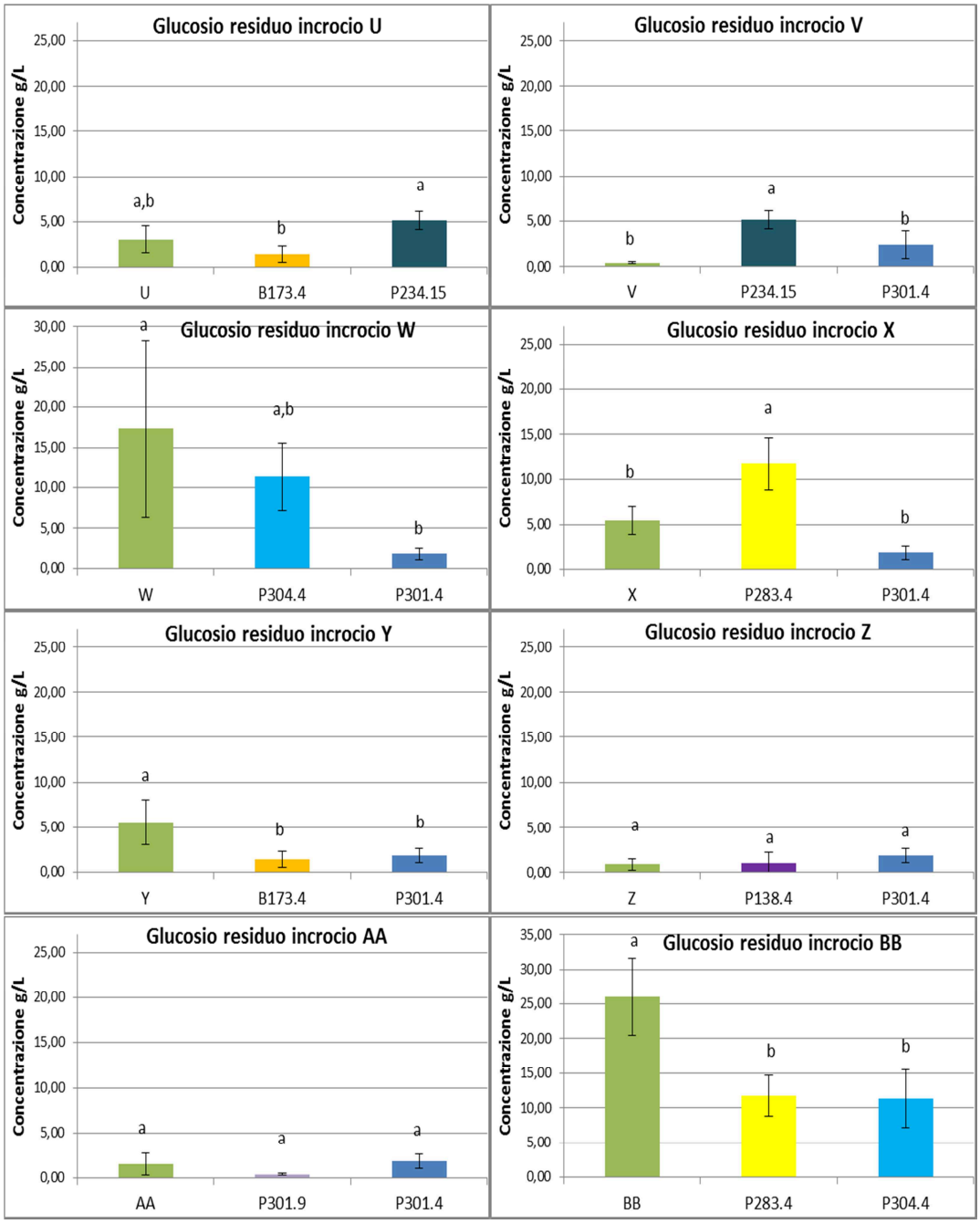


Figure 4.3.4 Residual glucose in single-strain and co-fermentations. Statistical analysis: one-factor ANOVA (p value < 0.05). Different letters indicate statistically significant differences.

4.3.2.2 Glycerol production

Glycerol is the most important chemical compound, produced by yeast in wine after ethanol and it is the most abundant among the alcoholic fermentation secondary compounds. Its content in wine is variable and ranging among 1-12 g/l. A good glycerol production is desirable as it gives structure and roundness to the wine, and plays an important role in defining the flavour and bouquet. Generally *S. cerevisaie* produces wines with high glycerol amount, about 4-8 g/l (Vincenzini et al. 2005). Glycerol is produced by yeasts at the beginning of the fermentation, in response to high sugars concentrations, to survive at the osmotic stress. This compound is produced during the glyceropyruvic fermentation (Ribèreau-Gayon et al., 2007). Therefore a low glycerol production is associated with a low ethanol production.

It is possible to affirm that the strains which produce the largest glycerol concentrations are better adapted to sugar and high osmotic pressures, and for this reason they possess a considerable fermentative vigour even in the presence of high sugar concentrations

The values of glycerol concentration produced at the end of fermentation by single strains, are reported in Figure 4.3.5, and vary between 3.99 and 5.26 g/l. The strains that produced the higher glycerol amount were B173.4 and P234.15 while the lower glycerol concentration was produced by strain P304.4.

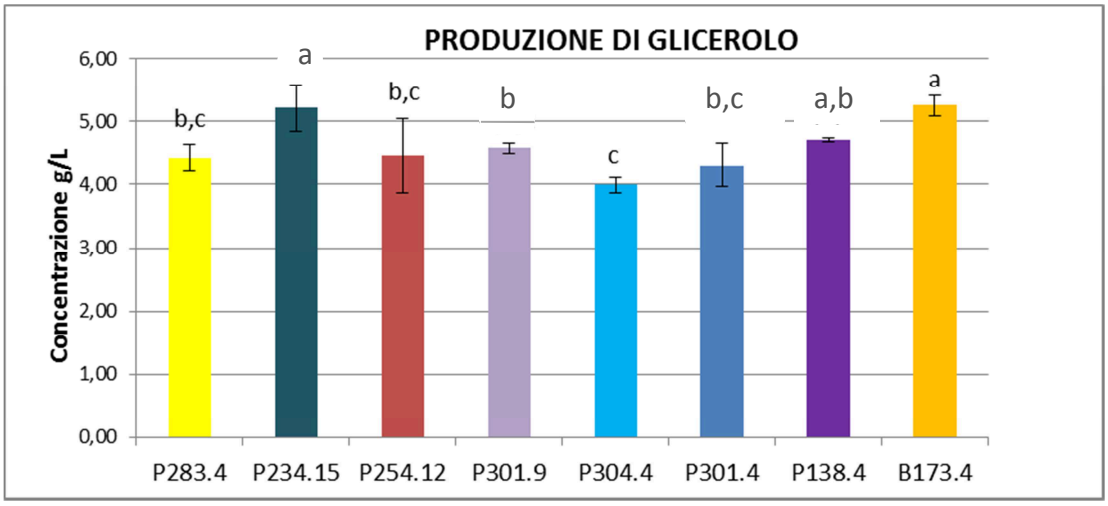
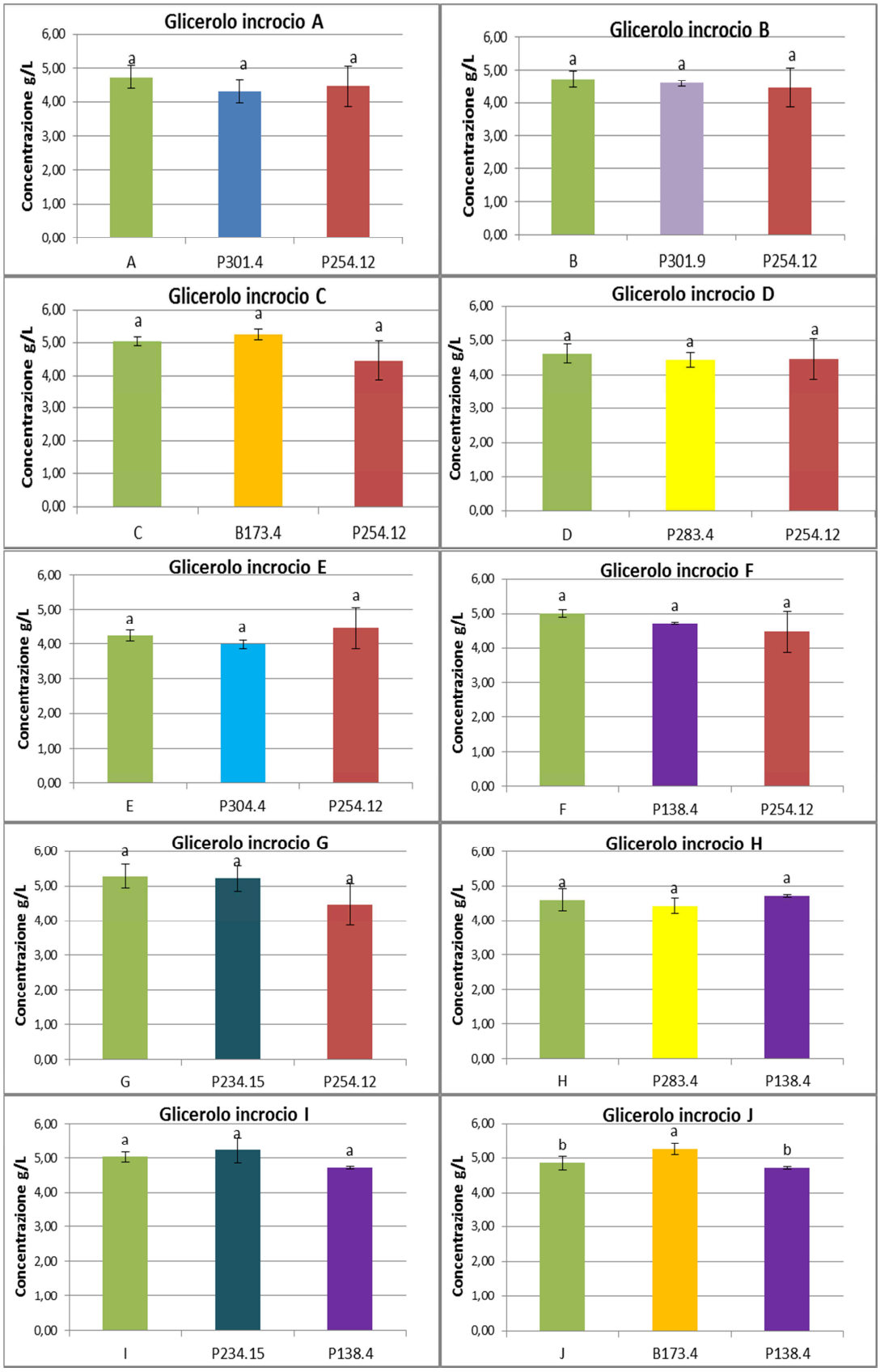
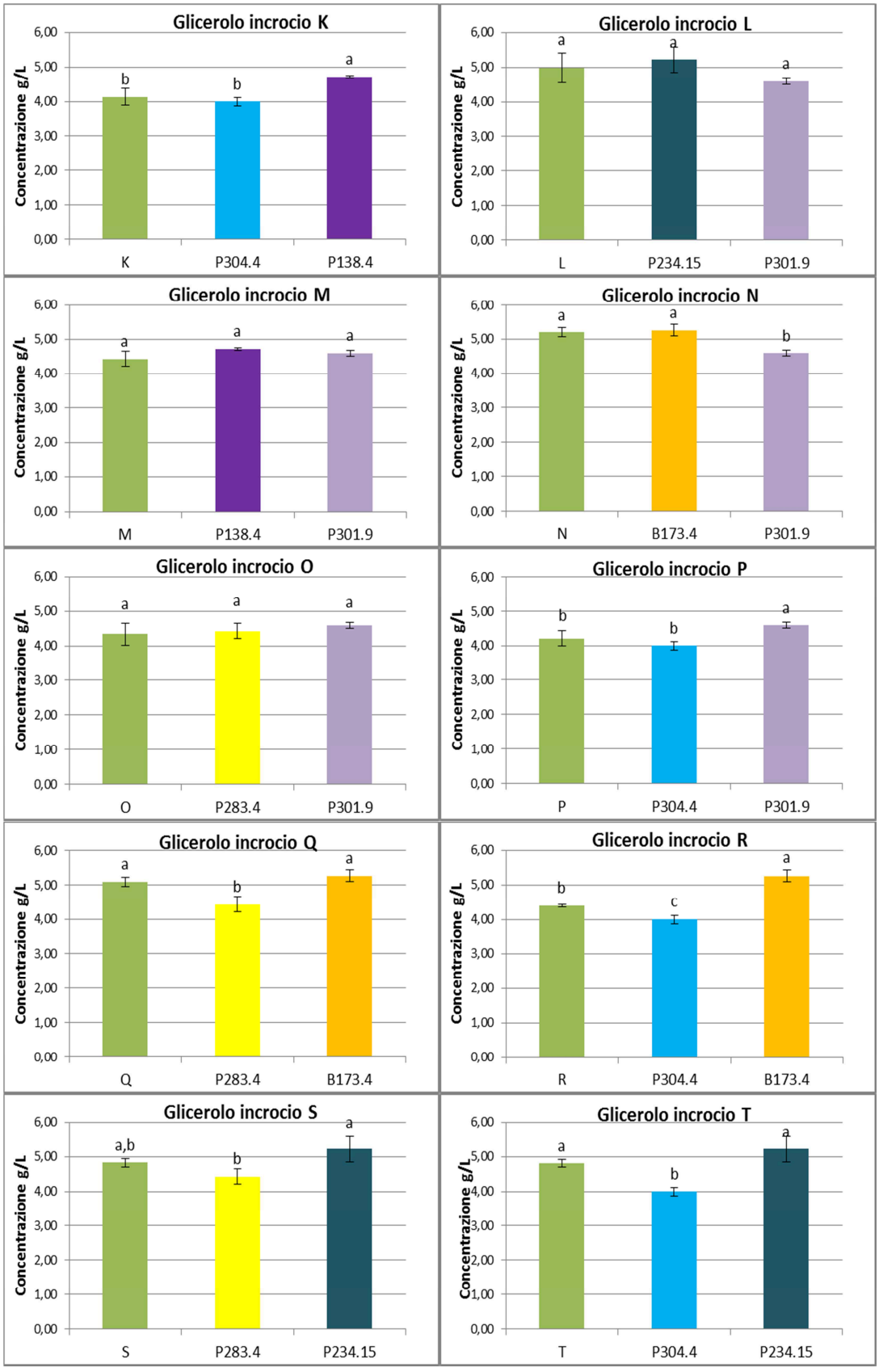


Figure 4.3.5 Glycerol production in single strains fermentations. Statistical analysis: one-factor ANOVA (p value < 0.05). Different letters indicate statistically significant differences.

The glycerol concentration measured at the end of the co-fermentation was compared with that produced by the respective single-strain fermentations (Figure 4.3.6). In 14 out of 28 co-fermentations no statistically differences have been detected between the glycerol production of single-strain and co-fermentations. However, 10 co-fermentations produced glycerol quantities statistically different to that of one of the single-strain fermentations. The co-fermentation R produced a glycerol concentration intermediate between the strains P304.4 and B173.4, which in single-strain fermentation produced respectively the lowest and the highest amount.


