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TESI DI DOTTORATO

Management of microbial resources for the production of typical Apulian wines

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“ . . . alcoholic fermentation is a process correlated with the life and organization of yeast cells, not with the death or putrefaction of the cells. Nor is it a phenomenon of contact, for in that case the transformation of the sugar would occur in the presence of the ferment without giving anything to it or taking anything from it (Pasteur, 1860)”

SUMMARY

ABSTRACT

Keywords: alcoholic fermentation, yeast, *Saccharomyces cerevisiae*, non-*Saccharomyces, Oenococcus oeni*, RFLP analysis, interdelta sequences, VNTR, co-inoculation, sequential inoculation**.**

BACKGROUND

Grape juice fermentation is a complex biochemical process in which wine yeasts play fundamental roles during their transformation of grape sugars into ethanol, carbon dioxide and hundreds of other secondary products.

The yeast ecology of the fermentation process is more complex than previously thought and non-*Saccharomyces* yeast species play relevant roles in the metabolic impact and aroma complexity of the final product.

To improve the chemical composition and sensory properties of wine, the inclusion of non-*Saccharomyces* wine yeasts, together with *Saccharomyces* strains as part of multi-starter fermentations, has been proposed as a tool to take advantage of spontaneous fermentation, while avoiding the risks of stuck fermentations.

AIM OF THE STUDY

The specific objective of this work was to isolate microbial strains (yeast and bacteria) representing the "virtuous" microbial biodiversity of several typical wine productions.

MATERIAL & METHODS

Yeast and bacteria were selected during spontaneous fermentations of several Apulian wines and from grape berries surface. The yeast strains were identified using restriction pattern analysis of the internal transcribed spacer region (5.8S-ITS), sequence of the internal transcribed spacer region (5.8S-ITS), species-specific primers, and interdelta analysis for *Saccharomyces cerevisiae* strains characterization. Technological characterization of selected *S. cerevisiae* and non-*Saccharomyces* strains was perform with in vitro, with a traditional polyphasic approach, and in vivo. The biodiversity of *O. oeni* strains isolated from wine undergoing spontaneous MLF, focusing attention on genetic biodiversity and on the different efficiency in degrading malic acid. For genotypic characterization, we utilized two different molecular tecniques: variable number tandem repeat (VNTR) and multilocus sequence typing (MLST). Furthermore, two strains of *S. cerevisiae* (named *S. cerevisiae* I6 and *S. cerevisiae* E4) were selected in

function of their fermentative behaviour. For microvinification assay, selected *O. oeni* strains were inoculated either with yeast (co-inoculum approach) or after the completion of AF (sequential inoculum approach).

RESULTS

A great biodiversity of yeast strains of oenological interest was observed. Indeed, strains belong to *H. uvarum, C. stellata, S. cerevisiae*, and *M. pulcherrima* were identified, with strains of *M. pulcherrima* and *H. uvarum* representing the major part of the yeasts analyzed.

The first step of technological characterization (e.g., killer activity, H_2S production, fermentation kinetics in synthetic medium and in must, cytofluorometric analysis) led us to select the most promising *S. cerevisiae* and non-*Saccharomyces* strains, mainly belong to *Hanseniaspora* and *Candida* species, and to assess the performance of possible co-inoculation approaches. However, using two classical strategies of inoculum and planned to promote the non-*Saccharomyces* 'expression', a strong competition took place with some of the non-*Saccharomyces* strains, compromising an efficient alcoholic fermentation. Delta analysis of *S. cerevisiae* strains shown a great biodiversity, distinguished 86 different delta profiles among 90 strains analyzed.

Among the 50 *O. oeni* strains isolated from different wines, Variable number of tandem repeats identified 30 profiles, of which 11 unique profiles, while 20 strains analyzed by Multilocus sequence typing ere differentiated in 8 different ST, of which 6 were new ST.

Different results related to the efficiency of malolactic fermentation (MLF) were observed when different association of yeast and bacteria were analyzed. For instance, some *O. oeni* strains performed an improved MFL when associated with *S. cerevisiae* I6. However, the improved MFL was undetectable, when the same *O. oeni* strains were associated to *S. cerevisiae* E4.

Overall, the finding reported are fruitful in order to manage microbial resources for typical wines production.

RIASSUNTO

Parole chiave: fermentazione alcolica, lieviti, *Saccharomyces cerevisiae*, non-*Saccharomyces, Oenococcus oeni*, analisi RFLP, sequenze interdelta, VNTR, coinoculo, inoculo sequenziale**.**

INTRODUZIONE

La fermentazione del succo d'uva è un processo biochimico complesso, in cui i lieviti svolgono un ruolo fondamentale trasformando gli zuccheri dell'uva in etanolo, CO_2 e altre centinaia di composti secondari.

L'ecologia dei lieviti, responsabili della fermentazione alcolica, risulta essere più complessa rispetto a quanto precedentemente pensato, dimostrando inoltre che i lieviti non-*Saccharomyces* svolgono un ruolo rilevante sull'impatto metabolico e la complessità aromatica del prodotto finito.

L'inclusione di lieviti vinari non-*Saccharomyces*, insieme a ceppi di *Saccharomyces* in multi-starter per la fermentazione alcolica è stato proposto come strumento per migliorare la composizione chimica e le proprietà sensoriali del vino, traendo vantaggio dalle fermentazioni spontanee ed evitando il rischio di arresti fermentativi.

SCOPO DEL LAVORO

L'obiettivo principale di questo lavoro è stato di isolare ceppi microbici (lieviti e batteri) rappresentanti la biodiversità della microflora "virtuosa" dei vini tipici pugliesi studiati.

MATERIALI E METODI

Lieviti e batteri sono stati isolati da fermentazioni spontanee di vini Pugliesi e da bucce d'uva. L'identificazione dei lieviti è stata ottenuta mediante analisi di restrizione e sequenziamento della regione compresa fra gli *internal transcribed spacers* e la frazione 5.8S del RNA ribosomiale (5.8S-ITS), inoltre i ceppi di *Saccharomyces cerevisiae* sono stati identificati mediante PCR specie-specifici e caratterizzati genotipicamente con l'analisi delle sequenze interdelta.

La caratterizzazione tecnologica dei ceppi selezionati di *S. cerevisiae* e non-*Saccharomyces* è stata condotta con in vitro, mediante un tradizionale approccio polifasico, e in vivo. In oltre è stata investigate la biodiversità dei ceppi di *O. oeni* isolati da vini in cui era in corso la fermentazione malolattica spontanea. In particolare si è deciso di focalizzare l'attenzione sulla biodiversità genetica e sulla diversa capacità di degradare l'acido malico. Per la caratterizzazione genotipica sono state utilizzate due tecniche molecolari: l'analisi *variable number tandem repeat* (VNTR) e l'analisi

Multilocus Sequence typing (MLST). Due ceppi di *S. cerevisiae* (*S. cerevisiae* I6 e *S. cerevisiae* E4) sono stati selezionati per le loro proprietà fermentative per successive esperimenti di microvinificazione, i batteri sono stati inoculati con due diversi tempi di inoculo, insieme al lievito (co inoculo) o alla fine della fermentazione alcolica (inoculo sequenziale).

RISULTATI

Fra i ceppi isolati ed identificati è stata osservata una grande biodiversità di ceppi di interesse enologico, infatti sono stati identificati ceppi di *H. uvarum* , *C. stellata, S. cerevisiae* e *M. pulcherrima*. In particolare la maggior parte dei ceppi analizzati appartengono ai generi *M. pulcherrima* e *H. uvarum.*

Il primo step della caratterizzazione tecnologica (attività killer, produzione di H_2S, cinetiche di fermentazione in terreno sintetico e mosto, analisi citofluorometriche) ci ha permesso di selezionare i ceppi di lieviti più promettenti, sia ceppi di *S. cerevisiae* che non-*Saccharomyces*, fra questi ultimi la maggior parte dei ceppi analizzati appartengono alle specie *Hanseniaspora* e *Candida*.

Inoltre ci ha permesso di valutare le performances di starter *multi-strain* (*Sacch*-non-*Sacch*.). Tuttavia, utilizzando due classiche strategie di inoculo, pianificate per promuovere l'espressione dei ceppi non-*Saccharomyces*, è stata notata una forte competizione fra i ceppi di *S. cerevisiae* e i non-*Saccharomyces,* compromettendo l'efficienza della fermentazione alcolica.

L'analisi PCR delle sequenze di S. cerevisiae ha mostrato una grande biodiversità, permettendo di distinguere 86 diversi biotipi su 90 ceppi analizzati.

Sono stati analizzati 50 ceppi di *O. oeni*, isolati da diversi vini, fra questi 50 ceppi la tecnica VNTR ha permesso di distinguere 31 profili differenti, di cui 11 profili unici, mentre i 20 ceppi analizzati con la tecnica MLST sono stati differenziati in 8 diversi ST, di cui 6 nuovi.

Diversi risultati relativi all'efficienza della FML sono stati osservati utilizzando diverse coppie di lieviti e batteri. Per esempio, alcuni ceppi di *O. oeni* hanno portato ad una fermentazione malolattica più efficiente quando coinoculati con *S. cerevisiae* I6, mentre altri *O. oeni* hanno svolto una fermentazione malolattica migliore se utilizzati con *S. cerevisiae* E4.

In generale, i risultati ottenuti permetteranno di gestire al meglio le risorse microbiche per la produzione di vini tipici pugliesi.

1. INTRODUCTION

Wine and fermented food wine has been with us since the beginning of civilization. Wine born more than 7,000 years ago from damaged grapes that spontaneously fermented in harvesting vessels.

Early inventions and innovations in grape and wine production were based on little or no knowledge of the biology of grapevines or the microbes that drive fermentation. In fact, it would be several thousand years before it was even known that microscopic organisms exist: using a primitive microscope, Antonie van Leeuwenhoek observed cells for the first time in 1680, and then scientific knowledge grows laying the foundation of our understanding of the biology of the microorganisms that drive fermentation (Chambers & Pretorious, 2010).

Under the generic term of "wine", there is a diversity of quality that is quite unique among the products and determined mainly by interaction between grapes, yeasts and technology. Wine is a natural product resulting from a number of biochemical reactions, which begin during ripening of the grapes and continue during harvesting, throughout the alcoholic fermentation, clarification and after bottling (Romano *et al*., 2003a).

Winemaking involves two different fermentation steps (alcoholic fermentation and malolactic fermentation), various microorganism, like yeast, bacteria and filamentous fungi, and also interaction among them (Fleet, 2007; Fugelsang & Edwards, 2007).

The chemical composition and the quality of a wine depends on several factors, that included the geographical conditions, viticultural practices, grape variety, winemaking practices and techniques and the microbial ecology of the grape and fermentation processes, among these microorganisms have a prominent role and they can influence the quality of the grapes before the harvest, during the fermentation and during the ageing and/or conservation of the wine (Cole and Noble, 1997; Ciani *et al*., 2009).

Yeasts, bacteria and filamentous fungi all contribute to the microbial ecology of wine production and the chemical composition of wine, although yeasts have the dominating influence because of their role in conducting the alcoholic fermentation (AF) (Fleet, 1993; Fugelsang, 1997).

AF, the primary wine fermentation, is a complex biochemical process in which wine yeasts play fundamental roles and transforms grape sugars into ethanol, carbon dioxide and hundreds of other secondary products.

In modern wineries, AF is usually conducted with selected microbial starter such as *Saccharomyces cerevisiae* strains, in contrast to traditional spontaneous fermentations carried out by the microflora present on the grapes and in the winery. Instead, spontaneous natural fermentation is carried out through a sequence of different yeast species, naturally present on grapes or in the winery.

Until 1980s, the contribution of yeasts to wine making was seen as a simplistic concept. Essentially, grape juice underwent a spontaneous AF that was dominated by strains of *Saccharomyces cerevisiae* (Fleet 2008).

Usually inoculated cultures of *S. cerevisiae* are able to suppress indigenous yeast, either non-*Saccharomyces* species or other *Saccharomyces* strains and to dominate fermentation process.

However, over the last few decades, significant advances have occurred in our comprehension of the ecology, physiology, biochemistry and molecular biology of the yeasts involved in AF.

Currently, it is known that the ecology of yeast during fermentation process is more complex than previously thought, and that non-*Saccharomyces* strains species play relevant roles in the aroma complexity of the final product (Ciani *et al*., 2009).

Also in recent years, there has been a growing demand for new and improved wine-yeast strains that are adapted to different types and styles of wines (Pretorius, 2000).

In this context, the inclusion of non-*Saccharomyces* strains, together with *S. cerevisiae* strains, as part of mixed and multi-starter fermentations, has been proposed as a tool to take advantage of spontaneous fermentation, to avoid the risks of stuck fermentations and also to improve the chemical composition and sensory properties of wine (Heard, 1999; Rojas *et al*., 2003; Romano *et al*., 2003; Ciani *et al*., 2006; Jolly *et al*., 2006).

During the last years the phenomenon of organic products, including “organic wine” (i.e a wine made from grapes that have been grown with as little human impact as possible, obtained from organically

growing grapes without the help of or need for synthetic fertilizers, synthetic plant treatments, or herbicides), had become increasing popular and the market for organic food continues to grow. In this context, the food biotechnological scientific community should offer an organic-friendly solution able to reconcile organic viewpoint with safe food fermentations. The use of commercial starter cultures might lead to losses in "unique qualities" while wild natural fermentations can result in fermentation arrests and/or production of undesired metabolites responsible for wine depreciation (Spano *et al*., 2010; Capozzi *et al*., 2011). Hence, yeasts naturally present on grape berries and must have a unique composition and these indigenous yeasts impart distinct regional and desired characteristics to wines, are well adapted to the conditions of a specific wine-producing area and can improve organoleptic quality of wines (Nielsen *et al*., 1996). Therefore, the formulation and the production of a multi-strain microbial starter that mimics the natural diversity and function of the biotechnological processes might be a reliable alternative to organic uncontrolled fermentations, increasing the organoleptic qualities of production and minimizing the risk of foodborne pathogens, microbial toxic compound productions, and microbial spoilage.

The secondary wine fermentation is the malolactic fermentation (MLF) that is the bacterial driven decarboxylation of L-malic acid into L-lactic acid and CO_2 and is carried out by lactic acid bacteria (LAB), mainly belonging to the *Oenococcus oeni* species.

MLF caused an increase in wine pH, from 0.2 to 0.5 units, a decrease in titratable acidity, which translates into a decrease in wine sourness, an increase of microbial stability, thanks to the removal of potential carbon sources which could be used by wine spoilage yeasts and bacteria, and the bacterial production of various secondary metabolites, which can improve the organoleptic properties of wine.

MLF is controlled by inoculating commercial starters at high concentrations, enough to ensure *O. oeni* survival and activity. Nevertheless, the induction of MLF by inoculation with commercial starter of *O. oeni* is not always successful, because wine is a very harsh environment for bacterial growth (Spano and Massa, 2006).

1.1 Yeast flora on the grape, in the winery, and in the must

Grape must is a non-sterile substrate that contains several types of microorganisms, among microorganism participating in winemaking process yeast have a prominent rule, in fact someone affirmed that "Yeast makes wine and bacteria refines it" (Torija *et al*., 2001).

A good knowledge of the yeast that make AF and of the kinetics of their growth throughout fermentation are essential steps in understanding how yeasts impact on wine quality and to developed new directions (Fleet *et al*., 2008).

Thousands of yeast have been identified, but only 15 species are usually found in wine (Ribéreau-Gayon *et al*., 2006).

Fresh grape juice contains different yeast species, belonging to the genera Hanseniaspora, *Pichia*, *Candida*, *Kluyveromyces*, *Metschnikowia* and *Saccharomyces*. At times could be found also yeast belonging to the genera *Zygosaccharomyces, Saccharomycodes, Torulaspora, Dekkera* and *Schizosaccharomyces* (Fleet & Heard, 1993; Fleet, 2003).

These yeast flora come from grape berry and winery environment, they reach the grapes by wind and insect dispersal and are present on the wines from the onset of fruit ripening (Lafon-Lafourcade, 1983) and usually the major part of non- *Saccharomyces* strains (especially species of *Hanseniaspora*, *Candida*, *Pichia* and *Metschnikowia*) begins AF of grape juice, but they are soon replaced from *S. cerevisiae* strains, that dominates the mid to final stages of fermentation. Most often being found only *S. cerevisiae* strains in fermented juice at end of AF (Fleet & Heard, 1993; Fleet, 2003).

Main of the yeast found on the grapes belong to the genera *Hanseniaspora uvarum* (anamorph *Kloeckera apiculata*), that represent more than 50% of the flora isolated from grape berries (Fugelsang & Edwards, 2007). Usually on grape berries may be found in lesser proportions other yeast, that are obligate aerobic or weakly fermentative yeasts with very limited alcohol tolerance, belonged to the genera *Candida*, *Cryptococcus*, *Debaryomyces*, *Hansenula*, *Issatchenkia*, *Kluyveromyces*, *Metschnikowia*, Pichia, and *Rhodotorula* (Fleet & Heard, 1993; Ribéreau-Gayon *et al*., 2006).

Instead, species more fermentative, *Saccharomyces cerevisiae* and *Saccharomyces bayanus*, are present in limited numbers.

The microflora isolated from grape can be influenced by several factors, mainly temperature, rainfall, altitude, ripeness of the crop and fungicides (Boulton *et al*., 1996).

The microflora usually recovered from the winery belonged to strains of *S. cerevisiae* (Fleet & Heard, 1993; Fleet, 2007), but yeast belonged to the genera *Brettanomyces*, *Candida*, *Hansenula, Kloeckera*, *Pichia*, and *Torulaspora* have also been isolated.

A series of microbiological analyses of the yeast flora associated with natural fermentation of grape juice revealed that in most enological areas, there is a sequential use of the substrate: during the first few hours of AF most of the yeast recovered from grape juice belonged to the genera *Hanseniaspora/Kloeckera,* while *Saccharomyces* yeasts (mainly *S. cerevisiae*) begin to develop about after 20 h. In spite of after 3–4 days, *Saccharomyces* yeasts predominate and are ultimately responsible for alcoholic fermentation (Ribéreau-Gayon *et al*., 2006; Pretorius, 2000). Furthermore, during the several steps of fermentation, could be isolated other yeast genera, such as *Candida*, *Pichia*, *Zygosaccharomyces*, *Schizosaccharomyces*, *Torulaspora*, *Kluyveromyces* and *Metschnikowia* (Fleet *et al*., 1984; Heard & Fleet, 1985, 1986).

The succession of yeast recovered during AF in due to the increasing presence of ethanol, the anaerobic conditions, the use of sulfites during harvesting and in the must, the decreasing concentration of sugar, and the greater tolerance of high temperatures shown by *S. cerevisiae* compared with other yeasts (Fleet & Heard, 1993; Fleet, 2007). Nevertheless, quantitative studies on AF showned that various strains of *Candida stellata* and *Kloeckera apiculata* can survive at significant levels (about 10^6–10^7 CFU/mL) not only during fermentation, but also for longer periods than thought previously (Fleet *et al*., 1984; Heard & Fleet, 1985).

The viability of non-*Saccharomyces* yeasts throughout fermentation is influenced by physico-chemical and microbiological factors. Some authors in their study showed that *K. apiculata* and *C. stellata* have

increased viability at the end of alcoholic fermentation due to an incresead tolerance to ethanol at lower temperatures, 10–15 °C (Heard & Fleet, 1988; Gao & Fleet, 1988).