Glicerolo incrocio A
Concentrazione g/L
6,00
5,00
4,00
3,00
2,00
1,00
0,00
a
a
a
A
P301.4
P254.12
Glicerolo incrocio B
Concentrazione g/L
a
a
a
B
P301.9
P254.12
Glicerolo incrocio C
Concentrazione g/L
a
a
a
C
B173.4
P254.12
Glicerolo incrocio D
Concentrazione g/L
a
a
a
D
P283.4
P254.12
Glicerolo incrocio E
Concentrazione g/L
a
a
a
E
P304.4
P254.12
Glicerolo incrocio F
Concentrazione g/L
a
a
a
F
P138.4
P254.12
Glicerolo incrocio G
Concentrazione g/L
a
a
a
G
P234.15
P254.12
Glicerolo incrocio H
Concentrazione g/L
a
a
a
H
P283.4
P138.4
Glicerolo incrocio I
Concentrazione g/L
a
a
a
I
P234.15
P138.4
Glicerolo incrocio J
Concentrazione g/L
b
a
b
J
B173.4
P138.4


Glicerolo incrocio K
Concentrazione g/L
6,00
5,00
4,00
3,00
2,00
1,00
0,00
b
b
a
K
P304.4
P138.4
Glicerolo incrocio L
a
a
a
L
P234.15
P301.9
Glicerolo incrocio M
a
a
a
M
P138.4
P301.9
Glicerolo incrocio N
a
a
b
N
B173.4
P301.9
Glicerolo incrocio O
a
a
a
O
P283.4
P301.9
Glicerolo incrocio P
b
b
a
P
P304.4
P301.9
Glicerolo incrocio Q
a
b
a
Q
P283.4
B173.4
Glicerolo incrocio R
b
c
a
R
P304.4
B173.4
Glicerolo incrocio S
a,b
b
a
S
P283.4
P234.15
Glicerolo incrocio T
a
b
a
T
P304.4
P234.15

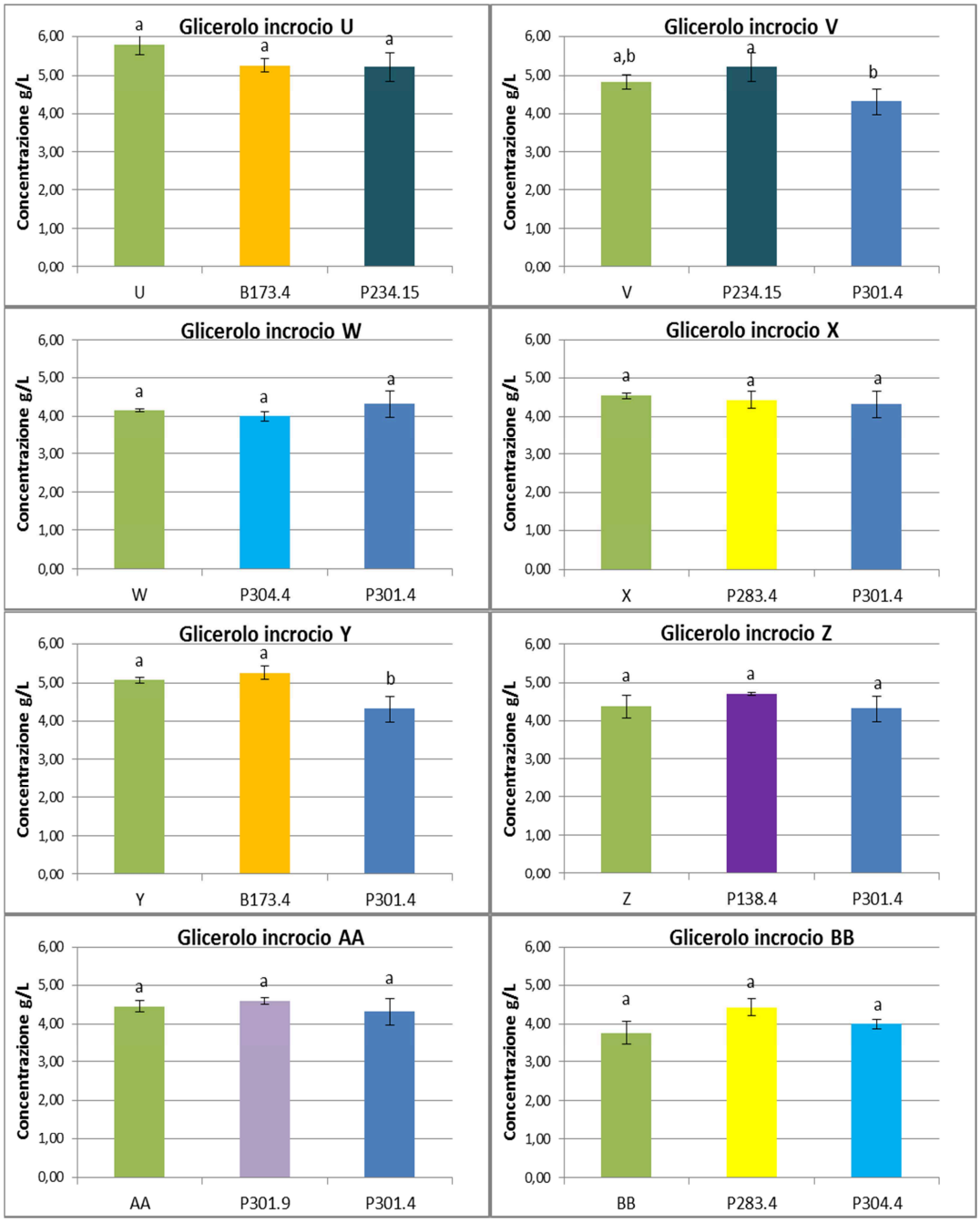


Figure 4.3.6 Glycerol production of single-strain and co-fermentations. Statistical analysis: one-factor ANOVA (p value < 0.05). Different letters indicate statistically significant differences.

4.3.2.3 Acetic acid production

Acetic acid is present in very small quantities in high-quality wines and in very high quantities in those that have undergone microbial alterations. When the concentration is higher than 0.6-0.7 g/l, the volatile acidity is perceptible and affects the quality of the wine accentuating the hardness and astringency. Acetic acid is formed during fermentation as a result of acetaldehyde oxidation and it is consumed by the yeast during alcoholic fermentation, to produce fatty acids and sterols. In Italy the maximum concentration set by the EU Reg. 1493/99 (with exemptions for some specific enological products) are of 18 meq/L (1.08 g/l acetic acid) for white and rosé wines and 20 meq/l (1.2 g/l of acetic acid) for wines red (Ribereau-Gayon *et al.*, 2006). In presence of high acetaldehyde concentrations and alkaline pH, the activity of the enzyme aldehyde dehydrogenase, cause the oxidation of acetaldehyde to acetic acid with NAD reduction.

The average values of acetic acid production at the end of fermentation by the single strains, ranged between 0.46 and 0.81 g/l (Figure 4.3.7). The acetic acid concentration found in synthetic must is generally higher than in natural musts fermentation. This is due to the synthetic must composition. In fact, the grapes lipid fraction is completely lacking, so in this artificial growing medium the synthesis of membrane lipids is more difficult, leading to higher production of volatile acidity. The strains that produced the highest amount of acetic acid were P254.12 and P234.15 while strains P301.4 and P138.4 were those with the lowest production.

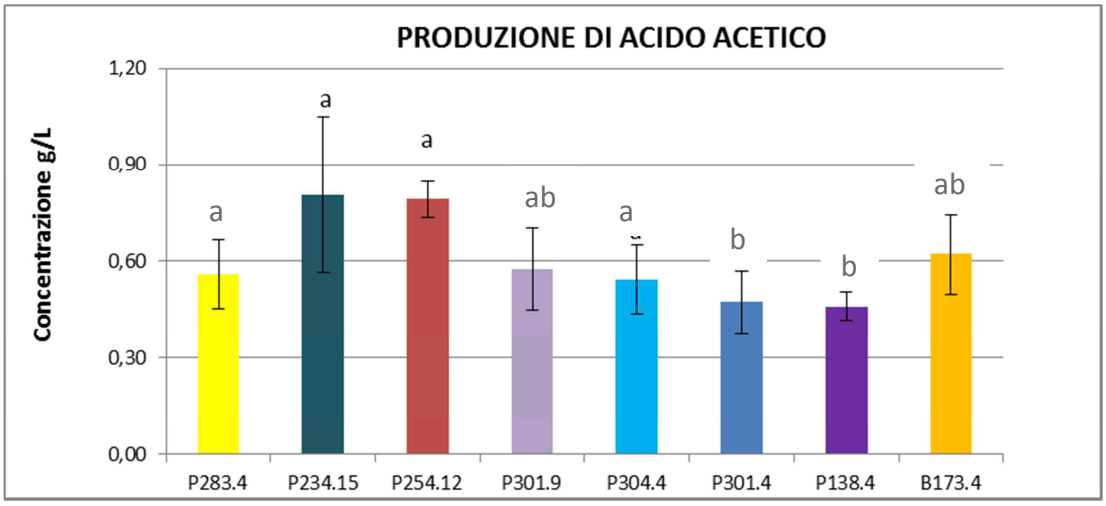
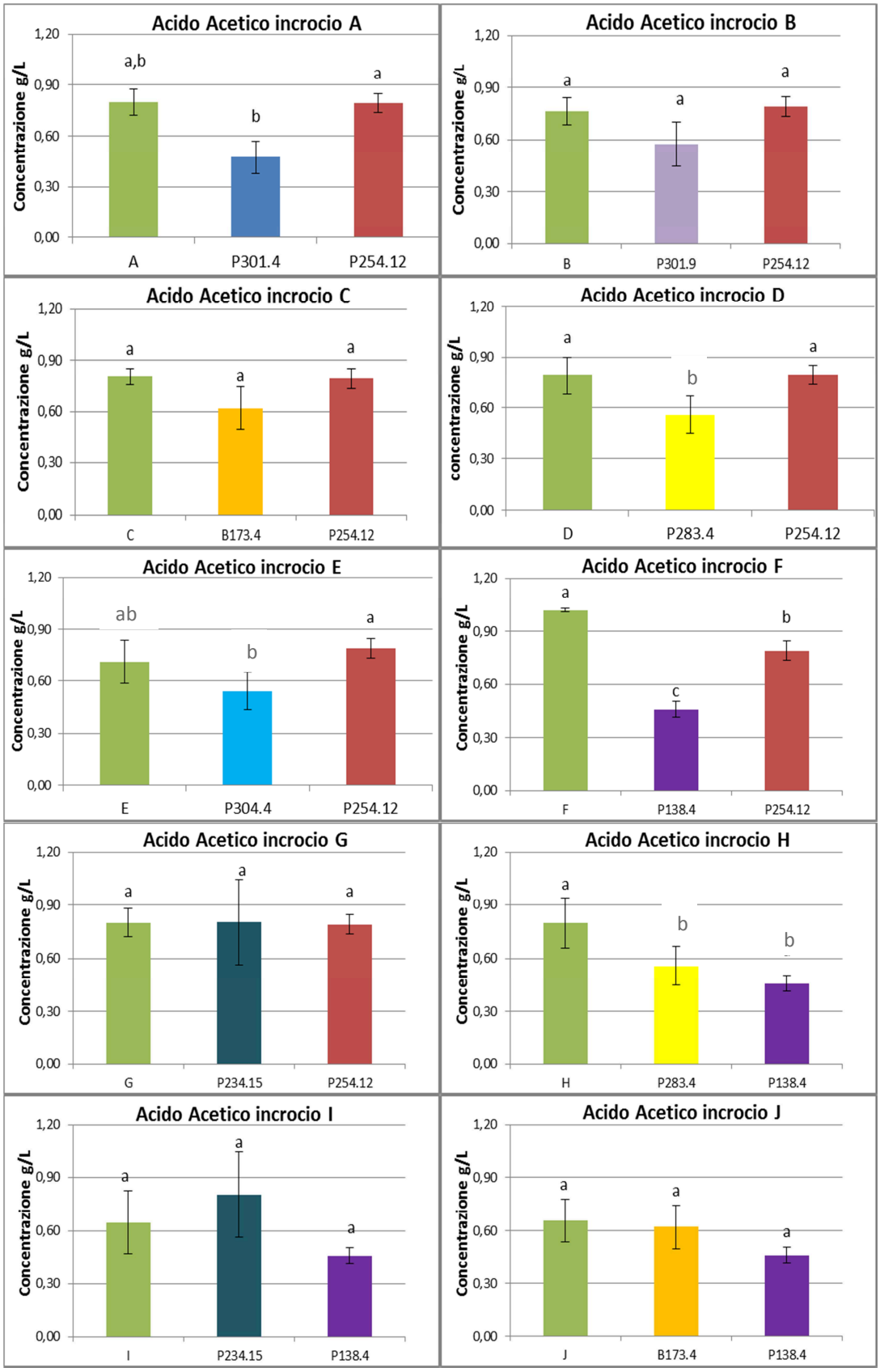
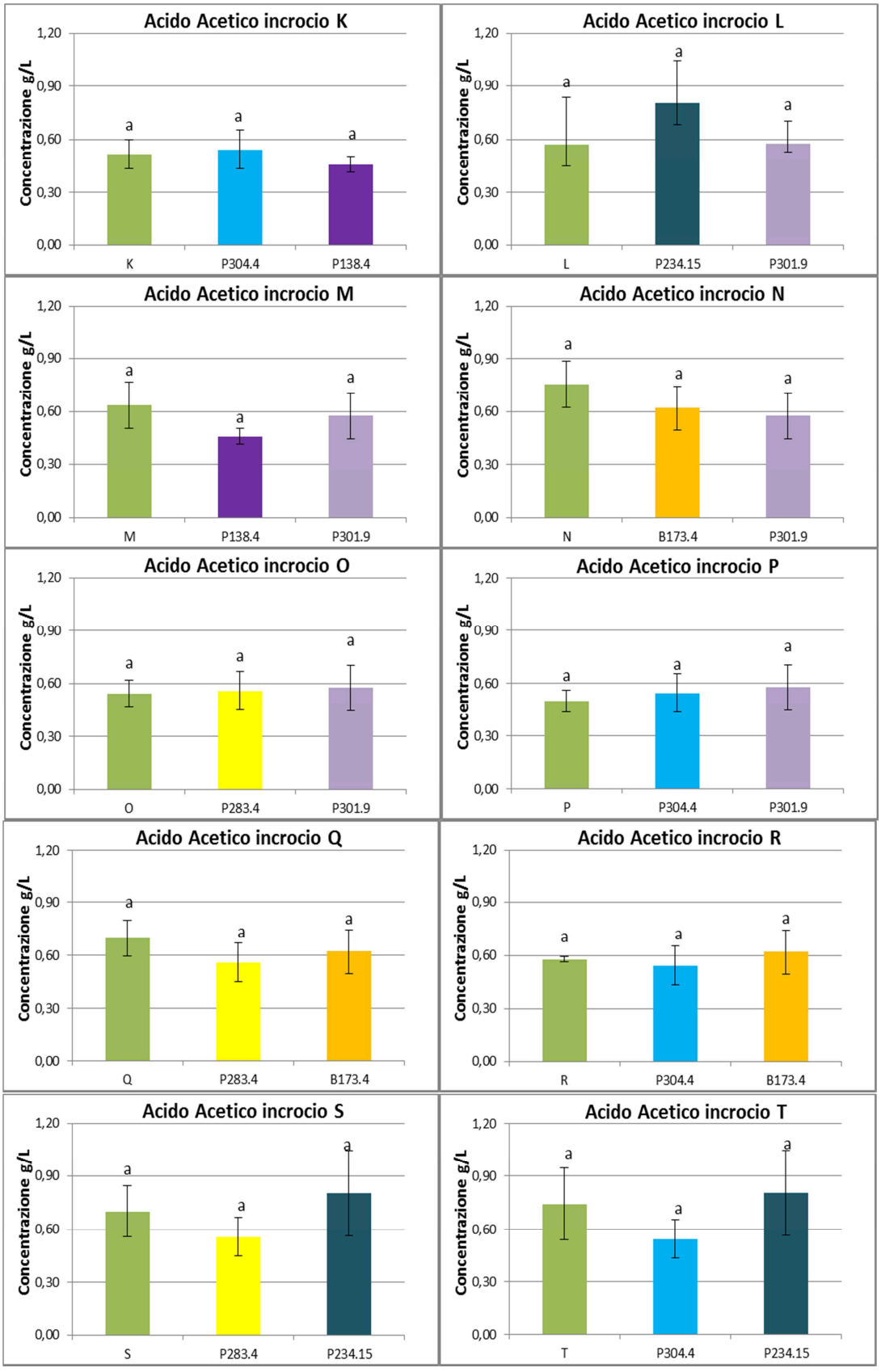


Figure 4.3.7 Acetic acid production in single-strain fermentations. Statistical analysis: one-factor ANOVA (p value < 0.05). Different letters indicate statistically significant differences.

In 20 out of 28 co-fermentations no statistically differences were found in acetic acid production respect to single-strain fermentations. Only in two co-fermentations, F (P138.4/P254.12) and H (P283.4/P138.4), a statistically increase in the acetic acid production was observed. The co-fermentations D (P283.4/P254.12), U (B173.4/P234.15), Y (B173.4/P301.4) and AA (P301.9/P301.4) have produced an acetic acid quantity statistically similar to that of the single-strain fermentation that showed highest acetic acid concentration between the two.