This behaviour has also been confirmed in grape juice fermented with starter cultures of *S. cerevisiae* (Heard & Fleet, 1985) or with mixed cultures using *K. apiculata* and *S. cerevisiae* (Erten, 2002). Furthermore, oxygen concentration influenced the survival of some non-*Saccharomyces* yeasts during fermentation, such as *Torulaspora delbrueckii* and *Klyveromyces thermotolerans* (Hansen *et al*., 2001). Cell–cell interactions are involved in inhibition of these two non-*Saccharomyces* species. A high amount of cells of *S. cerevisiae* inhibited the growth of *T. delbrueckii* and *K. thermotolerans* (Nissen & Arneborg, 2003; Nissen *et al*., 2003).

Several strains of *S. cerevisiae* are able to produce toxic compounds that could be the cause of the early death of *Hanseniaspora guilliermondii* in mixed fermentations (Pérez-Nevado *et al*., 2006). Ethanol, medium-chain fatty acids, acetic acid, acetaldehyde and their combinations have an important role in the inhibitory mechanism that occur in wine fermentation (Edwards *et al*., 1990; Bisson, 1999; Fleet, 2003).

Usually, inoculated cultures of *S. cerevisiae* are able suppress either indigenous non-*Saccharomyces* species and wild *Saccharomyces* strains or to dominate the fermentation, also the use of antiseptic agents, like SO_2, should guarantee the dominance of the inoculated strains.

However, several studies have shown that the growth of *K. apiculata* and *C. stellata* is not suppressed in grape juice inoculated with selected starter cultures of *S. cerevisiae* (Heard & Fleet, 1985; Mora *et al*., 1990), also other studies have revealed they are still viable during the various stages of grape juice fermented with starter cultures (Ciani & Rosini, 1993; Mannazzu *et al*., 2007).

Inoculated starter cultures may be even unable to dominate fermentation. This feature depends on several factors such as:

(1) the amount and viability of the inoculum, and its correct use;

(2) the metabolic and physiological characteristic of starter cultures;

(3) winemaking technologies (clarification procedures, temperature of fermentation and SO_2 addition) (Amerine & Cruess, 1960; Benda, 1982; Ciani & Rosini, 1993).

Usually, after the 1970s winemakers used inoculate grape juice with selected starter cultures of active dried yeast. This practice shortens the lag phase, guarantees a rapid and complete fermentation of sugars of the must and helps to create a much more reproducible final product (Bauer & Pretorius, 2000; Fleet & Heard, 1993).

However, is important to underline that the growth of the natural microflora is not completely suppressed during the first steps of vinification, so these strains can give a positive contribute to several properties of the wine (Querol *et al*., 1992). Consequently there is increasing interest in the use of mixed starter cultures of non-*Saccharomyces* strains, usually considered spoilage strain, cause they give a desirable contribute to the organoleptic quality of the wine, that complement the fermentative capacity of *Saccharomyces* yeasts (Fleet, 2008).

1.2 Spontaneous and induced fermentation of grape must

Microbial fermentations can be conducted with two different kind of process: batch processes or continuous processes. Major part of wines are produced by the 1st kind of process, batch fermentation, so the juice is placed in a vessel and the entire batch is kept there until complete fermentation, after about 5–10 days (Divies, 1993). However, continuous fermentations are much faster and more efficient.

Usually with batch fermentations, wine production may be performed either by spontaneous and natural fermentation or by starter culture fermentation (Pretorius, 2000).

Induced or inoculated fermentation is refer to the use of selected starter cultures used to induce AF, that guaranteed the outcome of the process, each in terms of fermentative process and quality of wine.

Until about the 1970-80s winemakers relied on natural or spontaneous fermentation of grape juice for the production of wine and the contribution of yeasts was seen as a relatively simplistic concept.

Several yeast species, recovered from grapes surface and winery environment, contributed to spontaneous fermentation of grape juice.

A series of microbiological analyses of the yeast flora associated with natural fermentation of grape juice revealed that in most enological areas, there is a sequential use of the substrate: during the first few hours

of AF the major part of yeast isolated belonged to the genera *Hanseniaspora/Kloeckera,* while *Saccharomyces* yeasts (mainly *S. cerevisiae*) begin to develop about after 20 h. In spite of after 3–4 days, *Saccharomyces* yeasts predominate and are ultimately responsible for alcoholic fermentation (Ribéreau-Gayon *et al*., 2006; Martini, 1993; Pretorius, 2000).

Farther, during the several steps of fermentation, could be isolated other yeast genera, such as *Candida*, *Pichia*, *Zygosaccharomyces*, *Schizosaccharomyces*, *Torulaspora*, *Kluyveromyces* and *Metschnikowia* (Fleet *et al*., 1984; Heard & Fleet, 1985, 1986).

Generally, apiculate yeast begin AF, but they are unable to complete it, then during 2nd steps of AF strains of *S. cerevisiae* dominated AF and completed it.

However, at the end of spontaneous AF we can have different situations (Zambonelli 1998):

- yeast strains enologically valid are able to consume grape sugars and to complete fermentation;
- stuck or sluggish fermentation due or at the lack of strains with a good fermentative behavior or to low temperature in presence of excellent strains;
- developing of spoilage yeast, such as *Schizosaccharomyces spp.* or *Brettanomyces spp.*, that give a negative contribute to organoleptic properties of wine.

Usually, natural or spontaneous fermentation may cause stuck or sluggish fermentation due to the presence of residual sugars in grape juice However, spontaneous fermentation, despite of unpredictability of the process and the risk of several microbiology problems, is quite diffused, especially in European countries and in Italy (Fleet, 2008; Rainieri & Pretorius, 2000; Mannazzu *et al*., 2002) and, in particular, for the production of certain great wines. Generally, a combination of artisanal and technological expertise is required for success with these fermentations.

Supporters of spontaneous fermentation affirmed that wines produced with this technique have a strong stylistic distinction, due to an improved complexity of wine aroma, flavor and texture, compared to those obtained by inoculation of selected starter cultures, that might lead to losses in “unique qualities” and in

flavor complexity, in addiction wines obtained with starter cultures are too standardized and ordinary in character (Pretorius, 2000 Rainieri & Pretorius, 2000; Mannazzu *et al*., 2002).

Nevertheless, starter culture fermentations, offer the advantages of a more predictable and rapid process, with greater consistency in quality. They are suitable for producing mass-market wines, and they had an increased diffusion in wine industry by the commercial availability of dried selected yeast strains that can be conveniently reconstituted for inoculation into grape juice (Degre, 1993; Manzano *et al*., 2006).

Several strains of *S. cerevisiae* and *S. bayanus* are available as starter commercial cultures, well selected for their properties, such as their ability to tailor specific wine character (Pretorius, 2000; Bisson, 2004).

The major complexity of wine produced with spontaneous fermentation is due to the combined action of different wild yeast species and strains, all these yeast give a specific qualitative fingerprint to final product, proportionally to their weight during fermentation (Lambrechts e Pretorius, 2000).

Several studies that compared spontaneous and inoculated fermentation shown significant differences on chemical composition of wine (Mora *et al.* 1990; Longo *et al.* 1992; Gafner *et al.* 1993; Lema *et al.* 1996). Moreover, several authors demonstrated that the use of commercial starter cultures caused a decrease of some metabolic compounds, such as higher alcohols, isoamyl acetate and ehyl acetate, instead found in appropriate concentrations in natural fermented wines (Mateo *et al.,* 1991).

Wine obtained with pure culture fermentation of non-*Saccharomyces* yeast shown several problems, due to their fermentative behavior or metabolite compounds production, for this reason generally are excluded their use as starter cultures.

Non-*Saccharomyces* yeast produced several spoilage compounds, such as acetic acid, acetaldehyde, acetoin and ethyl acetate, and also off-odours, usually due to the production of vinyl and ethyl phenols that are linked to the development of *Brettanomyces/Dekkera* spp. (Chatonnet *et al*., 1995).

In addition, the majority of the non-*Saccharomyces* strains lack of good fermentation aptitudes. In fact, usually they have low fermentation power, rate, and a low SO_2 resistance. The fermentation power refers to the maximum amount of ethanol produced in the presence of an excess of sugar. Nevertheless, in mixed multi-starters fermentations, like natural fermentations, some negative characteristic of non-*Saccharomyces* yeasts may not be expressed or could be modified and balanced by *S. cerevisiae* strains.

During the last decades several studies have been carried out to determine the oenological properties of non-*Saccharomyces* yeast and their role in winemaking (Romano *et al*., 1992, 1997; Ciani & Picciotti, 1995; Lema *et al*., 1996; Ciani & Maccarelli, 1998; Egli *et al*., 1998; Henick-Kling *et al*., 1998; Rojas *et al*., 2001; Zohre & Erten, 2002; Fleet, 2003; Jolly *et al*., 2003; Hermle *et al*., 2005; Domizio *et al*., 2007; Kim *et al*., 2008; Viana *et al*., 2008). Several authors highlighted the positive role of non-*Saccharomyces* yeasts in the chemical composition of wine (Herraiz *et al*., 1990; Moreno *et al*., 1991; Lema *et al*., 1996).

Some non-*Saccharomyces* yeast species can improve the chemical composition of wine and lead to a more complex aroma. Hence, there has been a re-evaluation of the role of non-*Saccharomyces* yeasts in winemaking (Fleet & Heard, 1993; Ciani, 1997; Esteve-Zarzoso *et al*., 1998; Heard, 1999; Fleet, 2008) and today more attention is being paid to the yeast ecology of fermentation, in order to better understand its impact on the chemistry and sensory properties of wine (Pretorius, 2000; Romano *et al*., 2003a; Swiegers *et al*., 2005).

There has been disputes on the use of spontaneous and inoculated fermentations, especially for the organoleptic properties of the final wine. Several authors, on the basis of sensory wine testing, asserted the advantages of both spontaneous or inoculated fermentations. It is generally recognized that spontaneous fermentation influenced the wine aroma and flavour may lack consistency for its uncontrability. Nevertheless, the total suppression of wild non-*Saccharomyces* yeast can reduce the aroma complexity of the final wines. Moreover, the use of selected starter cultures of *S. cerevisiae* strains can inhibit potential spoilage yeasts but also “positive” yeast species.