Acido Acetico incrocio A
Concentrazione g/L
1,20
0,90
0,60
0,30
0,00
a,b
b
a
A
P301.4
P254.12
Acido Acetico incrocio B
Concentrazione g/L
a
a
a
B
P301.9
P254.12
Acido Acetico incrocio C
Concentrazione g/L
a
a
a
C
B173.4
P254.12
Acido Acetico incrocio D
concentrazione g/L
a
b
a
D
P283.4
P254.12
Acido Acetico incrocio E
Concentrazione g/L
ab
b
a
E
P304.4
P254.12
Acido Acetico incrocio F
Concentrazione g/L
a
c
b
F
P138.4
P254.12
Acido Acetico incrocio G
Concentrazione g/L
a
a
a
G
P234.15
P254.12
Acido Acetico incrocio H
Concentrazione g/L
a
b
b
H
P283.4
P138.4
Acido Acetico incrocio I
Concentrazione g/L
a
a
a
I
P234.15
P138.4
Acido Acetico incrocio J
Concentrazione g/L
a
a
a
J
B173.4
P138.4


Acido Acetico incrocio K
Concentrazione g/L
1,20
0,90
0,60
0,30
0,00
a
a
a
K
P304.4
P138.4
Acido Acetico incrocio L
a
a
a
L
P234.15
P301.9
Acido Acetico incrocio M
a
a
a
M
P138.4
P301.9
Acido Acetico incrocio N
a
a
a
N
B173.4
P301.9
Acido Acetico incrocio O
a
a
a
O
P283.4
P301.9
Acido Acetico incrocio P
a
a
a
P
P304.4
P301.9
Acido Acetico incrocio Q
a
a
a
Q
P283.4
B173.4
Acido Acetico incrocio R
a
a
a
R
P304.4
B173.4
Acido Acetico incrocio S
a
a
a
S
P283.4
P234.15
Acido Acetico incrocio T
a
a
a
T
P304.4
P234.15

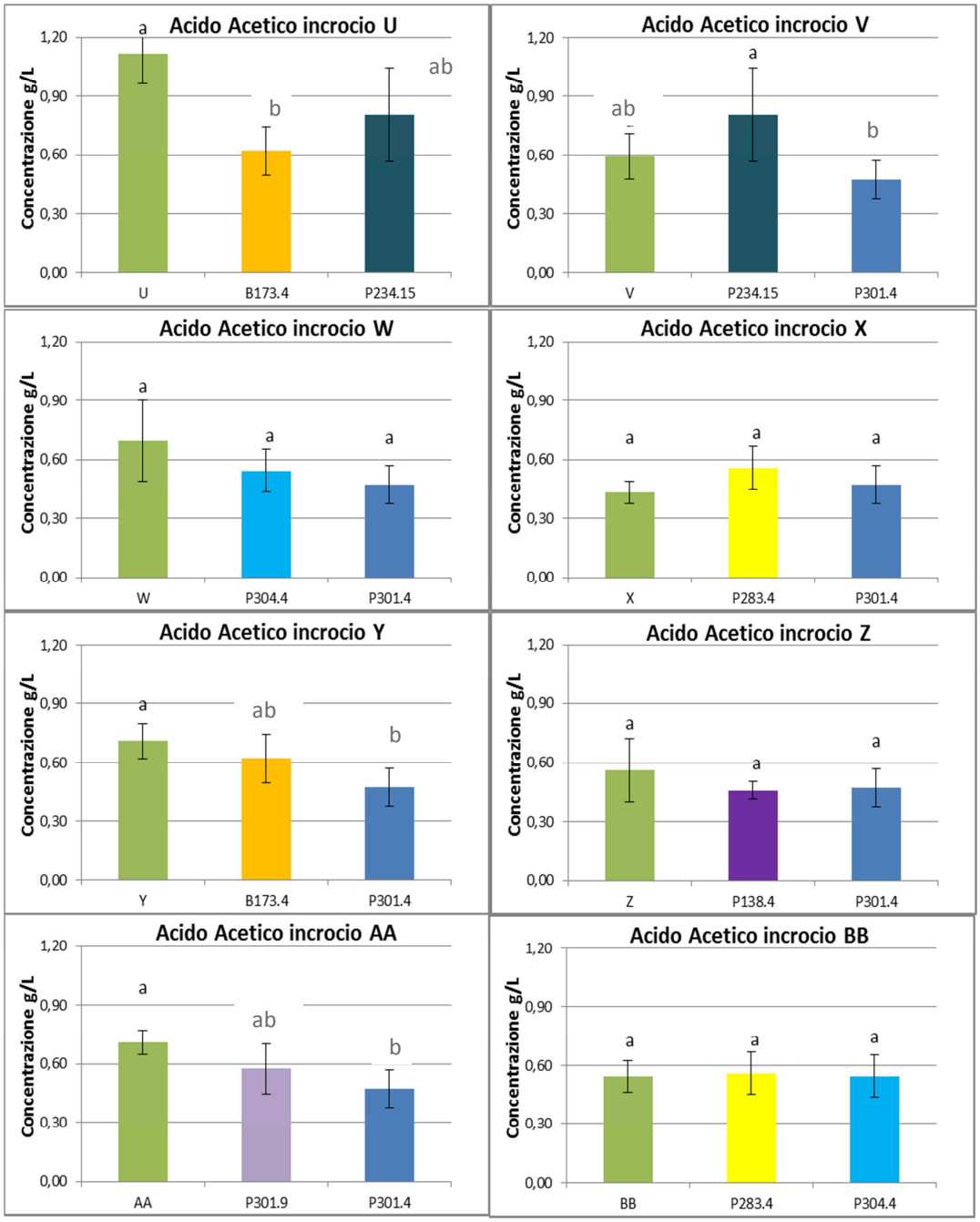


Figure 4.3.8 Acetic acid production of single strain and co-fermentations. Statistical analysis: one-factor ANOVA (p value < 0.05). Different letters indicate the statistically significant differences.

4.3.2.4 Succinic acid production

During the early stages of fermentation, pyruvate binds CO_2 by means of the biotin dependent enzyme pyruvate carboxylase, producing oxaloacetate. The oxaloacetate is transformed into succinate. In anaerobic conditions, however, the Krebs cycle cannot be closed, as the enzyme succinate dehydrogenase, which converts succinate to fumarate, requires FAD, coenzyme that operates only in the respiratory processes, constituting the complex II of the electron transport chain. The Kreb cycle is blocked and therefore an accumulation of succinic acid occurs reaching concentration values among 0.5 and 1.5 g/l. The NADH formed is re-oxidized by glyceropyruvic pathway (Vincenzini *et al*., 2005).
From a sensorial point of view, succinic acid gives an intense bitter and salty taste to the wine, stimulating salivary secretion. It also confers to the wines sapidity and vinosity (Ribereau-Gayon *et al*., 2006).

Succinic acid has not a great enological significance, but it is an indicator of the yeast adaptation to high sugar concentration during the earlier stage of the fermentation. High concentration of succinic acid indicates that the yeast is working in a sugar-stressing condition.

The succinic acid concentrations produced by strain during single-strain and co-fermentation are in the range between 0.29 and 0.40 g/l and the statistical analysis found no significant differences in single-strain fermentations (Figure 4.3.9)

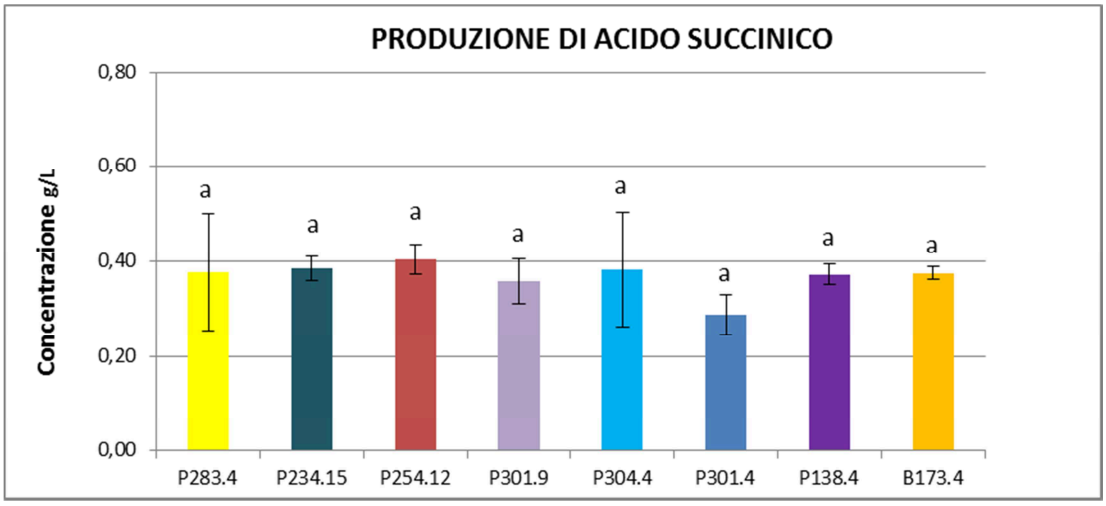
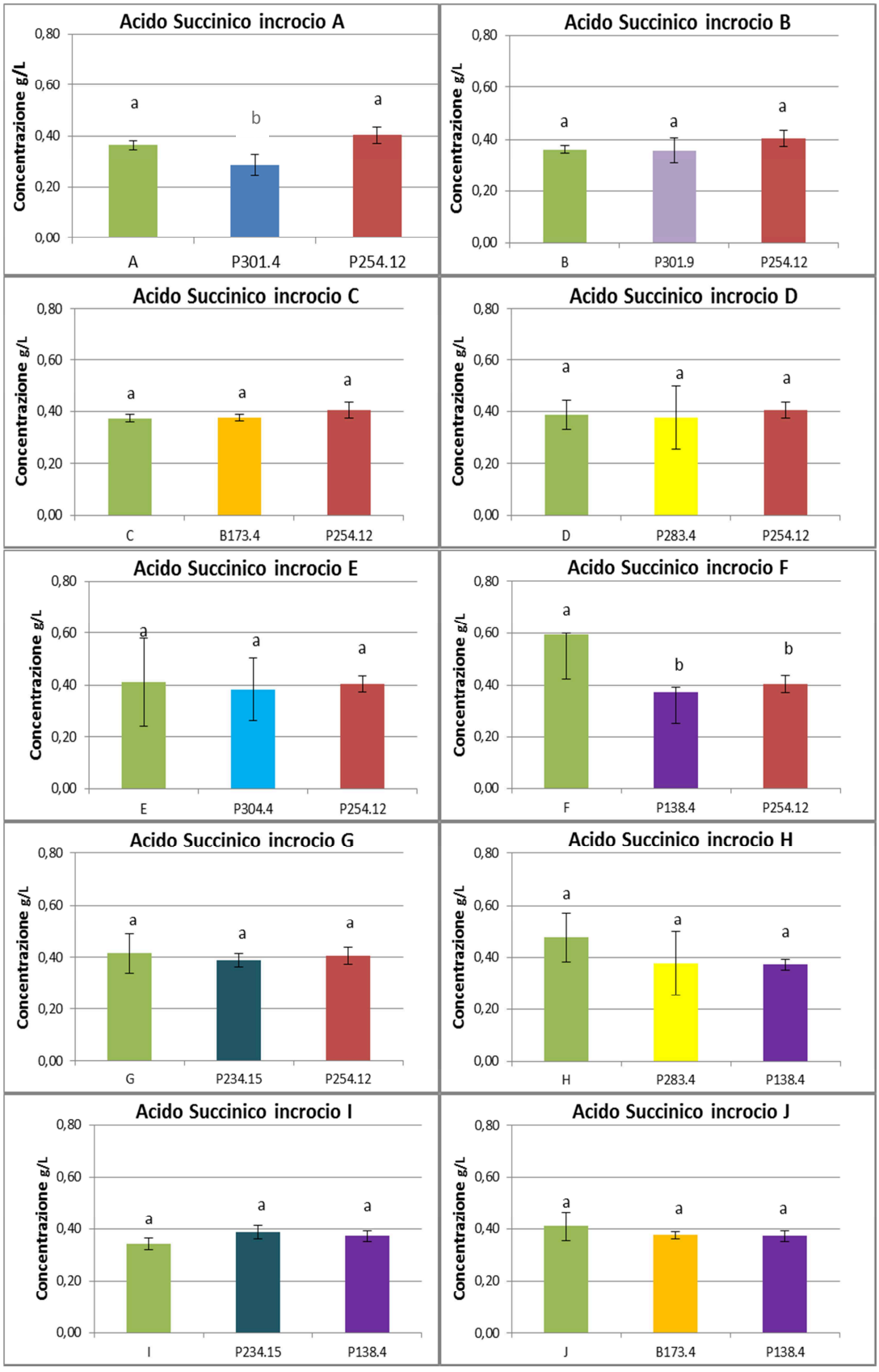
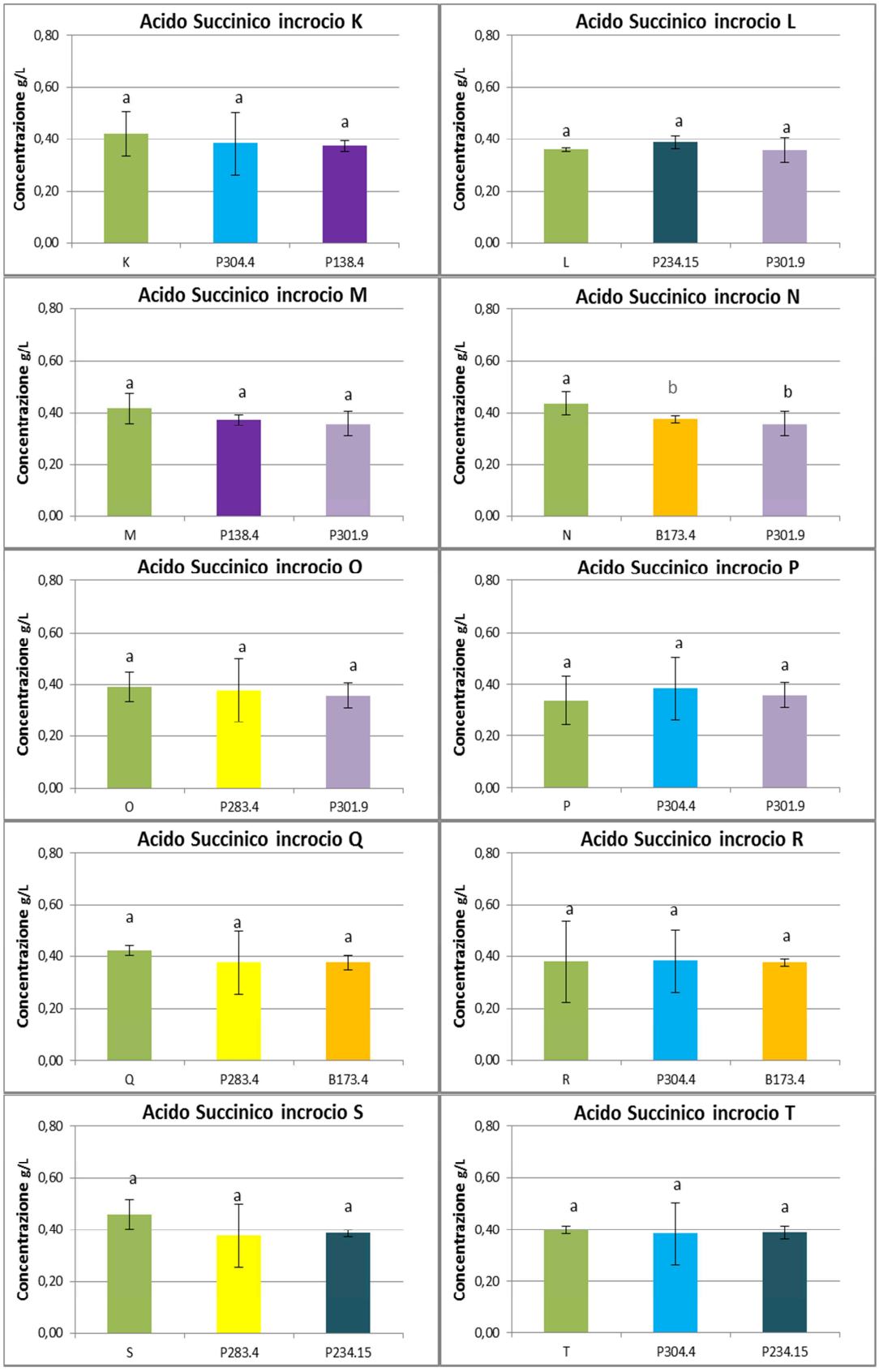


Figure 4.3.9 Succinic acid production in single-strain fermentations. Statistical analysis: one-factor ANOVA (p value < 0.05). Different letters indicate the existence of statistically significant differences.