Natural multistarter cultures is also an uncontrolled process, and need to be used under defined conditions. Still, the sequential or combined use of various yeast starter cultures for new fermentation technologies needs further studies.

Several species of non-*Saccharomyces* wine yeast have been proposed as starter cultures, due to their specific metabolic characteristics (Bely *et al*., 2008; Ciani *et al*., 2009; Moreira *et al*., 2008). Usually the activity of non-*Saccharomyces* yeasts in winemaking could be promoted by limiting or delaying the usem of *S. cerevisiae* starter cultures.

Recently, Bely *et al*., (2008) proposed the use of mixed and sequential inoculum of *T. delbrueckii–S. cerevisiae* in high sugar fermentation to improve the quality of wines and reduce the acetic acid content. Mixed *T. delbrueckii – S. cerevisiae* cultures (20:1 ratio) produced 53% and 60% reductions in the volatile acidity and acetaldehyde, respectively, instead sequential cultures showed lower effects on the reduction of these metabolites. The use of a multistarter fermentation process has also been proposed to simulate natural must fermentation, to confer greater complexity to a wine. Herraiz *et al*. (1990) studied the impact of pure, mixed and sequential cultures of *K. apiculata*, *T. delbrueckii* and *S. cerevisiae* on the volatile composition of wines and shown significant differences in the metabolism by *S. cerevisiae* in pure and mixed cultures. In addition, Ciani *et al*. (2006) studied several multistarter fermentations (mixed and sequential) of *T. delbrueckii* and *K. thermotolerans* inoculated together with *S. cerevisiae*.

A commercial blends of active dried yeasts of *S. cerevisiae/K. thermotolerans/T. delbrueckii*, commercialized as Vinflora® Harmony.nsac (Christian Hansen) and single non-*Saccharomyces* (*Zygosaccharomyces bailii*) are commercially available.

Furthermore, with the increasing demand for organic food the scientific community should offer an organic-friendly solution able to reconcile organic viewpoint with safe food fermentations.

Autochthonous yeasts, well adapted to a specific wine-producing area, can improve organoleptic quality of wines and should be used for the formulation and the production of a multi-starter cultures, with the aim to mimics the natural biodiversity as a valid alternative to organic uncontrolled fermentations.

1.2.1 Evolution and biodiversity of yeast during spontaneous fermentation

The complexity of sensorial properties of wine influenced its quality that depends from several factors, such as the techniques used in winemaking and the presence of different yeast during several steps of winemaking.

Fundamental steps to understand how yeast influenced wine quality and to have microbiologically stable product, are the knowledges related to the yeast that perform AF and their growth during the different steps of AF (Fleet 2008; Querol *et al.* 1992a).

On freshly crushed grape juice several yeast species, mainly belonging to the genera *Hanseniaspora* (anamorph *Kloeckera*), *Pichia*, *Candida*, *Metschnikowia*, *Kluyveromyces* and *Saccharomyces*, are recovered (Romano *et al*., 2003b). Sometimes, yeast species such as *Zygosaccharomyces*, *Saccharomycodes*, *Torulaspora*, *Dekkera* and *Schizosaccharomyces* are also observed (Fleet & Heard, 1993; Fleet, 2003; Fleet, 2008; Romano *et al*., 2003b).

Non-*Saccharomyces* wine yeast initiate spontaneous alcoholic fermentation of grape juice (especially yeast belonged to the genera *Hanseniaspora*, *Candida*, *Pichia* and *Metschnikowia*), but are very soon overtaken by *S. cerevisiae*, that dominates AF, from the mid to final stages of the process, often being the only species found in the fermenting juice at these times (Fleet & Heard, 1993; Fleet, 2003).

Until the 1980s ecological studies considered *Saccharomyces* species (mainly *S. cerevisiae* and *S. bayanus*) the yeasts of main relevance for AF and, logically, they became the species around which starter culture technology was developed (Degre, 1993). Further study shown that non-*Saccharomyces* yeast have a fundamental role during fermentation, their growth reach a population of 10^7 CFU/mL or more

during the early stages of fermentation before they died off. This amount of biomass was sufficient to affect the chemical composition of the wine and that the contribution of these yeasts to overall wine character was much more significant than thought previously (Degre, 1993).

Nevertheless, in particular circumstances, such as low temperatures, some non-*Saccharomyces* species did not die off and remained still viable until the end of fermentation with *S. cerevisi*ae (Heard & Fleet, 1988). In addiction Heard & Fleet (1985) shown that these indigenous non-*Saccharomyces* yeasts also grew in grape juice fermented with starter cultures of *S. cerevisiae* .

The generally assumptions that starter cultures dominate fermentation, inhibit the growth of non-*Saccharomyces* yeast and their influence on wine properties are, actually, not properly valid.

Indeed, several studies in various wine regions of the world attributed an important contribution of non-*Saccharomyces* species to the overall kinetics of yeast growth during both spontaneous and *S. cerevisiae*-inoculated wine fermentations (Lema *et al*., 1996; Egli *et al*., 1998; Granchi *et al*., 1999; Pramateftaki *et al*., 2000; Jolly *et al*., 2003a; Combina *et al*., 2005a,b; Zott *et al*., 2008). More recently, other authors confirmed these ecological results also using culture-independent molecular methods for yeast analysis (Cocolin *et al*., 2000; Mills *et al*., 2002; Xufre *et al*., 2006; Nisiotou *et al*., 2007).

Non-*Saccharomyces* yeast species introduce an element of ecological diversity in the AF that goes beyond *Saccharomyces* species and they require specific study to prevent any negative consequences, and to exploit their beneficial contributions (Ciani & Picciotti, 1995; Jolly *et al*., 2003b).

Culture-indipendent molecular techniques allows to differentiated strains within a species, so further ecological sophistication of the fermentation has been discovered. Usually, during fermentation process within each species there is a succession of different strains. Several authors suggested that more than 10 genetically distinct strains of *S. cerevisiae* contributed to AF (Sabate *et al*., 1998; Pramateftaki *et al*., 2000; Cocolin *et al*., 2004; Ganga & Martinez, 2004; Sipiczki *et al*., 2004; Santamaria *et al*., 2005).

Furthermore, within non-*Saccharomyces* yeast there is an evolution of strain diversity throughout fermentation (Schutz & Gafner, 1994).

It is now accepted that wine fermentations, whether spontaneous or inoculated, are ecologically complex and not only involve the growth of a succession of non-*Saccharomyce*s and *Saccharomyces* species but also involve the successional development of strains within each species.

Several factors, such as grape juice composition, pesticide residues, sulphur dioxide addition, concentration of dissolved oxygen, ethanol accumulation and temperature influenced the kinetics of yeast growth throughout wine fermentations, but little is known regarding how these factors might affect the dominance and succession of individual species and strains within the total population (Fleet & Heard, 1993; Bisson, 1999; Fleet, 2003; Zott *et al*., 2008).

It is generally recognized that the successional evolution of strains and species throughout fermentation depends from yeast different susceptibilities to the increasing concentration of ethanol, in fact usually the non-*Saccharomyces* species dying off earlier in the process because they are more sensitive to ethanol than *S. cerevisiae*.

Nevertheless, several authors recovered many wine yeast isolates belonging to the genera *Hanseniaspora*, *Candida* and *Kluyveromyces* with ethanol tolerances similar to that of *S. cerevisiae* (Mills *et al*., 2002; Pina *et al*., 2004; Xufre *et al*., 2006; Nisiotou *et al*., 2007).

In addition to ethanol, other factors, such as temperature of fermentation, dissolved oxygen content, killer factors, quorum-sensing molecules and spatial density influences affect the competitive interaction between yeast species and strains in wine fermentations (Yap *et al*., 2000; Fleet, 2003; Nissen *et al*., 2003; Hogan, 2006; Perez-Nevado *et al*., 2006).

Only few yeast species, over *S. cerevisiae*, are able to contribute during last steps of fermentation for their moderate fermentative behavior. Some strains of *Torulaspora delbrueckii* (formerly *Torulaspora rosei* or *Saccharomyces rosei*), *Zygosaccharomyces bailii* (formerly *Saccharomyces bailii*) and

Schizosaccharomyces (*Schiz. pombe*, *Schiz. japonicus*) that sometimes could replace *S. cerevisiae.* Other yeast, whose impact is marginal, are represented by *Saccharomycodes ludwigii*, *Metschnikowia pulcherrima* and some species of the genera *Brettanomyces*.

The participation of several species of yeast during spontaneous fermentation process compared to those inoculated, introduces an element of great biodiversity in the ecology of AF (Vincenzini *et al*., 2005). In addition, during the last years, the development and application of molecular methods for analysis of yeast populations, shown that spontaneous fermentation is characterized by a significant intraspecific biodiversity (Cocolin *et al*., 2002) as well high genetic polymorphism is observed in the population of *S. cerevisiae* that develops during spontaneous fermentation. In other words, the population of yeasts *S. cerevisiae* correlated to spontaneous fermentations consists of genotypically different strains, possibly with different phenotypic properties and, therefore, potentially capable of influencing, in proportion to their relative abundance, the aromatic characteristics of the final product (Romano *et al*., 2003b) .

Vincenzini *et al*., (2005) affermed that during spontaneous fermentation genetic polymorphism within yeast strains of *S. cerevisiae* is high, although only few (2-3) strains are able to dominate the process. Some predominant strains of *S. cerevisiae*, recovered from spontaneous fermentation in the same winery could occur over year, assuming that might be some correlation between strain and winery environment. Additionaly, some *S. cerevisiae* strains isolated from different winery of the same region could be very similar, highlighting a correlation between strains and enological region.

The practical implications of these experimental evidences influenced typical aspects of a wine and the intraspecific biodiversity, that itself influenced the properties of wine made from spontaneous fermentations with a blend of strains genetically different.

1.3 Starter culture and their selection

The quality of fermented food and beverages is correlated with the microorganisms used in their preparation. In addiction, also wine sensory properties arise from the direct action of these microorganisms on the substrate. Consequently, the exploitation of microorganisms, such as the yeasts and lactic acid bacteria responsible for wine fermentation (AF and MLF) is a constantly expanding branch of biotechnology.

Recently, there is an increasing emphasis on the importance of wine quality. Hence, to increase the competitiveness of wine industry, the wine must be standardized for its organoleptic properties that remain consistent year after year. The first step in winemaking (see **Figure 1**) is function of the type of wine produced. This step influence both the preparation and choice of the starter culture.

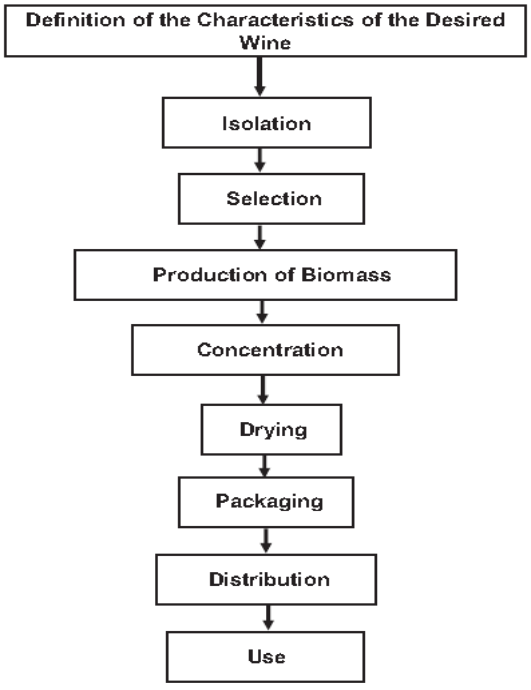


Figure 1. Example of production steps of starter cultures for use in winemaking.

The use of starter cultures of yeast offers several advantages compared with spontaneous fermentation in winemaking. These advantages depend on two principal factors: the techniques and the characteristics of the yeast strain used in winemaking. Fundamental characteristics of starter cultures are a more rapid onset

and progression of fermentation and a reduction in negative compounds for quality of wine, such as acetic acid associated with volatile acidity. Nevertheless, the different characteristics of each yeast strain contributes to final wine quality.

Criteria for the selection and development of starter culture for winemaking are object of several study (Degre, 1993; Rainieri & Pretorius, 2000; Mannazzu *et al*., 2002; Pretorius & Bauer, 2002; Bisson, 2004; Schuller & Casal, 2005).

Essentially these criteria belong to three categories:

1) properties that influenced the performances of fermentation process,
2) properties that determined the character and the quality of the wine,
3) properties of commercial production of wine yeast.

Within these several properties there are many factors, variable for their importance, some of them are essentially while other are more or less desirable.

Is generally recognized that a good wine yeast should develop a rapid, vigorous and complete fermentative process, with a high production of ethanol (> 8% v/v) and complete transformation of the sugars of grape juice. In addition, wine yeast should be able to tolerate high concentration of SO_2 (an antimicrobial agents usually added to grape and grape juice to inhibit the growth of undesirable yeast molds and bacteria), to well blend with grape juice, to produce a little amount of foam and to flocculate quickly at the end of fermentation. Usually these biotechnologically characteristics are well expressed in a range of temperature between 15-25°C, respectively for white or red wines. One of the fundamental characteristic of starter culture yeast is the ability to complete fermentation and not to cause stuck or sluggish fermentation (Bisson, 1999).

Another essential factor for the quality of wine is that the yeast must produce a balanced quantity of aromatic compounds, without an excess of undesirable volatile compounds, such as acetic acid, H_2S, SO_2 and ethyl acetate. Furthermore, at the end of AF, yeast should be unable to produce undesirable compounds after autolysis or to influence, negatively, wine color or its tannic compounds.

Summarizing, a good wine yeast starter culture should make a wine with a flavor clearly defined, devoid of sensorial defects and, finally, allows the consumers to perceive the individual characteristic typical of each grape cultivars (Lambrechts & Pretorius, 2000; Bisson, 2004; Swingers *et al*., 2005).

Within the important properties of yeast starter culture we should also consider selection and production cost (Degre,1993). Indeed, the production cost should be appropriate, with the aim to have a product accessible to the winery. For these reason yeast species must be cultivated on economics media, such as molasses. Furthermore, yeast must be resistant to several stress, such as dry, packaging and storage stress, they should be rehydrated and reactivated in the winery whithout influencing fundamental and desirable properties of wine yeast.

In addition to the fundamental criteria for wine yeast selection, development and production, there are new emerging criteria, due to an increasing demand for new style of wine, such as wine with a specific and clearly defined style or "healthy" (e.g., wine with a minor amount of ethanol, more antioxidant compounds, minor amounts of toxic compounds, like SO_2 and biogenic amines) (Bisson *et al*., 2002; Bisson, 2004).

Below are summarized the principal criteria for the selection of wine yeast starter cultures.

Fermentation power The 1st criteria used to select wine yeasts was a mixture of rapid fermentation, short lag phases, and almost complete consumption of the sugars present in the grape juice (Kraus *et al*., 1983). In fact, the original purpose of inoculating selected starter culture was to guarantee that the fermentation process was not too long, also to prevent stuck or sluggish fermentation. A complete and rapid fermentation process allows to obtain wines of appropriate quality without spoilage microorganisms.

Good fermentation power is correlate to the capacity of the strain to adapt to the stressful wine environment, such as a hyperosmotic environment, elevated concentrations of ethanol and scarcity of assimilable nitrogen.

Usually fermentation power is estimated in laboratory using natural musts or synthetic media, similar for its composition to grape juice, however in vitro analysis of fermentation power generally tends to be a poor predictor of the behavior of strains under industrial production conditions. Therefore, several studies

suggested further criteria based on the identification of properties that limit the survival of yeasts during winemaking. Zuzuarregui and del Olmo (2004a) using a multivariate analysis suggested that **resistance** to **ethanol** and **oxidative stress**, naturally be expected to have more relevance for the behavior of the strain during production of active dried yeast, could be used as a selection criteria for strains with good fermentation behavior, also they showed a correlation between the expression level of several stress-response genes and their fermentative power (Zuzuarregui & del Olmo, 2004b).

One of the most important factor affecting sluggish or stuck fermentation, after ethanol, is the lack of **assimilable nitrogen**, mainly in modern winery. The capacity to ferment under limited level of nitrogen depends on the characteristics of the strain, principally related to the activation of stress response mechanisms associated with entry into the stationary phase.

Therefore, nitrogen demand has been proposed as a selection criterion for industrial *Saccharomyces* strains. Another property that can influence the capacity to complete fermentation is the **sulfite resistance**, a common characteristic of *S. cerevisiae* strains, probably due to continued selection pressure for the common use of sulfite in wine (Romano & Suzzi, 1993) that is a character that will continued to be studied, especially when selection is not limited to *S. cerevisiae* strains.

In addition, **thermotolerance** is an important character in wine strains, particularly before the introduction of temperature control systems, since thetemperatures reached during exothermic fermentation could be suboptimal for growth. Usually *S. cerevisiae* strains are relatively heat-tolerant. Resistance to thermal stress can be a criterion for wine yeast selection, because it can influence the fermentative capacity and the survival of yeast dried during preparation for industrial use (Ivorra *et al*., 1999).

Another hereditary property that can influence the capacity of a strain to induce complete fermentation of grape juice is the **killer factor**. In *S. cerevisiae*, three killer toxins have been described: K1, K2, and K28 (Magliani *et al*., 1997). These toxins are encoded by double-stranded satellite RNAs (M1, M2, and M28), that are responsible for the synthesis of the corresponding toxin and for the immunity of the producing

strain to the toxin produced by that strain or by other cells that produce the same toxin. The toxin is able to kill strains that did not produce a killer factor or that produce a different killer factor.

The use of killer strains seems to favorite the establishment of inoculated strains during fermentation process, however there is no consensus regarding the true relevance of the killer factor under natural conditions. Among the killer factor K2 is the most interesting due to its activity and stability at low pH.

A good fermentation power is an important criterion for the selection of winemaking strains, but not alone. In fact, there are many additional selection criteria, based on the influence of **yeast metabolism** on the **primary** and **secondary aroma** of the wine.

The primary (varietal) aroma, essentially derived from the grape, but can also be influenced by yeast, thanks to the action of hydrolytic enzymes, that release terpenes from their glycosylated precursors, or facilitate the extraction of colors and aromas from the grapes.

The secondary aroma is correlated to the production of several esters of acetic acid and ethyl esters.

The different production of these compounds is correlated to the activity of two groups of enzymes, with antagonistic effects: alcohol acetyltransferases, which catalyze the synthesis of these compounds, and esterases, while catalyze their hydrolysis.

Another product of yeast metabolism that have a positive influence on the sensory properties of wine is glycerol, also increased glycerol levels are correlated with reduced concentrations of alcohol.

Consequently, the ever-growing demand for wines with lower alcohol content has led to a growing interest in strains that produce higher levels of glycerol and lower levels of ethanol.

However usually strains that produce higher levels of glycerol produce also high volatile acidity (due to excess acetic acid), one of the most easily detected wine flaws.

Another important defect in finished wine is due to hydrogen sulfide, which sensory threshold is very low. Various genes have been linked to the production of hydrogen sulfide (Spiropoulos & Bisson, 2000). The main source of sulfur for the formation of hydrogen sulfide can be either sulfate or sulfite, and depletion of nitrogen sources has been identified as one of the main determining factors for its formation.

Another undesirable product of yeast metabolism is urea, derived from the action of *Saccharomyces* spp. arginase that does not influence the sensory quality of wine, but could rise to the formation of ethyl carbamate, a toxic and carcinogenic compound.