Comparing the succinic acid concentration produced by co-fermentations with that produced by the single strains (Figure 4.3.10), 23 out of 28 co-fermentations produced a succinic acid concentration statistically similar to that produced by both single strains. Only in co-fermentations F (P138.4/P254.12), N (B173.4/P301.9) and U (B173.4/P234.15) a statistically significant increase in the succinic acid production when compared with single-strains fermentations was observed, while only in the co-fermentation A (P254.12/P301.4) the strains an amount of succinic acid similar to P254.12 strain. In the case of co-fermentation Z (P138.4/P301.4) the succinic acid production was intermediate between the two single strains. In co-fermentation F there was a significant increase in the production of both acetic and succinic acid compared to the single strains. These results lead to assume that the interaction between strains P138.4 and P254.12 affects the pyruvate metabolism.


Acido Succinico incrocio A
Concentrazione g/L
0,80
0,60
0,40
0,20
0,00
a
b
a
A
P301.4
P254.12
Acido Succinico incrocio B
a
a
a
B
P301.9
P254.12
Acido Succinico incrocio C
a
a
a
C
B173.4
P254.12
Acido Succinico incrocio D
a
a
a
D
P283.4
P254.12
Acido Succinico incrocio E
a
a
a
E
P304.4
P254.12
Acido Succinico incrocio F
a
b
b
F
P138.4
P254.12
Acido Succinico incrocio G
a
a
a
G
P234.15
P254.12
Acido Succinico incrocio H
a
a
a
H
P283.4
P138.4
Acido Succinico incrocio I
a
a
a
I
P234.15
P138.4
Acido Succinico incrocio J
a
a
a
J
B173.4
P138.4


Acido Succinico incrocio K
Concentrazione g/L
0,80
0,60
0,40
0,20
0,00
a
a
a
K
P304.4
P138.4
Acido Succinico incrocio L
a
a
a
L
P234.15
P301.9
Acido Succinico incrocio M
a
a
a
M
P138.4
P301.9
Acido Succinico incrocio N
a
b
b
N
B173.4
P301.9
Acido Succinico incrocio O
a
a
a
O
P283.4
P301.9
Acido Succinico incrocio P
a
a
a
P
P304.4
P301.9
Acido Succinico incrocio Q
a
a
a
Q
P283.4
B173.4
Acido Succinico incrocio R
a
a
a
R
P304.4
B173.4
Acido Succinico incrocio S
a
a
a
S
P283.4
P234.15
Acido Succinico incrocio T
a
a
a
T
P304.4
P234.15

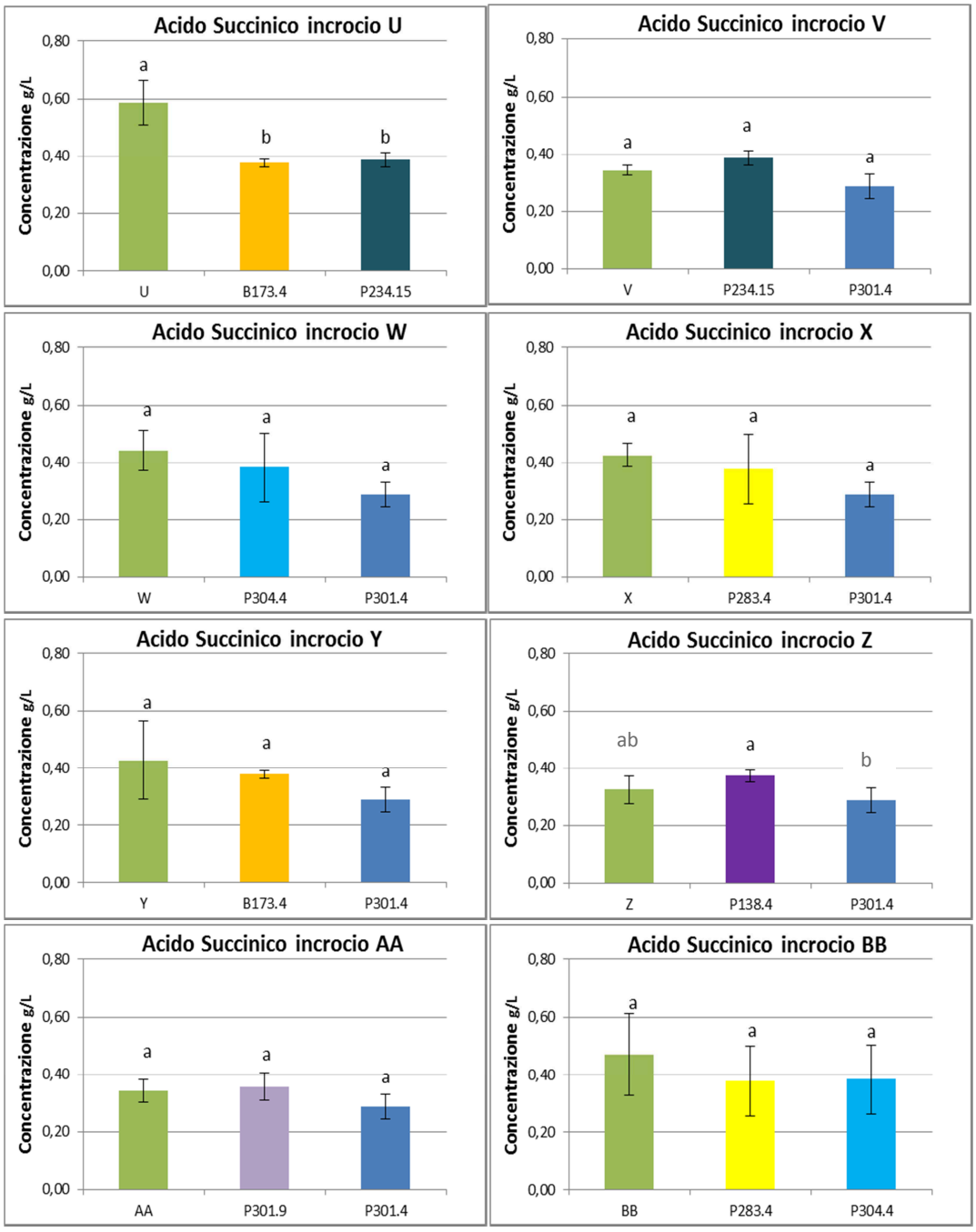
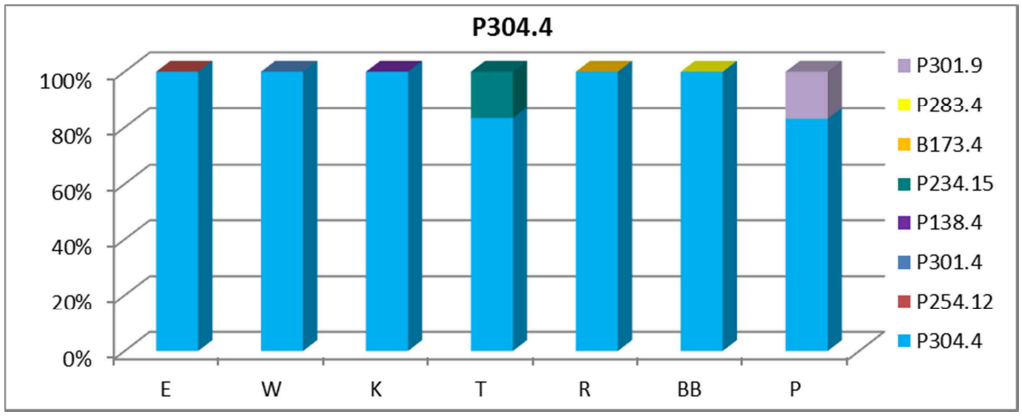
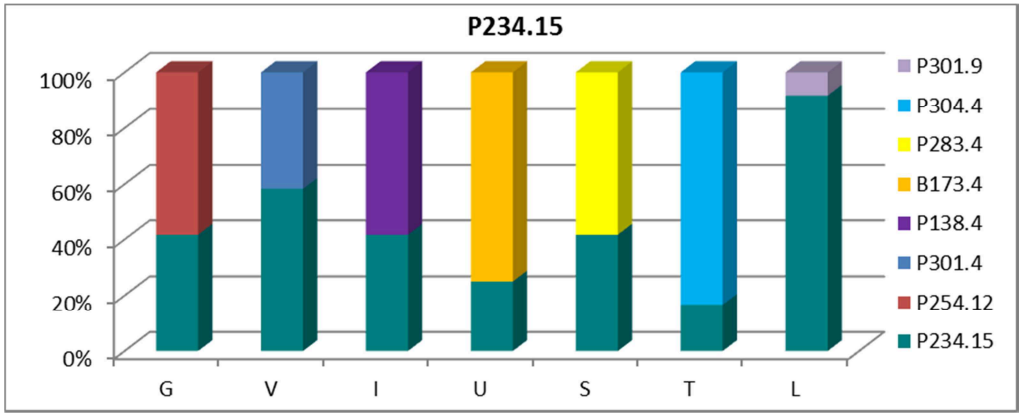


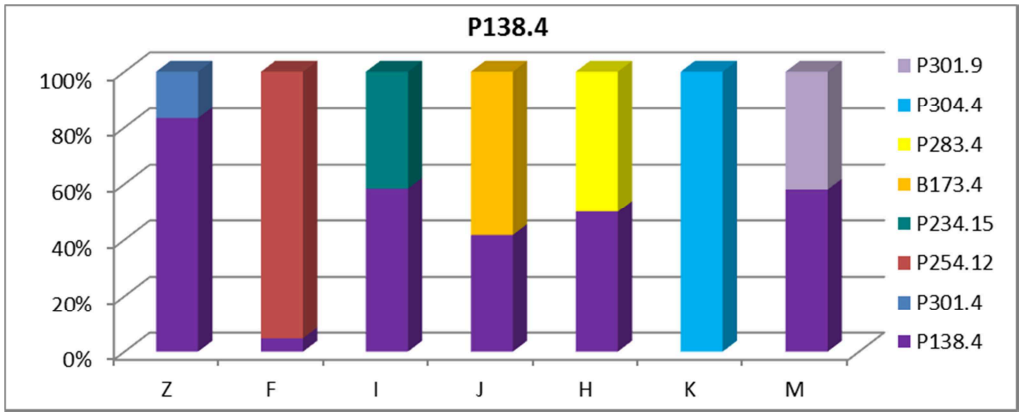
Figure 4.3.10 Succinic acid production of single-strain and in co-fermentations. Statistical analysis: one-factor ANOVA (p value < 0.05). Different letters indicate statistically significant differences.

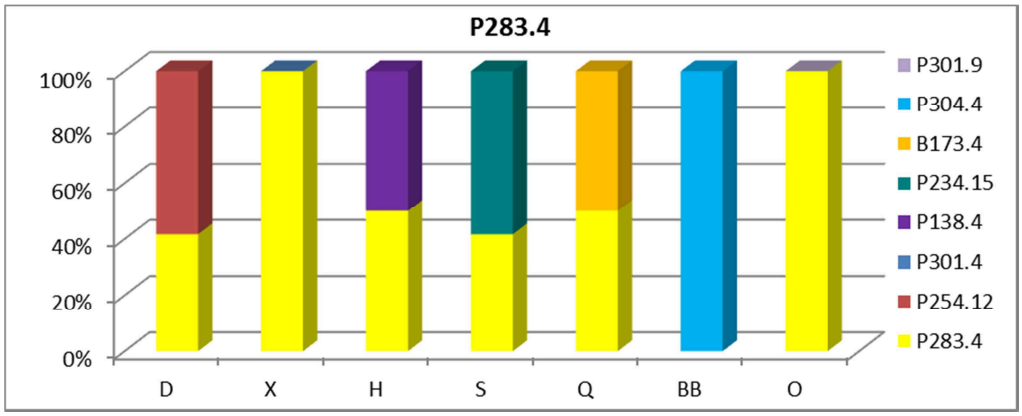
4.3.3. Strain colonization

In order to investigate the strain ability to colonize synthetic must, the fermentations were sampled when alcohol reached about 6,5%, to analyze the implantation level of each strain using for yeast identification the interdelta. For each fermentation 12 isolated were analyze. The strain P304.4, which has neutral killer phenotype, was the best competitor as it completely displaced the co-inoculated strain in five co-fermentations (E, K, R, W and BB) and it was present at higher percentage than the co-inoculated strain in two co-fermentations (P e T). This strain determined a decrease of the fermentation rate every time it was co-inoculated, except for the co-inoculation T. The other two strains (P234.15 and P138.4) with neutral phenotype never dominated the co-fermentation and generally were present in the fermenting must at similar percentage to the co-inoculated strain. As regards the strains exhibiting the killer phenotype, P283.4 and B173.4 inhibited the growth of sensitive strains while, when inoculated with the other strains, maintain the same concentration. Generally P254.12 showed a co-dominance behavior, in two cases (when co-inoculated with P301.4 and P138.4) it was present at higher percentage. All of these three strains were not able to compete with P304.4. The two sensitive strains (P301.4 and P301.9) showed very different behavior. P301.4 was not able to compete with 4 out 7 strains when was tested in co-fermentations (B173.4, P283.4, P304.4 and P301.9); it was present at lower percentage when co-inoculated with P254.12 and P138.4, while showed co-dominance ability with P234.15 strain. P301.9 co-dominated with the strains P254.12 and P138.4; but was not able to compete in co-fermentation with P234.15, P304.4, P283.4 and B173.4 (strains with killer phenotype). When co-inoculated with the other sensitive strain P301.4, P301.9 dominated completely the fermentation.


P304.4
100%
80%
60%
40%
20%
0%
E
W
K
T
R
BB
P
P301.9
P283.4
B173.4
P234.15
P138.4
P301.4
P254.12
P304.4


P234.15
100%
80%
60%
40%
20%
0%
G
V
I
U
S
T
L
P301.9
P304.4
P283.4
B173.4
P138.4
P301.4
P254.12
P234.15


P138.4
100%
80%
60%
40%
20%
0%
Z
F
I
J
H
K
M
P301.9
P304.4
P283.4
B173.4
P234.15
P254.12
P301.4
P138.4


P283.4
100%
80%
60%
40%
20%
0%
D
X
H
S
Q
BB
O
P301.9
P304.4
B173.4
P234.15
P138.4
P301.4
P254.12
P283.4

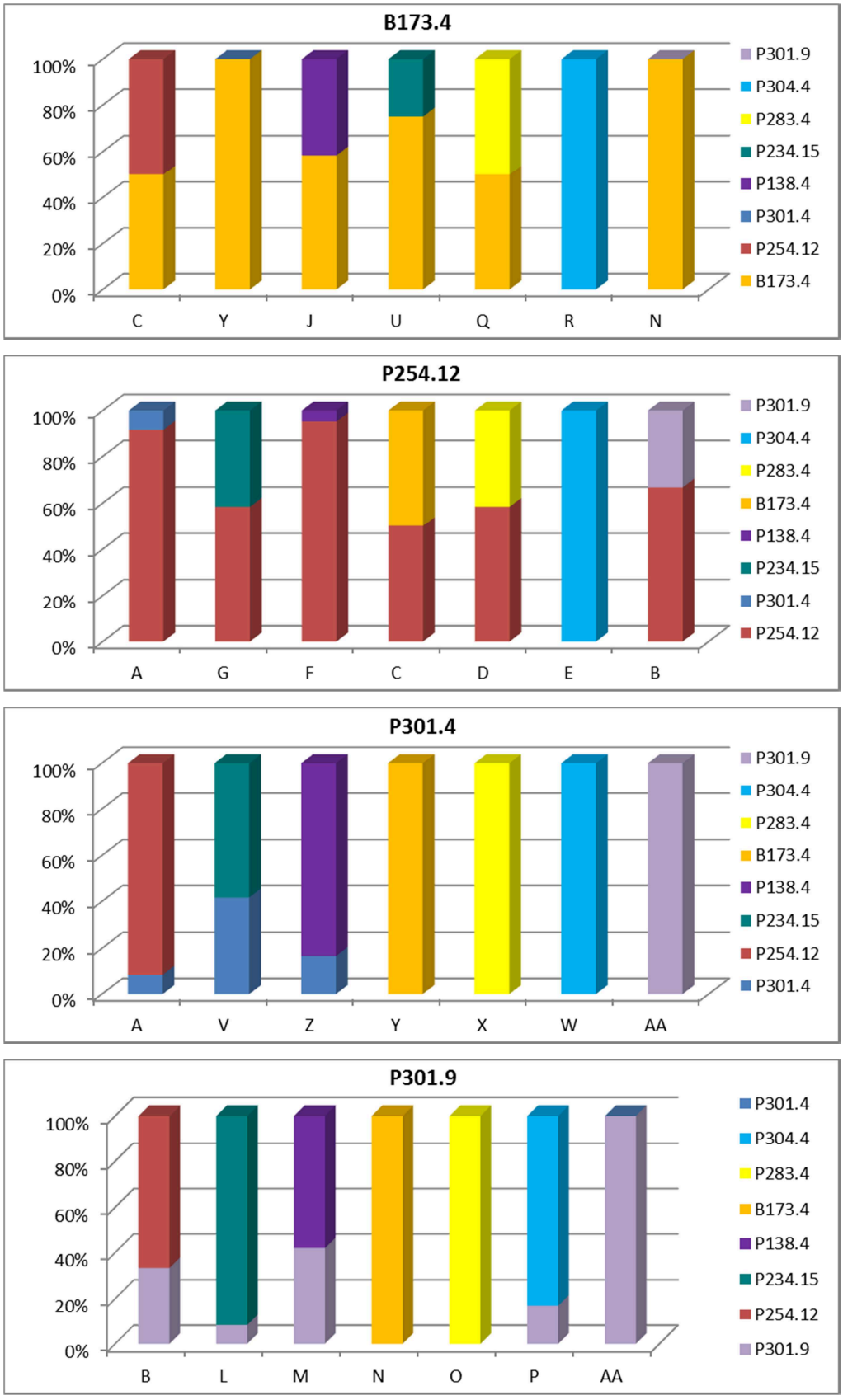


Figure 4.3.11 Presence of co-inoculated strains.