The tasting is one of the most difficult and important steps to select industrial yeast strains. In addition, chemical analysis can confirmed the detection of undesirable flavors or aromas that influenced primary and secondary aroma.

Specific selection criteria may also be used according to the specific style of vinification, for example flower formation, ethanol tolerance, and autolytic capacity are essentially for the production of wines aged on lees.

Finally, successful marketing of wine yeast strains will depend largely on their behavior under industrial production conditions, particularly in terms of genetic stability, growth on molasses, and survival and metabolic activity following drying and rehydration.

1.4 Autochthonous yeast and valorization of typical wine

In modern wineries is well diffuse the use of commercial starter culture to induce fermentation. However, there is a discussion about the use of this commercial starter, due to the deficiency of some desirable traits, which are provided by spontaneous fermentation (Fleet and Heard, 1993).

In recent years, there is increasing demand for autochthonous yeast, in particular with the aim to select starter cultures that are potentially better adapted to the growth in a specific grape must, reflect the biodiversity of a given region, which support the idea that native yeast strains can be correlated with a "*terroir*" (Lopes *et al*., 2002; Versavaud *et al*., 1995; Torija *et al*., 2001; Sabate *et al*., 1998).

The main objection to the use of selected starter cultures is the standardization of wine quality, a characteristic useful for table wine, but undesirable for fine wines. Nevertheless, *S. cerevisiae* strains show an high biodiversity among their technological properties, the selection of starter culture lead to strains very similar, that can be distinguished only for some characteristic, such as type of growth or disacidificant activity (Zambonelli *et al*., 2004).

Moreover, the preservation of spontaneous microflora is essential to obtain starter cultures able to develop the typical flavor and aroma of wines originating from different grapevine cultivars (Pretorius, 2000), and to ensure the conservation of gene pools of technological importance. Therefore, exploring the biodiversity of indigenous fermentative strains can be an important contribution towards the understanding and selection of strains with specific phenotypes (Capece *et al*., 2010).

In this context, the use of autochthonous strains, well selected with the aim to produce a specific kind of wine, has been proposed as a tool to take advantage of spontaneous fermentation, to avoid the risks of stuck or sluggish fermentations and to increase the sensory properties of wine. Furthermore, during the last years, the phenomenon of organic products, such as "organic wine", show an increasing demand. Therefore, there are many studies to offer new organic-friendly solution, able to reconcile organic viewpoint with safe food fermentations (Suzzi *et al*., 2012; Mendoza *et al*., 2011; Settani *et al*., 2012; Capece *et al*., 2010). The use of commercial starter cultures might lead to losses in "unique qualities" while wild natural fermentations can result in fermentation arrests and/or production of undesired metabolites responsible for wine depreciation (Spano *et al*., 2010; Capozzi *et al*., 2011). Hence, yeasts naturally present on grape berries and must have a unique composition and these indigenous yeasts impart distinct regional and desired characteristics to wines, are well adapted to the conditions of a specific wine-producing area and can improve organoleptic quality of wines (Nielsen *et al*., 1996). The formulation and the production of a multi-strain microbial starter that mimics the natural diversity and function of the biotechnological processes might be a reliable alternative to organic uncontrolled fermentations, increasing the organoleptic qualities of production and minimizing the risk of foodborne pathogens, microbial toxic compound productions, and microbial spoilage.

1.5 Multistarter fermentation

The inclusion of non-*Saccharomyces* wine yeasts, together with *Saccharomyces* strains as part of mixed and multistarter fermentations, has been proposed as a tool to take advantage of spontaneous

fermentation, while avoiding the risks of stuck or sluggish fermentations (Heard, 1999; Rojas *et al*., 2003; Romano *et al*., 2003a; Ciani *et al*., 2006; Jolly *et al*., 2006; Fleet, 2008).

Neverthless is still recognized that non-*Saccharomyces* strains show a low fermentative aptitude, because they are unable to completely use sugars present in must and consequently produce low quantity of ethanol, although they present several enological properties fundamental for wine organoleptic properties. For example several authors demonstrated that some *Hanseniaspora/Kloeckera* strains produce high level of desirable volatile compounds and more extracellular enzymes, such as glucosidases or proteases, than *Saccharomyces* strains (Zironi *et al.,* 1993; Capece *et al.,* 2005; Mendoza *et al.,* 2007; Moreira *et al.,* 2008), *C. stellata* strains produce high amount of glycerol (Ciani & Comitini, 2006), *K. thermotolerans* show an increased production of lactic acid (Mora et al., 1990), *T. delbrueckii* produce low quantity of acetic acid (Ciani *et al*., 2006; Bely *et al*., 2008) and *Schizosaccharomyces* spp. cause a decrease of wine acidity trough the metabolism of malic acid (Gao & Fleet, 1995).

Over the last few years, as a consequence of the re-evaluation of the role of non-*Saccharomyces* yeasts in winemaking, there have been several studies that have evaluated the use of controlled mixed fermentations using *Saccharomyces* and different non-*Saccharomyces* yeast species from the wine environment (Fleet & Heard, 1993; Ciani, 1997; Esteve-Zarzoso *et al*., 1998; Heard, 1999; Fleet, 2008).

Several non-*Saccharomyces* species are been used as starter cultures for a long time, due to their metabolic characteristics. It is possible to promote their activity in winemaking by limiting or delaying the use of selected starter cultures of *S. cerevisiae*.

The first use of a selected multistarter approach was proposed several years ago by Castelli (1955) that demonstrated that the sequential use of *T. delbrueckii* and *S. cerevisiae* reduce the acetic acid content of wine. Bely *et al*. (2008) studied the influence of mixed or sequential *T. delbrueckii–S. cerevisiae* cultures in high sugar fermentation, and demonstrated that mixed cultures, at a 20:1 ratio, caused a reduction of 53% and 60% in the volatile acidity and acetaldehyde, respectively, while sequential cultures showed lower effects on the reduction of these metabolites.

Several *Schizosaccharomyces pombe* strains are able to reduce malic acid in grape juice and/or wine, Rankine (1966) proposed the sequential inoculation of *S. pombe* and *S. cerevisiae* to improve the competition between the yeasts and to reduce or eliminate the negative sensorial characteristics due to *S. pombe*.

An alternative process was obtained inoculating *S. cerevisiae* with immobilized *S. pombe* cells (Magyar & Panyik, 1989; Ciani, 1995), in which *S. cerevisiae* used almost all of the sugar available, while *S. pombe* used malic acid. Under these conditions, the negative effects of *S. pombe* on the wine quality were limited or eliminated.

Other authors suggested the use of a strain of *Issatchenkia orientalis* to degrade malic acid rapidly (Seo *et al.*, 2007; Kim *et al*., 2008). Moreover, *K. thermotolerans* strains have positive oenological characteristics, such as low production of volatile acidity and high production of fixed acidity, i.e. lactic acid form. In fact, Kapsopoulou *et al*.(2005, 2007) demonstrated that mixed cultures of *K. thermotolerans* and *S. cerevisiae* cause a 70% increase in titratable acidity and consequently a reduction of 0.3 pH units.

The use of a multistarter fermentation process has also been proposed to simulate natural must fermentation and to confer greater complexity to a wine.

Several studies investigated the mixed or sequential use of apiculate yeast (*K. apiculata*, *T. delbrueckii* and *K. thermotolerans*) and *S. cerevisiae* (Herraiz *et al*., 1990; Zironi *et al*., 1993; Ciani *et al*., 2006), showing evident differences in the metabolism by S. cerevisiae in pure and mixed cultures.

Blends of active dried yeasts of *S. cerevisiae/K. thermotolerans/T. delbrueckii* denominated "Vinfloras Harmony.nsac" (Christian Hansen) and single non-*Saccharomyces* (*Zygosaccharomyces bailii*) have become commercially available.

Moreira *et al*., (2008) demonstrated that mixed cultures of *H. uvarum/guilliermondii* and *S. cerevisiae* enhance the production of desirable compounds, *H. guilliermondii* increased the 2-phenylethyl acetate in wine, while *H. uvarum* increased the isoamyl acetate.

Mixed starter cultures have also been proposed to increase the level of glycerol in wine (Ciani & Ferraro, 1996). Sipiczki *et al*., (2005) used an imobilized strain of *C. stellata*, recently reclassified as *Starmerella bombicola* or *Candida zemplinina*, to enhance the organoleptic properties of wine.

Candida cantarellii, another fermenting species of the wine environment, has also been proposed in multistarter fermentations, to enhance glycerol and to develop wines with particular characteristics. Garcia *et al*. (2002) suggested the use of a mixed culture of *Debaryomyces vanriji* and *S. cerevisiae* to increase volatile compounds, particularly geraniol, in Muscat wine. Additionaly, Anfang *et al*. (2009) used mixed cultures of *S. cerevisiae* and *Pichia kluyveri* to increase varietal thiol concentrations in Sauvignon Blanc. Also *Pichia fermentans* has been proposed for multistarter fermentation with *S. cerevisiae* (Clemente- Jimenez *et al*., 2005), however here only sequential use of non-*Saccharomyces* and *S. cerevisiae* strains, with the inoculation of *S. cerevisiae* after 2 days, has positive influence on several volatiles and by-products.

Non-*Saccharomyces* yeasts present several oenological characteristic missing in *S. cerevisiae* species and can have additive effects on wine organoleptic properties. Controlled mixed cultures of *S. cerevisiae* and non- *Saccharomyces* strains can enhance the wine flavor and aroma through metabolic interactions between the yeast species (Languet *et al*., 2005).

1.6 Chemical compound produced during alcoholic fermentation and influence of yeast on chemical composition and flavor of wine

Grape juice is a complex medium containing several different nutrients necessary for the growth of yeast strains, each *Saccharomyces* spp. or non-*Saccharomyces* spp. Nevertheless, each must has a typical composition that is crucial for the characteristics of the final product, also influences the growth dynamics of the yeast.