4.3.4 Principal component analysis (PCA)

All the collected data has been used to perform a principal component analysis (Fig. 4.3.12). The first two components account for 82.79% of the total variance. The first principal component (F1) accounted for 53.36% of the total variance and significantly correlates ($\alpha < 0.001$) with fermentation performances in synthetic must (fermentative vigour, glucose consumed after 7 days of fermentation and at the end, fermentation time) and glycerol production. The second principal component (F2) explained 29.43% of the total variance and correlats with glycerol, acetic and succinic acid production ($\alpha < 0.001$)

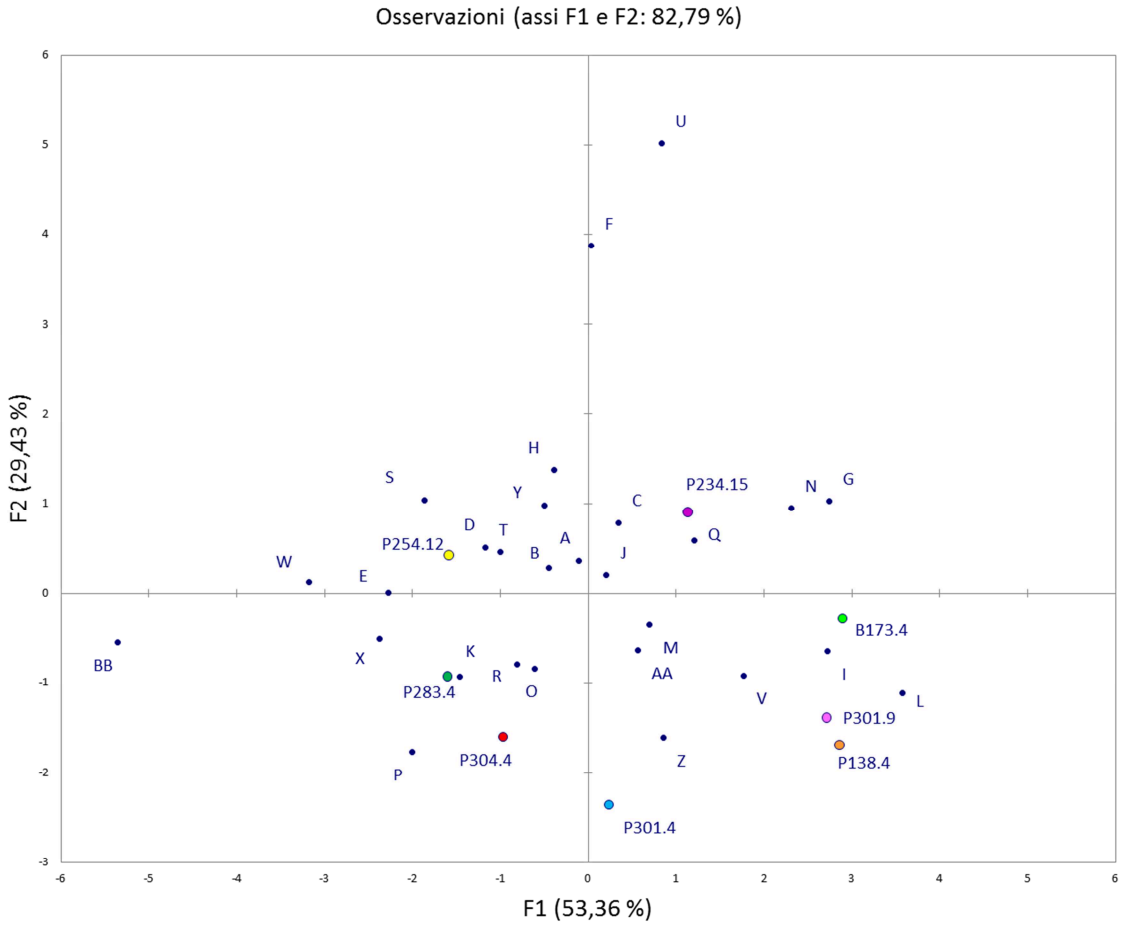


Figure 4.3.12 Principal component analysis (PCA) biplot of mean values of fermentative performances and secondary compounds determined in single-strain (colored circles) and co-fermentations (blue circles). F1, explains 53.36% of the variation while F2 explains 29.43%. Co-fermentations: (**P301.9**: B, L, M, N, O, P, AA); (**P301.4**: A, V, Z, Y, X, W, AA); (**P254.12**: A, G, F, C, D, E, B); (**P138.4**: Z, F, I, J, H, K, M); (**P234.15**: G, V, I, U, S, T, L); (**B173.4**: C, Y, J, U, Q, R, N); (**P283.4**: D, X, H, S, Q, BB, O); (**P304.4**: E, W, K, T, R, BB, P).

Samples on the right hand side of the biplot can be considered the best fermenter showing the higher values of fermentative vigour and glucose consumed after 7 days and the lower glucose residue and fermentation time. Moreover the samples plotted in the 4th quadrant also produce a low level of organic acids such as acetic and succinic acids. As regards P304.4, together with P283.4 and P254.12, the strains showed poor fermentation performance in term of glucose residue and fermentation time. The co-fermentations performed with P304.4 strain (E, W, K, T, R, BB, P) were distributed in the left side of the PCA plot even when co-inoculated with good fermenter strains (5 out of 7) therefore highlighting the worsening of the fermentation performances with respect to single-strain fermentations. In all cases P304.4 was able to dominate the fermentation or was present at very high percentage. Most of mixed fermentation involving P283.4 (D, H, O, S, X, BB) and P254.12 (A, B, D, E), were located in the left side of the PCA plot. Regarding P283.4 the co-fermentation in which this strain dominated, X and O were located very near to P283.4 single-strain fermentation, whereas the co-fermentation BB was very far from both single-strain fermentations. In four cases P254.12 genetic profile was identified in about 50% of the isolates from co-fermentations, nevertheless in two cases (B, D) the co-fermentations were located near the single strain whereas the other co-fermentation (C, G) were plotted in the right side of the PCA plot.

Among the co-fermentations performed with P173.4, P138.4 and P234.12, all good fermenters, 5 out of 7 co-fermentations were distributed on the right hand side of the plot, indicating that the positive fermentation performance were maintained in the co-fermentation, as well. It is interesting to note that in the fermentation Y (B173.4/P301.4), where the killer strain B173.4 was present and totally dominated on the sensitive strain P301.4, was very far from both single strain fermentations. The co-fermentation M resulted from the pairwise strain inoculation of two strains, P138.4 and P301.9, with similar performance (single strain fermentations very close each other in the plot), was plotted very far from the two single strains, but in the same quadrant.

Regarding the sensitive strains P301.9 and P301.4, even though the former totally dominated the mixed fermentation, the performance of the co-fermentation (AA) was more similar to P301.4. Again, the fermentation behavior of L (P234.15/P301.9) was very close to P301.9 single-strain fermentation even though the P234.15 strain almost dominated the

co-fermentation. The PCA analysis confirmed the improvement of the fermentation performance in the case of co-fermentation G (P234.15/P254.12) with respect to the single-strain fermentations shifting the co-fermentation to the right side of the plot. .

In two cases (F and U) the acetic and succinic acid production of pairwise strain inoculation resulted higher than respective single strain fermentations, indicating a potential metabolic interaction among strains. Therefore, yeasts modify their metabolism during growth in co-fermentation, where interaction among strains composing mixed starter cultures can determine the sharing of some secondary metabolites (Cheraiti et al., 2005; Howell et al., 2006; King et al., 2008).

4.4 CONCLUSIONS

This work evaluates the fermentation activity of 8 vineyard strains, belonging to the species *S. cerevisiae*, in co-fermentation in synthetic must.

By pairwise strain inoculation, 28 mixed fermentations were performed in triplicate. Considering the co-fermentation kinetics with respect the single-strain fermentations 5 trends can be evidenced: 13 co-fermentations show kinetics very similar to one of the two co-inoculated strain; 6 very similar to both single-strain fermentations, in 6 cases the co-fermentation shows a trend intermediate between the two single-strain fermentations; in only one case the co-fermentation performance was improved compared to both single-strain fermentations; finally in only two cases co-fermentations show kinetics worse than the two single strains.

Among the co-fermentations studied ten mixed fermentation and their respective single strains show a glucose residual quantity less than 5 g/l, therefore it is possible to affirm that both the single strains and the co-cultures have successfully completed the fermentation. Interestingly the co-culture G has a residual sugar statistically lower than the P234.15 and P254.12 strains, this data confirms its better fermentative performance than the single-strain fermentations.

In order to assess the strain colonization ability during co-fermentations, colonies from isolation plates were identified by interdelta genetic profile. The presence of the couple killer/ sensitive strain in 4 out of 6 cases resulted in the almost complete elimination

of sensitive strain, although, surprisingly, the co-fermentation kinetics did not reproduce those of the killer strains (in 2 cases the kinetics retraced those of the sensitive strains and in the other two the co-fermentation kinetics were intermediate). This result indicates that, despite the dominance of the killer strain, a perturbation of the kinetics occurs due to co-presence of the two strains, even if for a limited time.

Regarding the co-fermentation involving the two sensitive strains, the complete dominance of one strain was observed. This co-inoculation kinetic was worse than that of both single-strain fermentations.

The strain P304.4, which has neutral killer phenotype, was the best competitor as it completely displaced the co-inoculated strain in 5 of 7 cases and it was present in higher percentage on the other 2 cases.

These results highlight the existence of other factors than the killer character implicated in the phenomenon of the must colonization. Furthermore, the presence of a second strain deeply influences the fermentation kinetics, improving or sometime worsening the fermentation trend.

CHAPTER 5

The role of nitrogen uptake on colonization ability of three vineyard *S. cerevisiae* strains

5.1 INTRODUCTION

Many factors affect the microbial ecology of wine production, of which the chemical composition of the grape juice and the fermentation processes are most significant. In complex microbial ecosystems, containing mixtures of different species and strains, there is the possibility that interactions will occur between individual microorganisms, themselves, and this will also determine the final ecology. Wine production presents such an ecosystem where, in relation to yeasts, there is the potential for yeast–yeast interactions, yeast–filamentous fungi interactions, and yeast–bacteria interactions. The interactions between individual microorganisms, are neutralism, commensalism, synergism, antagonism, parasitism, and competition. From the perspective of practical winemaking, the relevant outcomes of these interactions are whether or not they enhance or inhibit the growth of any particular species or strain. Within the wine ecosystem, there are numerous mechanisms whereby one yeast may influence the growth of another yeast. (Fleet GH 2003)

Early growth of yeasts in grape juice decreases or strips it of nutrients, making the resultant wine less favourable as an environment for any further microbial growth. Yeasts growth generates an array of metabolites, some of which will be toxic to other species ,as ethanol and short chain fatty acids. Carbon dioxide production and purging of the juice/wine strips it of oxygen, thereby limiting the growth of aerobic species. Some species may produce inhibitory peptides, proteins or glycoprotein, such as killer toxins, and enzymes that destroy other species by lysis of their cell walls. The quorum sensing is a mechanism by which microbial cells communicate with each other and regulate population growth. It was shown that bacterial populations regulate their behaviour and expression of certain properties through the production of low molecular- mass signaling molecules (Whitehead et al., 2001). These molecules, called quorum sensing signals, are produced

throughout growth and, when their concentration reaches a certain threshold, they activate or inhibit gene expression to modify the behaviour of the whole population. In the case of yeasts, there is evidence that bicarbonate (Ohkuni et al., 1998), acetaldehyde (Richard et al., 1996) ammonia (Palkova et al., 1997) and farnesol (Hornby et al., 2001) may act as cell communicating molecules.

More recently, there has been a growing interest towards controlled mixed fermentations that use more than one selected yeast strain. This thus aims to improve specific traits, or in general to enhance the complexity and particular characteristics of the resulting wines. In this context, yeast interactions have a fundamental role to obtain the desired product characteristics. These interactions can affect the metabolite production and/or the microbial growth (M. Ciani & F. Comitini, 2015).

Ethanol production by *S. cerevisiae* is considered to be a major factor that governs the growth and influence of non-Saccharomyces species during fermentation. Generally, the species of *Hanseniaspora, Candida, Pichia, Kluyveromyces, Metschnikowia* and *Issatchenkia* found in grape juice are not tolerant of ethanol concentrations exceeding 5–7%, and this explains their decline and death as the fermentation progresses beyond the mid-stage (Heard and Fleet, 1988; Gao and Fleet, 1988). The increase in ethanol concentration during alcoholic fermentation could also explain the sequential growth of strains within a species. Strains of *S. cerevisiae*, as well as those of other species, vary in their tolerance to ethanol stress (Fleet, 1992; Bauer and Pretorius, 2000; Bisson and Block, 2002). Strains with higher ethanol tolerance are more likely to dominate at later, rather than earlier stages of fermentation. This behaviour has been demonstrated experimentally, along with the interactive effect of fermentation temperature (Torija et al., 2002), and becomes an important consideration in designing mixtures of yeast strains (oligo-strains) for use as cultures to enhance the complexity of wine flavor (Grossman et al., 1996).

Together with ethanol, other factors can have strong selective pressure in mixed wine fermentation. In particular, the production of medium-chain fatty acids and high amounts of acetic acid can negatively affect the growth of a co-fermenting yeast species.

Cell-to-cell contact appears to be also involved in the interactions between *S. cerevisiae* and other non-*Saccharomyce*s species, such as *Torulaspora delbrueckii,*

Hanseniaspora uvarum and *Kluyveromyces thermotolerans* (now reclassified as *Lachanchea thermotolerans*) (Arnebort N *et al*., 2005).

Another mechanism that regulates the presence and dominance of yeast species during wine fermentation is the involvement of oxygen. Reduced oxygen availability under grape juice fermentation might have an important role as a selective factor in mixed cultures. Indeed, low tolerance to low available oxygen exhibited by *K. thermotolerans* and *T. delbrueckii* could in part explain their relative competitiveness, and consequently their rapid death in the presence of *S. cerevisiae* (Hansen EH *et al*., 2001).

There is good evidence that killer interactions may determine species and strain evolution during fermentation although many winemaking variables affect the expression of killer and killer-sensitive phenotypes. Killer strains of *S. cerevisiae* sometimes predominate at the completion of fermentation, suggesting that they have asserted their killer property and taken over the fermentation. Killer strains have been found within wine isolates of *Candida, Pichia* and *Hanseniaspora* and some of these can assert their killer action against wine strains of *S. cerevisiae* (Fleet and Heard, 1993).

Nutrient availability and nutrient limitation are likely factors that modulate the yeast ecology of fermentation, as one yeast species or strain produces or utilises a nutrient relevant to another species or strain. Non-*Saccharomyces* species growing early in the fermentation could utilise amino acids and vitamins, and limit the subsequent growth of strains of *S. cerevisiae*. There are reports that *Kloeckera apiculata* could strip the grape juice of thiamine and other micronutrients, leading to deficient growth of *S. cerevisiae* (Bisson, 1999; Mortimer, 2000). However, some non-*Saccharomyces* species, such as *Kl. apiculata* and *M. pulcherrima* are significantly proteolytic (Charoenchai et al., 1997; Dizzy and Bisson, 2000) and could generate amino acids for use by *S. cerevisiae*. The early death and autolysis of these non-*Saccharomyces* yeasts (Hernawan and Fleet, 1995) is another possible source of nutrients for *S. cerevisiae*, and spoilage yeasts.