The end-product of fermentation process are mainly secondary metabolic compounds produced by wine yeast during AF. These by-product of AF are very important for the finished wine, in fact they influenced the flavor, the aroma and the final bouquet of the wine.

Each yeast strains are involved at various level in the fermentation process, with the transformation of grape juice in wine, due to their complex metabolic activities. The characteristic of each wine depend from the yeast that dominated AF.

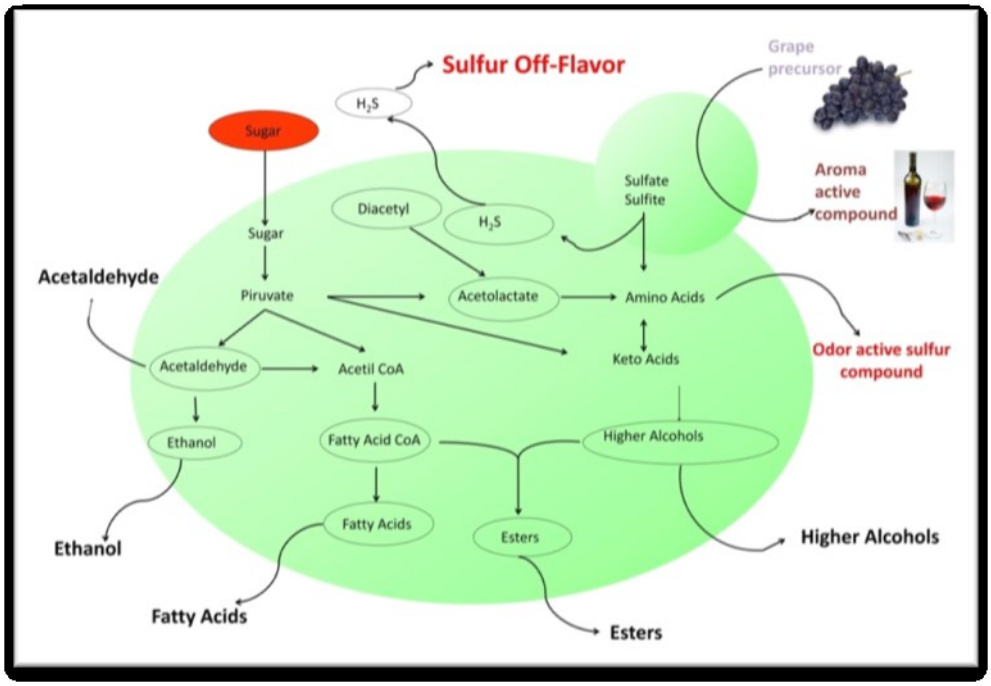


Figure 2. A schematic representation of the formation of aroma compounds by yeast (adapted from Lambrechts & Pretorius, 2000).

Ethanol is the principal product of fermentation process, it represents about the 10-15% of wine and its amount is correlated by the grape sugars concentration and by the fermentation power of yeast. In addition, the smell of ethanol influenced the perception of aromatic compounds, the aroma and flavor of wine, instead its taste, lightly sweet, reduces the acidity of wine.

Together with organic acids, alcohol helps to preserve the microbiology stability of wine, thanks to their antiseptic and sterilizing action. In addition, the alcohol content is still the benchmark to evaluate the commercial value of wines.

Several secondary compounds present in wine also influenced the organoleptic properties of wine. In fact, generally, yeast are able to produce many secondary products during the fermentation process, but their level is different for each species and also for each strains of yeast (Lambrechts & Pretorius, 2000).

The glycerol is the most common product of AF, after ethanol and CO_2, and contributes to the overall mouthfeel of wine. Usually its concentration is over a range of 1- 12 g/l, dry wines contain about 5 g/l of glycerol (Ribéreau-Gayon *et al*., 1998) and derives mainly from yeast metabolic activities, also from chemical-physic parameters of must (composition, amounts of sugars and nitrogen assimilable, temperature, pH, acidity, sulfitation, oxygen concentration,…).

Higher glycerol concentrations enhance the desirable complexity of the wine, however, little attention has been given to the interaction of glycerol and various flavor compounds and the role that glycerol plays in the formation of the aroma profile (Styger *et al*., 2011).

The level of glycerol produced is a character species and strains dependent, that allows to identify several phenotype thanks to the level of glycerol produced, in fact the comparison of the level of glycerol produced through the fermentation process by different yeast species are very variable, so this property are specie-dependent (Romano *et al*., 1997).

Comparing *Saccharomyces* strains to non-*Saccharomyces*, apiculate yeast produced minor amount of glycerol, about 2-3,5 g/l), while other non-*Saccharomyces* spp., such as *C. stellata*, produce more glycerol (Ciani & Picciotti, 1995). From a technologically point of view these variability among the same yeast strain confirmed the important rules of yeast on wine flavor and bouquet (Romano *et al*., 2003).

Acetic acid is most important volatile compounds of wine, it is produced with different levels by every wine yeast strains. Over a threshold, specific for each kind of wine, this compound has a negative effect on organoleptic properties of wine (Ribéreau-Gayon *et al*., 2000).

Generally, acetic acid in wine is present over a range of 0.3-0.6 g/l, more high concentration are undesirable, because it imparts negative organoleptic properties to wine. The production of acetic acid is species and strains dependent, although correlated to the initial amount of sugars, nitrogen availability and pH.

Is usually recognized that apiculate yeast produced a high amounts of acetic acid, for this reason they are generally defined as spoilage yeast. Nevertheless, several study on apiculate yeast shown a great variability strain-dependent (Romano, 2002).

S. cerevisiae strains produce low amount of acetic acid, however the specie that has the lower production of acetic acid is *Torulaspora delbrueckii* (Castelli, 1969), with average level of 150 mg/l.

Glycerol and acetic acid are correlated with osmotic stress of wine, in fact to an higher content of sugar in grape juice correspond an higher concentration of acetic acid and glycerol produced during fermentation process, so sweet musts usually have an higher volatile acidity (Romano, 2005).

Another important compounds produced by yeast metabolic activity is the hydrogen sulfite (H_2S), a compound with rotten egg aroma, which has an acceptable limit of between 10 and 100 µg/L. This compound derived from sulfate naturally present in the medium and elemental sulfur introduced by fungicides. Its formation by yeasts is linked to nitrogen and sulfur metabolism (Henschke & Jiranek, 1993). In fact, it has been observed that deficiencies in easily assimilable sources of nitrogen are a major cause of hydrogen sulfide formation by yeasts and these levels can vary according to the initial concentration of nitrogen in the must and the strain under consideration (Mendes-Ferreira *et al*., 2009).

Therefore, low hydrogen sulfide production is an important criterion in the selection of new yeasts for the industry. Various studies have identified the genes involved in the production of hydrogen sulfide and recently a yeast strain has been identified that produced little or no hydrogen sulfide (Linderholm *et al*., 2010; Linderholm *et al*., 2008; Mendes-Ferreira *et al*., 2010).

Higher alcohols are an important flavor and aroma compounds produced from yeast metabolism. They are mainly represented by n-propanol, isobutanol, amyl alcohol active, isoamyl alcohol and 2-phenylethanol, derived principally from the catabolism of amino acids (treonine, valine, isoleucine, leucine, phenylalanine), but they can derived also from the metabolism of glucose, in fact the content of amino acids isn't correlated to higher alcohols in wine. Usually, small amounts of higher alcohols influence positively the wine aroma, thanks to fruit notes, however high level (> 300-350 mg/l) could influence negatively wine flavor. For example, high concentration of isoamylic alcohol impart a characteristic burning smell, while 2-phenyletanhol has an rose odor, also if present in high level has a positive impact on wine (Swiegers *et al*., 2005). Generally higher alcohols are present in wine over a range of 100-500

mg/l, their level is affected by several factors, such as the media composition (oxygen availability, nitrogen sources, temperature and sugars) and yeast species or strain used in fermentation process.

Compound	Amino acid	Concentration in wine (mg/l)	Odor
Isovaleraldehyde	Leucine	Traces	Fruitly, nut-like
Isobutyraldehyde	Valine	Traces	Slightly apple-like
2-Methylbutyraldehyde	Isoleucine	NR	Green (herbaceous), malty
Isobutyric acid	Valine	Traces	Sweet, apple-like
Isovaleric acid	Leucine	<3	Rancid, cheese, rotten fruit
2-Methylbutanoic acid	Isoleucine	NR	Fruitly, waxy, sweaty fatty acid
Isoamyl alcohol	Leucine	45-490	alcohol
Isobutanol	Valine	40-140	Fruitly, alcohol, solvent-like
Amyl alcohol (active)	Isoleucine	15-150	Marzipan (almond)
Isoamyl acetate	Leucine	0.03-8.1	Banana, pear
2-Phenyl acetate	Phenylalanine	0.01-4.5	Rose,honey,flowery
Ethyl isovalerate	Leucine	0-0.7	Apple, fruity
Isobutyl acetate	Valine	0.01-0.8	Banana
Ethyl 2-methylbutanoate	Isoleucine	0-0.9	Strawberry, pineapple

Table 1. Branched-chain amino acid metabolites and their odor characteristics, Concentrations from Lambrechts and Pretorius (2000), NR not reported.

Isobutanol and n-propanol also in small quantity influence wine flavor and aroma, in addition they shown a solvent action to other odorous compounds (Romano, 2005).

Acetaldehyde is another important aroma compound, formed from pyruvate that represents more than 90% of the total aldehyde content of wine. Acetaldehyde levels is between 10-300 mg/l, generally reach the maximum level when the rate of fermentation is at its fastest, then decreases towards the end of fermentation, only to slowly increase again thereafter (Lambrechts & Pretorius 2000). Also the content in acetaldehyde can be used as a marker of the degree of oxidation of wine, generally at low levels this compound imparts a pleasant fruity aroma to wine and other beverages, at higher concentration is undesirable, already a concentration of 100-125 mg/l has a pungent irritating odor, reminiscent of green grass or apples (Liu & Pilone, 2000). In addition, SO_2 can induce the synthesis of acetaldehyde, that is too a precursor metabolite for acetate, acetoin, and ethanol synthesis. Acetaldehyde is also extremely reactive and readily binds to proteins or individual amino acids to generate a wide range of flavor and odor compounds.