Saccharomyces cerevisiae yeasts require a relatively high level of nutrients to complete the fermentation of grape must, typically producing 12–15% v/v ethanol. Assimilable nitrogen has been identified as a key nutrient that is often suboptimal in many grape musts surveyed worldwide. A minimal concentration of more than 140 mg/L is often quoted as necessary for the fermentation of low-solids (filtered), low-temperature (<15°C),

anaerobic musts of moderate sugar level (20%) (Bell and Henschke 2005). Nitrogen sources are rapidly accumulated by yeast in the early stages of 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 M. *et al*. 2007).

Nitrogen component plays a predominant role in the fermentation process. Grape must contains a variety of nitrogen compounds, among which the most important are amino acids, ammonium ion, and small peptides. These nitrogen compounds, excluding proline, constitute what is called yeast assimilable nitrogen. Nitrogen affects yeast cells in two aspects: biomass production during fermentation and the fermentation rate (Varela C. *et al*., 2004). Therefore, the nitrogen content exerts an action on fermentation by regulating both its rate and its end. In fact, the lack of nitrogen has been pointed as one of the main reasons of stuck or sluggish fermentations (Bisson L.F. 1999; Taillandier P. *et al*., 2007). Stuck and sluggish fermentations are detrimental for wine quality as they leave residual sugars that would increase microbial instability and change the organoleptic properties of the final wine. The nitrogen content also affects other pathways in yeast, in particular, through the redox status of the cells, which affects the production of ethanol and other metabolites such as glycerol, acetic acid, and succinic acid (Albers E. *et al*., 1996; Radler F. 1993; Camarasa C. *et al*., 2003). Finally, other metabolites very relevant to wine quality are the volatile compounds and *Saccharomyces cerevisiae* produces different concentrations of those depending on fermentation conditions. Among these conditions, the quality and quantity of the nitrogen sources are critical in the formation of some aromatic molecules (Gutiérrez A. *et al*., 2013).

Although nitrogen concentration is a relevant factor, it is also important to underline that not all the nitrogen sources support equally yeast growth. In complex mixtures of amino acids and ammonium, such as grape must, wine yeasts have preference for some nitrogen sources, and the pattern of the preferential uptake of the nitrogen sources is determined by different molecular mechanisms. In *S. cerevisiae*, the mechanism is known globally as nitrogen catabolite repression (NCR). The NCR allows cells to detect the presence of the best sources of nitrogen by limiting the use of those that do not allow for the best growth. The detection of the rich nitrogen sources triggers a signaling chain that culminates with the activation of genes involved in the transport and metabolism of these

rich sources and the suppression of those genes involved in the transport and use of poorer sources. Once the richest sources of nitrogen (ammonium, glutamine, and asparagine) are consumed, yeast metabolism activates the utilisation of the poorer sources of nitrogen (arginine, glutamate, alanine, etc.) (Mas A. *et al*., 2014).

In this work the nitrogen request of the wine industrial strain QA23 were investigated in comparison with those of three autochthonous *S. cerevisiae* strains isolated directly from vineyard. We monitored amino acids and ammonium consumption during fermentation in synthetic must supplemented with high and low nitrogen level. The expression level of some of the genes under nitrogen catabolite repression was monitored in order to correlate nitrogen metabolism, fermentation rate and nitrogen request. Finally, pairwise strain fermentations in synthetic must supplemented with high and low nitrogen concentration were set up. All collected data regarding nitrogen demand and population dynamics were used to understand the possible role of nitrogen availability in colonization dynamics during must fermentation.

5.2 MATERIALS AND METHODS

5.2.1 Yeast strains

For this study the commercial wine yeast QA23 and three autochthonous strains were used. The autochthonous yeasts were isolated from the vineyards in the winemaking area of Prosecco Superiore di Conegliano Valdobbiadene DOCG shown in Table 5.2.1

Strain	**Species**	**Origin**
QA23	*Saccharomyces cerevisiae*	LALLEMAND S.A., Canada
P301.4	*Saccharomyces cerevisiae*	Vineyard
P254.12	*Saccharomyces cerevisiae*	Vineyard
P304.4	*Saccharomyces cerevisiae*	Vineyard

Table 5.2.1 Yeast strains used in this work

5.2.2 Culture media and growth condition

Media

YPD (Yeast Extract/Peptone/Dextrose)

10 g L-1 yeast extract (OXOID)

20 g L-1 vegetatone peptone (DIFCO)

20 g L-1 glucose (PROLABO)

Adjust to volume with distilled water. Sterilize by autoclaving at 121 ° C for 15 minutes.

Synthetic nutrient medium (SNM) HNC - LNC

Macroelements

100 g glucose

100 g fructose

0,155 g $CaCl_2 \cdot 2H_2O$

0,2 g NaCl

0,75 g KH_2PO_4

0,25 g $MgSO_4 \cdot 7H_2O$

0,5 g K_2SO_4

0,46 g $(NH_4)Cl$ for HNC

0,09 g $(NH_4)Cl$ for LNC

3 g tartaric acid

0,5 g citric acid

5 g malic acid

Microelements

4 mg $MnSO_4 \cdot H_2O$

4 mg $ZnSO_4 \cdot 7\ H_2O$

1 mg $CuSO_4 \cdot 5H_2O$

1 mg KI

0,4 mg $CoCl_2$

1 mg H_3BO_3

1 mg $(NH_4)6Mo_7O_{24} \cdot 4H_2O$

Vitamins

20 mg Myo-inositol

2 mg Nicotinic acid

1,5 mg Calcium Panthotenate

0,25 mg Thiamine hydrochloride

0,25 mg Pyridoxine hydrochloride

0,003 mg Biotin

Anaerobiosis factors

1,50 g ergosterol

0,5 ml oleic acid

50 ml tween 80

50 ml ethanol

Amino acids
3,70 g leucine
5,80 g threonine
1,40 g glycine
38,60 g glutamine
11,10 g alanine
3,40 g valine
2,40 g methionine
2,90 g phenylalanine
6,00 g serine
2,50 g histidine
1,30 g lysine
1,00 g cysteine
46,80 g proline
1,40 g tyrosine
13,70 g tryptophan
2,50 g isoleucine
3,40 g aspartic acid
9,20 g glutamic acid
28,60 g arginine

Prepare the amino acids in a 1 litre aqueous solution and use 13,09 ml per litre of must HNC and 2,62 ml per litre of must LNC. Dissolve all components in distilled water, adjust the pH with KOH of the resulting solution to pH 3.2.

Solution

Ringer Solution for dilutions (1/4 *strenght*; Dept. of Health & Social Security, 1937). Dissolve one tablet preparation (LAB M, International Diagnostics Group) in 500 ml of deionized water. Sterilize by autoclaving at 121 ° C for 15 minutes.

Growth conditions

The yeast strains were grown at 25°C, the liquid cultures, used for inoculing the fermentations, were shaken (130rpm) during incubation.

5.2.3 Single-strain fermentation in synthetic must

5.2.3.1 Yeast inoculum preparation

Yeasts were grown on YPD solid medium at 25 ° C for 3 days. The cultures obtained were used to inoculate 30 ml of YPD liquid medium. The tubes were left incubated for 24 hours at 25 °C to reach the stationary phase (about 10^7-10^8 cells/ml).

Cell concentration was measured by microscopy count with Thoma chamber using a phase contrast microscope (Olympus).

5.2.3.2 Test preparation

Two fermentation trials were set up in synthetic must with different nitrogen content. The yeast-assimilable nitrogen (YAN) content in high nitrogen condition (HNC) was 300 mg N/l: ammoniacal nitrogen (NH4 Cl) 120 mg N/l and amino acids 180 mg N/l. The low nitrogen condition (LNC) contained 1/5-fold (60 mg N/l) the YAN present in HNC. The proportions of the different amino acids and ammonium were maintained in the HNC and LNC synthetic musts..

Based on the yeast concentration present in the pre-culture, for each strain, the suitable volume to obtain a final concentration of $2x10^6$ cells/ml in 200 ml of medium was used.

Fermentations were performed in 250 ml glass bottles containing 200 ml of SNM and fitted with closures that enabled the carbon dioxide to escape and the samples to be removed. Fermentations were done in triplicate at 25 °C with continuous orbital shaking (150 rpm). Fermentations were monitored by measuring the changing in the medium density. Yeast cell concentration was determined by plate counts on YPD medium. Cell samples were collected throughout the fermentation at different time points. Cells were harvested by centrifugation, frozen in liquid nitrogen and stored at -80 °C. Supernatant was also stored at -20 °C to analyze the content of nitrogen and some fermentation products.

5.2.4 Co-fermentation in synthetic must

5.2.4.1 Yeast inoculum preparation

Yeasts were grown on YPD solid medium at 25 ° C for 3 days. The cultures obtained were used to inoculate 30 ml of YPD liquid medium. The tubes were left incubated for 24 hours at 25 °C to reach the stationary phase (about 10^7-10^8 cells/ml).

Cell concentration was measured by microscopy count with Thoma chamber using a phase contrast microscope (Olympus).

5.2.4.2 Test preparation

Two fermentation trials were set up in synthetic must with different nitrogen content. The yeast-assimilable nitrogen (YAN) content in high nitrogen condition (HNC) was 300 mg N/l: ammoniacal nitrogen (NH4 Cl) 120 mg N/l and amino acids 180 mg N/l. The low nitrogen condition (LNC) contained 1/5-fold (60 mg N/l) the YAN present in HNC. The proportions of the different amino acids and ammonium were maintained in the HNC and LNC synthetic musts.

Based on the yeast concentration present in the pre-culture, in co-fermentation trial, for each strain, the suitable volume to obtain a final concentration of $1x10^6$ cells/ml in 150 ml of medium was used. In single-strain fermentation, the inoculum final concentration was $2x10^6$ cells/ml, while in co-fermentation where all the four strains were present, for each strain, cell concentration was of $5x10^5$ cells/ml.

Fermentations were performed in 200 mL glass bottles containing 150 mL of SM and fitted with closures that enabled the carbon dioxide to escape and the samples to be removed. Fermentations were done in triplicate at 25 °C with continuous orbital shaking (150 rpm). Fermentation kinetics was monitored by daily measuring the weight loss. The fermentations were considered completed when the daily weight loss was lower than 0,1 g within 24 hours.

Yeast isolation was carried out on YPD medium at three sampling points (12, 24, 48 hours) and 20 colonies, randomly chosen from each sample, were submitted to amplification of inter-δ region.

5.2.5 Amplification of inter-δ region.

5.2.5.1 Sample preparation for DNA amplification

Yeast colonies (1–2mm diameter), grown for 1–3 days, were picked up with a sterile toothpick from YPD plates and resuspended in 20 µL of sterile deionized water in 0.5mL tubes. Two microlitres of the suspension were used for PCR amplification.

5.2.5.2 PCR-amplification of delta sequences

The various components of the reaction mixture were used in the following final concentrations:

Table 5.2.2 PCR master mix composition

Delta 12	1 µM
Delta 21	1 µM
dNTPs (Amersham)	200 µM
Taq polimerasi (Promega)	0,02 U/µ
Buffer	1X
DNA	2 µl cellular suspension

Primers utilized are reported below (table 5.2.3).

Table 5.2.3 Primers for ITS1-ITS4 amplification

Name	Length	Sequence (5'-3')	Source
Delta 12	19 nt	TCAACAATGGAATCCCAAC	Legras *et al*.,2003
Delta 21	20 nt	CATCTTAACACCGTATATGA	Legras *et al*.,2003

The thermal protocol was as follow:
initial incubation at 95°C for 4 min to allow cell lysis and DNA denaturation, followed by 35 cycles composed of denaturation at 95°C for 30 s, annealing at 46°C for 30 s and extension at 72°C for 90 s. A final extension step was added at 72°C for 10 min.

Amplified samples were run on 1% agarose gel with 0,1 µg/ml of ethidium bromide. The running was performed with TBE 0,5X (44,5 mM Tris, 44,5 mM boric acid, 1 mM EDTA) on a potential difference of 100 V.

Digital images were acquired with an EDAS290 image capturing system (Kodak, Rochester, NY).

5.2.6 Nitrogen content analysis

Analysis of ammonia and individual amino acids was determined by diethyl ethoxymethylenemalonate (DEEMM) derivatization (Gómez-Alonso, Hermosín-Gutiérrez, & García-Romero, 2007), using the Agilent 1100 Series HPLC (AgilentTechnologies, Germany). Separation was performed in an ACE HPLC column (C18-HL), particle size 5 µm (250 mm × 4.6 mm) thermostated at 20 °C. The concentration of each compound was calculated using internal (L-2-aminoadipic acid, 1 g/l) and external standards, and expressed as mg N/l. The software used was Agilent ChemStation Plus (Agilent Technologies, Germany).

5.2.7 Real-time quantitative PCR

Total RNA was isolated from yeast samples as described by Sierkstra et al. (1992) and resuspended in 50 µl of diethyl pyrocarbonate-treated water. cDNA was synthesized from total RNA using Superscript^{TM} II RNase H^{-} Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) in a GenAmp PCR System 2700 (Applied Biosystems, Foster City, CA, USA). The protocol provided by the manufacturer was used. The PCR primers used in this study are *ACT-F*, TGGATTCCGGTGATGGTGTT, and *ACT-R*, CGGCCAAATCGATTCTCAA (*ACT*, for actine gene); *GAP1-F*, CTGTGGATGCTGCTGCTTCA, and *GAP1-R*, CAA-CACTTGGCAAACCCTTGA (*GAP1*, for general amino acid permease gene); and *MEP2-F*, GGTATCATCGCTGGCCTAGTG, and *MEP2-R*, CAACGGCTGACCAGATTGG (*MEP2*, for ammonium permease gene) The real-time quantitative PCR reaction was performed using SYBR® Green I PCR (Applied Biosystems, USA). The quantity of the studied gene was normalized with actin gene, as housekeeping gene. In the PCR reaction, the final reaction volume was 25 µl, the final concentration of each primer was 300 nM,

together with 1 µl of the cDNA previously synthesized from total RNA. All PCR reactions were mixed in 96-well optical plates and cycled in a GeneAmp 5700 Sequence Detection System (Applied Biosystems, USA) under the following conditions: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles at 95 °C for 15 s and at 60 °C for 60 s. The threshold was positioned to intersect the exponential part of the amplification curve of positive reactions, as recommended by Applied Biosystems. Each sample had two controls, which were run in the same quantitative PCR: NAC (No Amplification Control; sample without reverse transcriptase reaction) to avoid the interference by contaminant genomic DNA and NTC (No Template Control; sample without RNA template) to avoid interference by primer-dimer formation. Relative gene expression 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 of the gene of interest and Ct of the housekeeping gene (ACT1); and ΔΔCt is the difference in ΔCt at time = t and ΔCt at time = 12 h in the non-limiting condition (300 mg N/L). All samples were analyzed in duplicate and the expression values were averaged by the analysis software (Applied Biosystems, USA) (Gutiérrez A.*et al*.2013).

5.2.8 Chemical analysis

The glucose, fructose, glycerol, acetic acids and ethanol were quantified using commercial enzymatic kits (Roche Diagnostics, Germany). The pH was determined using a pH-meter Crison MicropH 2000 (Crison, Barcelona, Spain).

5.2.9 Statistical analysis

The software XLSTAT, vers.7.5.2, was used to performe the analysis of variance (one-way ANOVA), followed by the Tukey "post-hoc" test. The analysis was conducted by comparing the averages of three independent replications and differences were considered statistically significant for p-value less than 0.05. Hierarchical clustering was performed using MeV MultiExperiment Viewer, and Pearson correlation metrics and group clustering based on group averages (average linkage).

5.3 RESULTS

5.3.1 Fermentation kinetics and yeast growth in presence of high and low nitrogen content

Fermentations in synthetic must with low (60 mg/l, LNC) and high nitrogen content (300 mg/l ; HNC) were carried out with the industrial *S. cerevisiae* strain QA23 and the following autochthonous vineyard strains: P301.4, P304.4 and P254.12. In presence of HNC (Figure 5.3.1) sugar consumption during exponential and early stationary phase was significantly higher in vineyard strains than in QA23. Afterwards QA23 was faster, closing the fermentation at the same time of the rest of the strain.

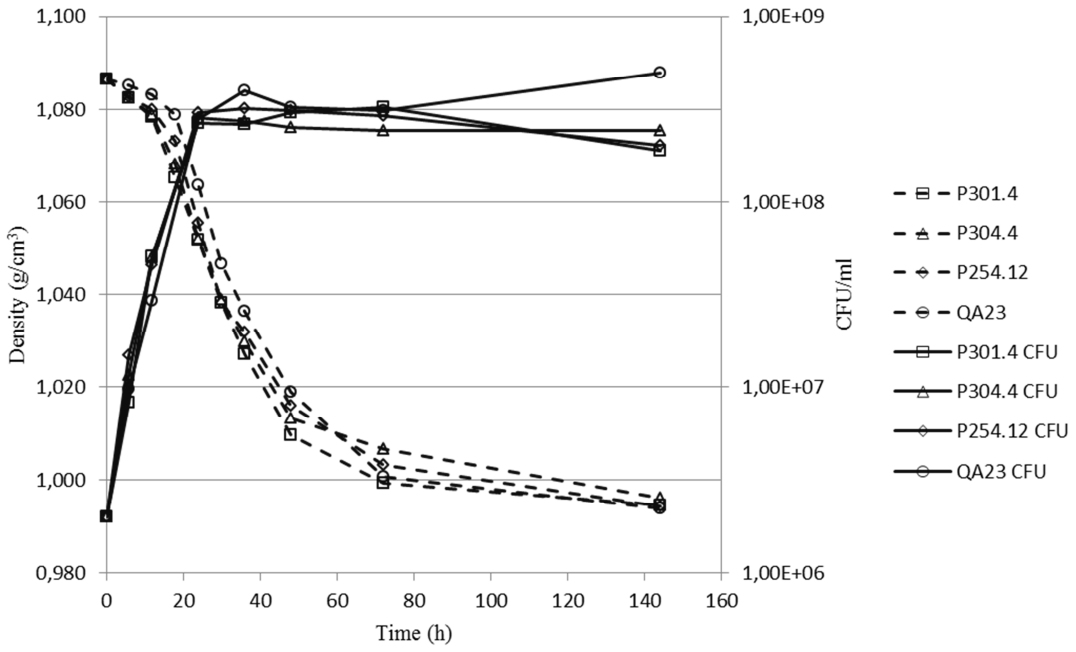


Figure 5.3.1. Must density and population evolution during fermentations in HNC.

When LNC was tested (Figure 5.3.2), although all the strains slowed notably down the fermentation rate, no significant difference in sugar consumption was found during exponential growth phase. When middle stationary phase was reached P304.4 was significantly slower than the rest of the strains showing a reduced culturable cell

concentration during the stationary phase, as well. Qa23, the fastest yeast closed the fermentation 140 hours earlier than P304.4 that showed a fermentation time of 15 days.

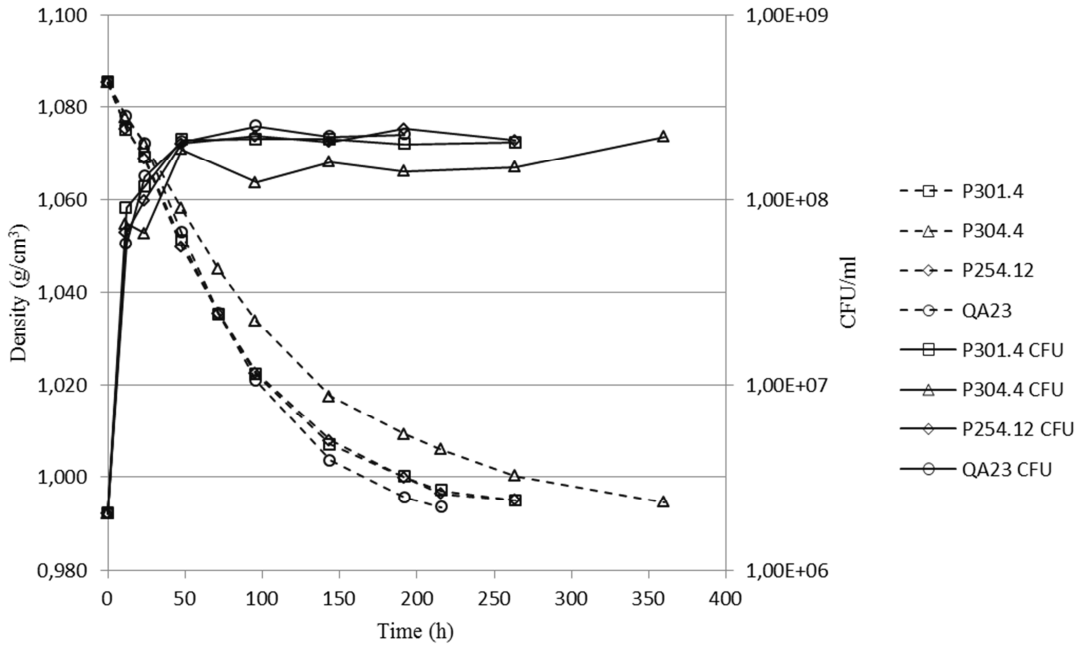


Figure 5.3.2. Must density and population evolution during fermentations in LNC.

No significant differences were found at the end of fermentation in ethanol and acetic acid production at both HNC and LNC. In the latter condition glycerol production (Figure 5.3.3) was significantly higher in P254.12 and P304.4, no significant differences were found when HNC was tested

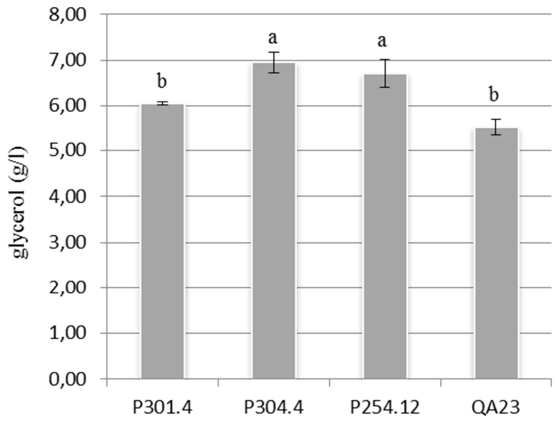


Figure 5.3.3 Glycerol production during fermentations in LNC. Statistical analysis: one-factor ANOVA (p value < 0.05). Different letters indicate statistically significant differences.

5.3.2 Nitrogen consumption and expression of genes under nitrogen catabolic repression

Consumption of ammonium and yeast assimilable nitrogen (YAN) was monitored throughout these fermentations. In HNC (Figure 5.3.4) in the first 12 hours the former was assimilated more rapidly by P301.4, the strain that showed poor fermentation performance when grow in LNC. No differences were found among the rest of the strains. Afterwards, as expected, QA23 consumed nitrogen more slowly than the other strains that used all the NH_4^+ amount within 24 hours. The YAN consumption retraced the same kinetics. During exponential growth phase amino acid uptake was faster in P301.4 with no significant differences in YAN consumption among the other strains. After 24 hours in these strains YAN dropped to 4,4 g/l, a residual concentration mainly composed by cysteine.

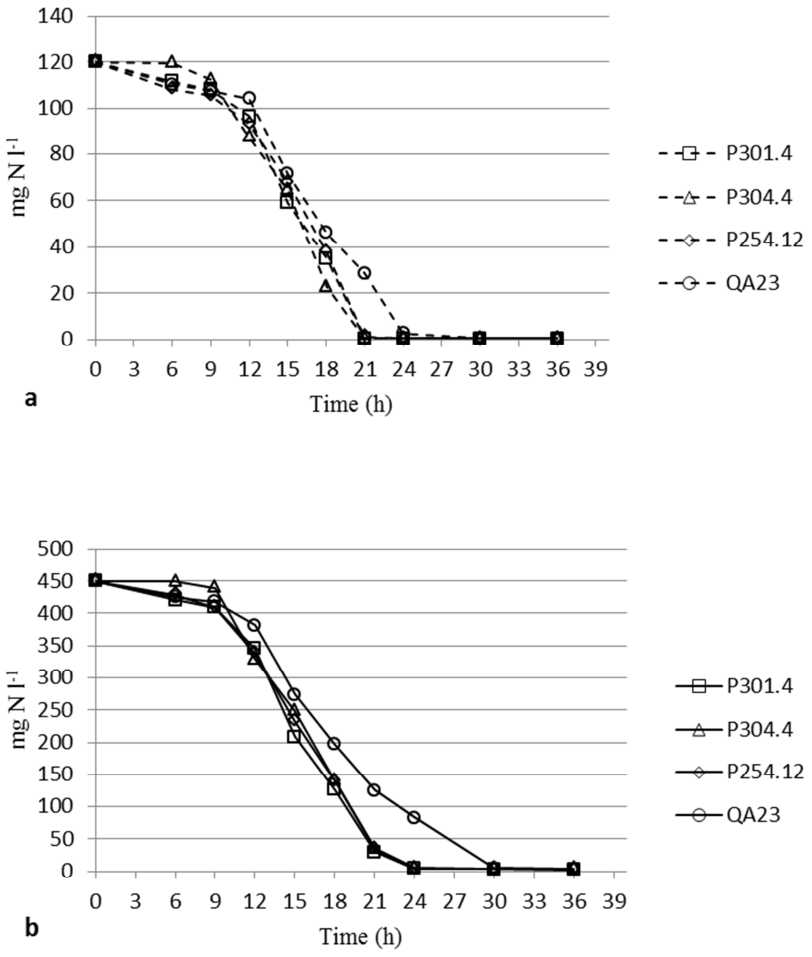


Figure 5.3.4. Ammonium (a) and yeast assimilable nitrogen (YAN) (b) concentration during fermentations in HNC.

In presence of LNC all the strains consumed ammonium very rapidly, within 12 hours (data not showed). At the same sampling point YAN was depleted by all vineyard strains. While QA23 still showed a consistent residue (10 mg/l)

In both conditions, for each amino acid the consumption trend was determined by means of HPLC analysis. In HNC the MEV analysis of amino acid consumption (Figure 5.3.5) showed a conserved pattern among strains, with some amino acids that were more rapidly consume (methionine, threonine, leucine, serine, glutamine, histidine, aspartic acid) than the others. The amino acid consumption trend evidenced differences between the vineyard group and the commercial strain. After 12 hours the vineyard strains completely consumed the following amino acids: serine, histidine, threonine, valine, isoleucine, leucine, phenylalanine, glutamic acid, and glutamine. QA23 showed similar trend, but in the case of valine and glutamine the consumption is reduced (30% and 75% of the initial concentration, respectively). At the same sampling point the concentration of aspartic acid, tyrosine, methionine, tryptophan and arginine dropped to 50% in the vineyard strains. Within this group tyrosine and methionine concentrations were reduced to 75% by QA23. The vineyards strains reduced to 30% and 25% alanine and lysine contents respectively, whereas QA23 left 70% of alanine in the medium. At this sampling time, all the strains did not consume cysteine and glycine. Ammonium concentration dropped to 56,4% (66,8 mg/l N left in the medium) in QA23, 73,2% (41,2 mg/l) in P301.4, 87,1 (19,71 mg/l) in P304.4, and 91,3 (13,36 mg/l) in P254.12, Although the last two nitrogen concentrations were not statistically different (ANOVA test) these results evidenced larger ammonium consumption in vineyard strain that in the commercial yeast. After 24 hours vineyards strains completely consumed glycine, tyrosine, arginine and alanine, whereas in Qa23 fermentation 13 mg/l of arginine was still present. In LNC after 12 hour NH_4^+ was completed depleted in all fermentations and amino-acid concentrations was notably reduced. Vineyard strains completely consumed the same amino acids that were depleted after 12 hours in HNC, together with glycine, alanine, and the most abundant arginine, totally consume only after 24 in HNC. The consumption of the remaining amino acids was similar to that in HNC. At the same sampling point QA23 did not use glycine tyrosine and consumed 75% of methionine, 65% of valine and alanine, 50% of phenylalanine and tryptophan. All the strains left cysteine concentration unchanged in the medium as previously found in HNC.

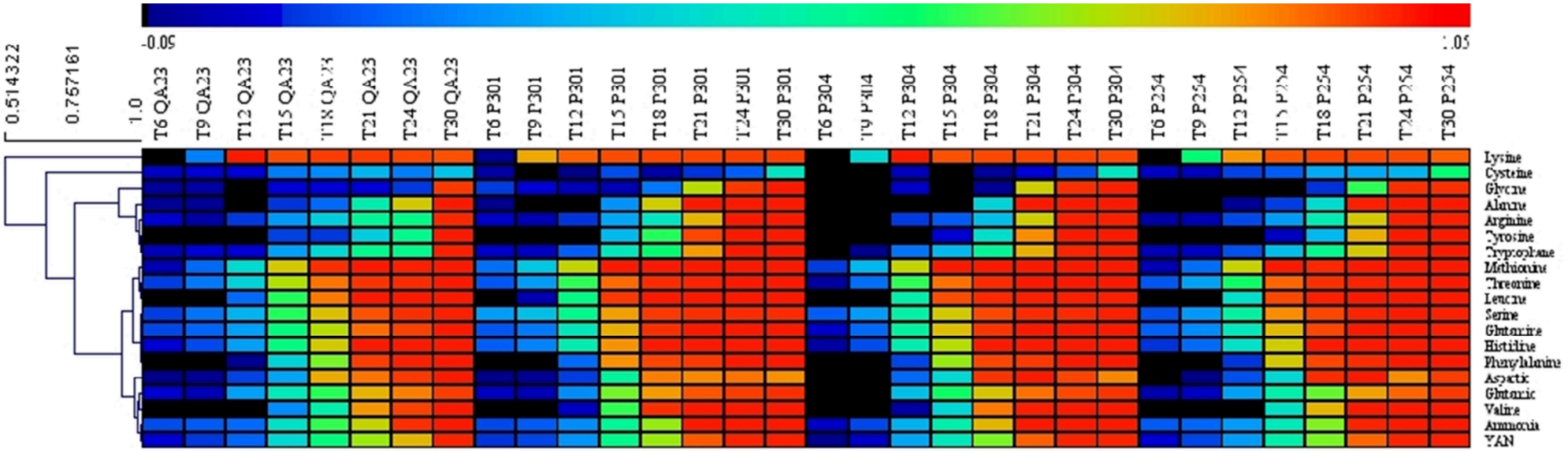


Figure 5.3.5 Hierarchical clustering of nitrogen sources (ammonium and amino acids) consumption at different sampling points during fermentations in HNC. Pearson correlation coefficient was used. Heatmap color reflects the normalized nitrogen content. Blue = high content, red = low content and black = initial nitrogen content of the must.

To evaluate the effect of different assimilation rate on nitrogen metabolism the expression of MEP2 and GAP1 genes was investigated during fermentation in both HNC and LNC (Figures 5.3.6 and 5.3.7). The general amino-acid permease GAP1 and the ammonium permease MEP2 are activated when NH_4^+ is very low. The former enhances the assimilation of amino acids, increasing the transport of those that do not have specific permeases that are constitutively expressed. The latter allows the complete consumption of ammonium (Beltran G *et al.* 2004). In both HNC and LNC P304.4 and P301.4, the two vineyards strains that mostly slow down the fermentation rate in LNC, showed the earliest and the strongest activation of both genes. In HNC this was particularly evident at 72 hours, while in LNC since 48 hours the expression level was very high.

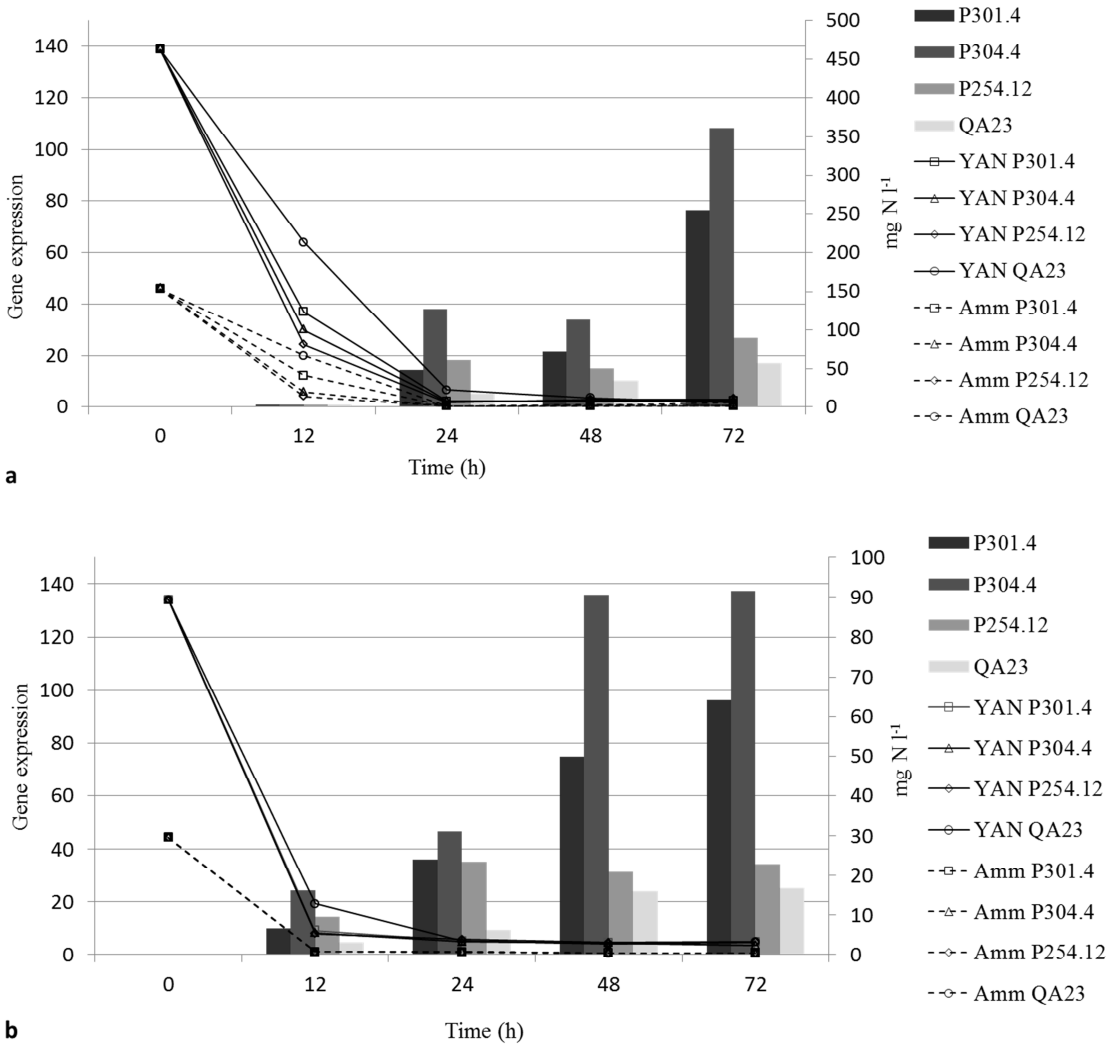


Figure 5.3.6 Expression of MEP2 gene, ammonium and yeast assimilable nitrogen (YAN) consumption during fermentations in HNC (a) and LNC (b). Gene expression values are normalized using as reference the housekeeping gene ACT1 (Actine). The ratio value 1 was set up as 100%.

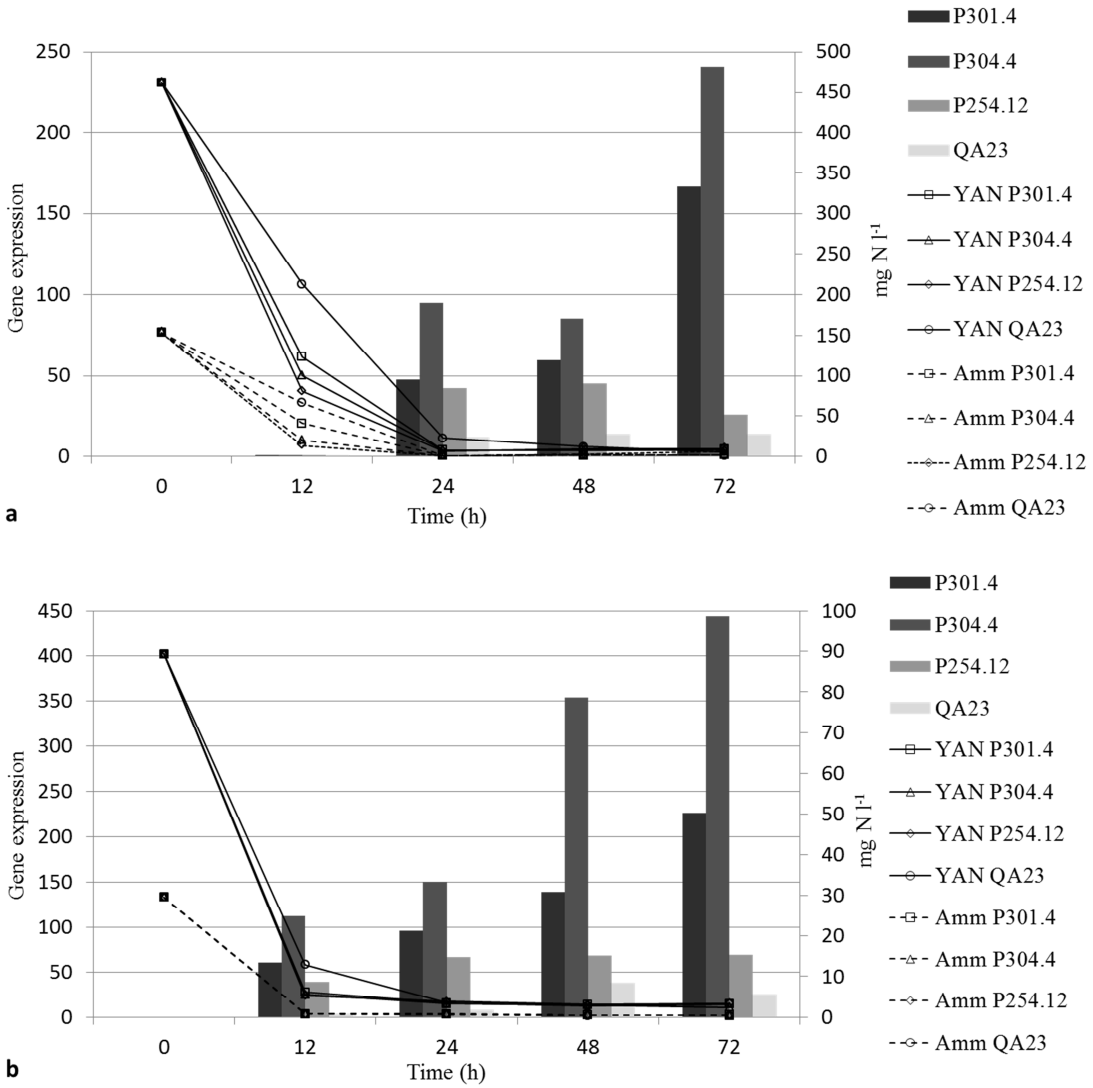


Figure 5.3.7 Expression of GAP1 gene, ammonium and yeast assimilable nitrogen (YAN) consumption during fermentations in HNC (a) and LNC (b). Gene expression values are normalized using as reference the housekeeping gene ACT1 (Actine). The ratio value 1 was set up as 100%.

5.3.3 Pairwise strain fermentations and yeast dynamics

In order to investigate if different nitrogen needs could influence colonization ability during must fermentation, pairwise strain fermentations were performed in synthetic must at HNC (MS300) and LNC (MS60). Each time Qa23 was co-inoculated with one of the vineyard strains. Single strain fermentations were also run, as control. Moreover, in a set of trial the four strains were inoculated together at the same cell concentration. In HNC sugar consumption of the single strain fermentations confirmed the faster fermentation rate

of P304.4 and P301.4, the strains with the highest nitrogen demand, with respect to QA23 (Table 5.3.1). As expected QA23, increased fermentation rate later, consuming during late stationary phase the highest amount of sugars. In the pairwise fermentations the sugar consumption during the exponential phase, was variable, but when late stationary phase was reached sugar consumption was lower than that found in QA23 single fermentation. This was particularly evident for the strain P304.4 the one with the highest nitrogen demand. When LNC was present, at late stationary phase, P304.4 tested in single fermentation consumed less sugar that QA23, as expected, and showed a population size statistically smaller than the commercial strain. In most of the case in the pairwise strain fermentations no differences were found in sugar consumption and population size with respect to QA23.

Fementation	Sugars consumption (g/100ml)			Population size (CFU/ml)
	HNC		LNC	LNC
	Exponential phase	Late stationary phase	Late stationary phase	Late stationary phase
QA23	5,62±0,18 bc	17,06±0,33 a	18,90±0,09 a	1,18E+08±1,11E+07 a
P301.4	6,26±0,10 a	16,02±0,11 ab	18,45±0,05 ab	5,97E+07±6,11E+06 d
P254.12	6,16±0,28 ab	16,18±0,36 ab	18,06±0,28 ab	1,03E+08±4,00E+06 ab
P304.4	6,26±0,04 a	14,70±0,17 cd	17,74±0,28 bc	6,53E+07±1,25E+07 cd
QA23-P301.4	4,69±0,07 d	16,41±0,85 ab	16,75±0,88 c	8,46E+07±4,91E+06 bc
QA23-P254.12	6,05±0,12 ab	15,57±0,27 bc	18,10±0,42 ab	1,08E+08±4,00E+06 ab
QA23-P304.4	5,73±0,40 abc	14,55±0,53 cd	18,29±0,22 ab	7,76E+07±6,85E+06 cd
QA23-P301.4-P254.12-P304.4	5,23±0,05 cd	14,11±0,26 d	18,04±0,16 ab	7,82E+07±1,18E+07 cd

Table 5.3.1 Sugars consumption and yeast population size in single-strain and co-fermentations in HNC and LNC. Data are means ± standard deviations of three trials. Values displaying different letters (a, b, c, d) within each column are significantly different according to the Tukey test (0.05 %).

In the pairwise strain fermentations the presence of the inoculated strains was monitored by means of interdelta analysis on 20 colonies randomly chosen at three sampling points, 12, 24, 48 hours after strains inoculation (Tables 5.3.2 and 5.3.3).

In HLC, in most of the fermentations, QA23 dominated and the strain presence was always very high, ranging from 44 to 100%.

Co-fermentation	Strain presence		
	T12	T24	T48
QA23-P301.4	QA23(100), P301.4(0)	QA23(100), P301.4(0)	QA23(100), P301.4(0)
QA23-P254.12	QA23(75), P254.12(25)	QA23(70), P254.12(30)	QA23(95), P254.12(5)
QA23-P304.4	QA23(70), P304.4(30)	QA23(70), P304.4(30)	QA23(50), P304.4(50)
QA23-P301.4-P254.12-P304.4	QA23(44), P301.4(0), P254.12(30), P304.4(26)	QA23(44), P301.4(0), P254.12(32), P304.4(24)	QA23(48), P301.4(4), P254.12(8), P304.4(40)

Table 5.3.2 Strains frequency evaluated at three sampling points (12, 24 and 48 hours) during co-fermentations in HNC.

Co-fermentation	Strain presence		
	T12	T24	T48
QA23-P301.4	QA23(90), P301.4(10)	QA23(95), P301.4(5)	QA23(80), P301.4(20)
QA23-P254.12	QA23(40), P254.12(60)	QA23(50), P254.12(50)	QA23(45), P254.12(55)
QA23-P304.4	QA23(60), P304.4(40)	QA23(45), P304.4(55)	QA23(40), P304.4(60)
QA23-P301.4-P254.12-P304.4	QA23(30), P301.4(0), P254.12(30), P304.4(40)	QA23(40), P301.4(0), P254.12(25), P304.4(35)	QA23(35), P301.4(10), P254.12(25), P304.4(30)

Table 5.3.3 Strains frequency evaluated at three sampling points (12, 24 and 48 hours) during co-fermentations in LNC

Surprisingly strain P301.4 was not found among the tested colony in HNC, and its concentration was very poor also when LNC was tested, indicating a very limited colonization ability. This could be due to the presence of killer toxins produced by QA23. In fact P304.4 is the only strain, within the vineyard group, sensitive to the killer toxin K2 produced by wine yeasts (data not showed). In HNC, among the vineyard strains, P304.4, showed the highest colonization ability particularly when the stationary phase was reached (48 hours) achieving 50% of presence. In mix fermentation, with the simultaneous presence of all the vineyard strains, QA23, although the most abundant, halved the cell concentration. In LNC the vineyard strains were generally more competitive. Qa23 was clearly the dominant strain only when P301.4 was present. In presence of P254.12 during early exponential (12 hours) only 40% of the colonies possessed the genetic profile ascribed to QA23, the presence increased up to 45% during stationary phase. In presence of P304.4 an opposite trend was observed Qa23 cell concentration decreased from 60% to 40%. In mix fermentation the presence of Qa23 was lower than in HNL.

5.4 DISCUSSION

The *S. cerevisiae* strain QA23 is one of the most studied wine industrial strain.

This yeast is well known to have a low nitrogen demand and strong ethanol resistance during vinification and, as all commercial wine strains, was selected for the excellent ability to dominate the microbiota during fermentation. The autochthonous yeasts P301.4, P304.4 and P254.12 were isolated by single grape bunch fermentation. In each fermentation, co-domination of at least 3 strains was found. In the case of P304.4 nine different strains were present simultaneously. In this work the four yeasts were tested in single strain fermentation using synthetic musts with low and high nitrogen content, to evaluate the request related to such macronutrient. When high nitrogen concentration was present only small differences were found among the fermentation kinetics. Notwithstanding, QA23 sugar consumption during exponential and early stationary phase was significantly slower than those found for the other yeasts. On the contrary strong difference in the fermentation kinetics were evidenced when low nitrogen was present, indicating P304.1, the strains with highest nitrogen demand, followed by P301.4. When ammonium and amino acids consumption was determined, strong differences between vineyard strains and the commercial QA23 were found confirming the lower nitrogen demand of strain QA23. In particular in HNC after 12 hours P304.4, P254.12 showed a highest ammonium consumption (around 90% depletion) compared to P301.4 (70%) and QA23 (56%). It interesting to note that the two strains (P304.4, P254.12) showing the higher nitrogen demand evidenced a significantly higher glycerol production. This could be due to higher pyruvate quantity that escapes the alcoholic fermentation to satisfy amino acid biosynthesis. When nitrogen is poor, pyruvate is re-routed to alternative metabolism such as glyceropyruvic fermentation.

Regarding amino acid assimilation the pattern seems to be very conserved among the strains, indicating that the main difference between QA23 and the vineyard group is the assimilation rate. All the strains showed the same preference in amino-acid consumption: methionine threonine, leucine, serine, glutamine are consumed faster. Glycine consumption started later and cysteine contents remained unchanged. To evaluate the effect of different assimilation rate on nitrogen metabolism the expression of MEP2 (ammonium permease) and GAP1 (general amino acids permease) genes was investigated. *Saccharomyces*

cerevisiae is able to use different nitrogen sources for growth but not all nitrogen sources support growth equally well. *S. cerevisiae* selects nitrogen sources that enable the best growth by a mechanism called Nitrogen Catabolite Repression (NCR) (Wiame J.M. et al. 1985; Magasanik B. 1992). Good nitrogen sources such as glutamine, or ammonium decrease the level of enzymes required for utilization of poorer nitrogen sources (Ter Schure, E.G. *et al*., 2000). Amino acids are transported into the cell by general and specific transport systems. The general high-capacity permeases like GAP1 and AGP1 or the specific proline permease PUT4 are nitrogen-regulated and become down-regulated at the transcriptional as well as the posttranslational level, in response to high-quality nitrogen sources like ammonium (Forsberg H. & Ljungdahl O. 2001). However, specific permeases like the histidine permease (HIP1), the lysine permease (LYP1) and the basic-amino-acid permease CAN1 are expressed constitutively (Ter Schure, E.G. *et al*., 2000). The MEP genes related to ammonium uptake are also subjected to nitrogen control. These genes are expressed when low ammonium concentrations are present in the growth medium, but at high concentration of a good nitrogen source (including ammonium) all three MEP genes are repressed. With a poor nitrogen source, MEP2 expression is much higher than MEP1 and MEP3 expression (Marini A.M. *et al*., 1997) The data confirmed that the amino acids assimilation rate is different among the strains. In particular in HNC the strains that grew and fermented faster during exponential grow phase, namely P304.4 and P301.4, where those removing earlier the nitrogen catabolite repression. In order to investigate if different nitrogen needs could influence colonization ability during must fermentation, pairwise strain fermentations were performed in synthetic must at HNC and LNC. Results suggested a strong implication of nitrogen assimilation ability on must colonization. In fact, the colonization ability of the vineyard strains in LNC was always higher than that found in HNC. Moreover, in HNC the strain with highest nitrogen demand, P304.4, is the one that strongest opposed to QA23 colonization. During the fermentation P304.4 colonization ability increased, indicating better performance when nitrogen was depleted. The increasing competitiveness found in vineyard strains as nitrogen was reduced could be due to the higher nitrogen assimilation rate possessed by these yeasts. As in natural must up to 10-20 strains can be present simultaneously during fermentation, those with the highest nitrogen demand could quickly remove nitrogen that is no longer available, reducing cell growth and

fermentation performance of the less demanding yeasts. Many strategies are known to be adopted by yeasts to dominate enological environment, among them the killer toxin production is one of the most studied. In this work we demonstrated that also yeast nitrogen assimilation attitude do strongly influence the possibility of strain to grow and colonize grape must.

CHAPTER 6

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