Other important odorant formed from acetaldehyde are diacetyl, acetate and acetoin. Diacetyl is mainly formed by lactic acid bacteria during MLF, but yeasts are also able to synthesize this compound during the fermentation process.

Low concentrations of diacetyl (threshold value, 8 mg/l) addes yeasty, nutty, toasty aromas to wine, but at high concentrations, it shown a typic buttery aroma, usually associated with a lactic character (Romano & Suzzi, 1996; Bartowsky & Henschke, 2004). In addition Almy & De Revel (2008) shown that this compound can react with cysteine with a negative effect on wine aroma due to sulfur compounds. However, the majority of diacetyl is further metabolized to acetoin and 2,3-butanediol none of which has a strong odor, with their detection threshold values of about 150 mg/l in wine (Romano & Suzzi, 1996).

2,3-butanediol concentration in wine varied from 0.2 to 3 g/l. Generally non-*Saccharomyces* yeast, such as *Hanseniaspora/Kloeckera* spp or *C. stellata* spp., produce high amount of acetoin during first step of AF, then this compound is used by *S. cerevisiae* or to synthesis the 2,3-butandiol or other compounds, also etanhol.

The production of acetoin is correlated to several factors of which the most important is the specie of yeast that dominate the fermentation process, other parameters are pH, temperature, media, oxygen concentration, viability and concentration of yeast (Romano, 2005).

Esters are other important compounds originated from amino acids, they are synthesized during AF by yeast. Several factors are involved in their level in wine, like the composition of grape juice and fermentation conditions, however the parameter most important is the yeast that conduces the fermentation process.

Ethyl acetate is the most important ester for the wine aroma, it confers a typical fruity flavor and is produced by yeast metabolic activity, is a properties specie-dependent, in fact non-*Saccharomyces* yeast produced higher quantity of ethyl acetate, about 30-120 mg/l, instead *S. cerevisiae* produce less amount (Rojas *et al*., 2001; Romano 2005).

Generally, when its range is between 50-80 mg/l has a positive effect on organoleptic properties of wine, while higher level (about 120-150 mg/l) has a negative effect.

Another group of esters important for their fruit and floreal notes are the ethyl esters, such as ethyl butanoate, ethyl hexanoate, ethyl octanoate, and acetate esters, such as isoamyl acetate, propyl acetate, hexyl acetate, phenethyl acetate.

Among ethyl esters ethyl butanoate has fruit notes of blueberries and blackberries, ethyl hexanoate has exotic notes of pineapple and ethyl octanoate has a toasted fragrance, similar to bread crust. While among acetate esters the isoamyl acetate has a sweet flavor, similar to banana, and the phenilethyl acetate has characteristic flavor of rose and honey.

Is generally recognized that yeast can releases in wine several polysaccharides from their cell wall, due to their autolysis after prolungated contact with wine. In addition, during alcoholic fermentation yeast releases several macromolecules, mainly mannoprotein.

The level and the type of polysaccharides released in the wine are correlated to yeast strains and to fermentation conditions.

The release of polysaccharides is due to yeast growth, during the first step of AF, then at the end of AF died yeast undergo to autolysis after a prolunged contact with wine (Llaubères *et al*., 1987), however other parameters are essential for polysaccharides release, such as environmental factors (pH, sugar concentration, torbidity and temperature). There is a direct correlation between the initial amount of polysaccharides in grape juice and those arise from yeast wall, in fact if grape juice is more turbid, so rich in macromolecule, there will be less polysaccharides release in wine form yeast. Some authors suggested that a grape juice lesser turbid and rich in sugars causes an increasing in cell wall porosity, with a major release of mannoproteine by yeast (Guilloux-Benatier *et al*., 1995).

In addition, pH of grape juice in a parameter strictly correlated with the release of polysaccharides and mannoproteine in wine, usually a low pH causes a decrease of the amount of colloid. Nevertheless, an increase of the temperature during fermentation power incentives the solubility of cell wall polysaccharides, and also their amount in wine. Therefore, temperature is a parameter very important, also at the end of AF, for yeast autolysis.

Mannoproteine released from yeast in wine are very important for the organoleptic properties of wine, in fact, they can improve the flavor and aroma of final wine, and the microbial stability of wine, promoting the beginning of MLF.

1.6.1 Influence of *Saccharomyces cerevisiae* strains on wine

The yeast species is a fundamental factor in determining the wine quality and composition (Benda, 1982; Herraiz *et al*., 1990; Romano, 1997; Brandolini *et al*., 2002). *S. cerevisiae* is the principal actor of the winemaking, it become the dominant yeast during the 2nd step of AF, when alcohol concentration increase, due to its more tolerance to ethanol. Several studies shown that in addition to the production of ethanol, this yeast generates many secondary metabolites essential for wine quality (Fleet, 1990; Lema *et al*., 1996; Lambrechts and Pretorius, 2000; Fleet and Heard, 1993).

Generally, spontaneous fermentation are characterized by several *S. cerevisiae* strains (Frezier Dubourdieu, 1992; Querol *et al*., 1992b; Polsinelli *et al*., 1996), that produce different quantities of by-products, consequent impact positively or negatively wine flavor and aroma.

The wine organoleptic properties are due to several compounds, mainly due to yeast metabolism, such as organic acids, higher alcohols, esters, and, to a lesser extent, aldehydes, also fatty acids, compounds arise from them or sulfur compounds affect wine quality (Boulton *et al*., 1996).

By-products arising from yeast metabolism can have both a positive or negative impact on wine quality, primarily according to their amount, for example acetaldehyde, acetic acid, ethyl acetate and some higher alcohols when present at high concentrations, nevertheless the most "negative" aroma compounds are the reduced sulfur compounds, hydrogen sulfide, organic sulfides and thiols.

Generally, *S. cerevisiae* strains shown a strong polymorphism within this species (Giudici *et al*., 1990; Romano *et al*., 1993, 1994; Henschke, 1997; Pretorius, 2000) and it is widely recognized that *S. cerevisiae* strains, producing different amounts of secondary compounds, impart desirable or undesirable characteristics on the flavour and aroma of the wines.

Romano *et al*. (2002) studying 115 *S. cerevisiae* autochthonous strains, isolated from Aglianico del Vulture, a typical wine of Basilicata region (Southern Italy) emphasized the important role of the *S. cerevisiae* strain on the final composition of the wine.

An important variable for strain differentiation are the different levels of higher alcohols produced. Higher alcohols constitute a minor component in wine due to their low amount, compared to the quantity of the total substances contained in wine, however they may undoubtedly influence certain sensory qualities of white wines. Higher alcohols influence positively the desirable aroma complexity of wine, but when their concentrations exceed 400 mg/L (Rapp & Versini, 1991), these compounds have a negative effect on wine quality. Total concentration of higher alcohols can be affected by numerous factors, such as climate conditions, must composition, juice turbidity, temperature and fermentation procedure.

Among highers alcohols isoamyl alcohol is the most abundant in wine, represent more than 50 % of total higher alcohol content, and it is the principal responsible of fragrant component of higher alcohols.

Several authors demonstrated that *S. cerevisiae* is characterized by high production of isoamyl alcohol, however their level is an individual strain characteristic, fundamental for characterizing strains for industrial purpose (Romano *et al*., 2003; Majdak *et al*., 2002).

Several parameters can influence the production of higher alcohols, such as temperature and fermentation conditions, usually cryotolerant yeasts (*S. uvarum*) produce 4-fold (216 mg/L compared to 45 mg/L) more phenethyl alcohol than mesophilic *S. cerevisiase* yeasts. These cryotolerant yeasts also produced twice as much isobutyl and isoamyl alcohols as the mesophilic yeasts.

Another parameter important for wine properties is the volatile acidity that usually lies between 500 and 1000 mg/L, about 10-15% of the total acid content. Normally more than 90% of the volatile acid of wine consists of acetic acid (0.2- 2 g/L) (Henschke & Jiranek, 1993; Radler, 1993). Its flavour threshold is about 0.7-1.1 g/L, and values between 0.2 and 0.7 g/L are considered optimal (Corison *et al.,* 1979).

Generally, *Saccharomyces* strains are classified into three distinct classes for acetic acid production:

- low (0.0 to 0.3 g/L)
- medium (0.31 to 0.60 g/L)

- high (more than 0.61 g/L)

S. cerevisiae strains produce a range of acetic acid between 0.3-1.2 g/l (Hanneman, 1985; Fleet & Heard, 1993), nevertheless its production is affected by sugar concentration, pH and nitrogen. Hanneman (1985) analized 100 strains of various genera, within them 13 strains of *S. cerevisiae* produced more than 1 g/L of acetic acid in a synthetic medium.

Esters are a group of volatile compounds, arise from yeast metabolic activity, that impart a mostly pleasant smell. The fresh, fruity aroma of young wines derives in large part from the presence of the mixture of esters produced during fermentation, which is why it is usually called fermentation aroma/bouquet.

S. cerevisiae usually produces high quantity of esters, such as isopenthyl acetate, ethyl hexanoate, ethyl octanoate, ethyl decanoate and isoamyl acetate, compared to those produced by *S. uvarum* strains.

S. cerevisiae strains produce relatively high levels of acetaldehyde, from 50 to 120 mg/L, while other wine yeasts, such as *K. apiculata*, *C. stellata*, *H. anomala* and *M. pulcherima*, produce low levels, 0-40 mg/L, (Fleet & Heard, 1993). Romano et al. (1994) divided 86 *S. cerevisiae* wine strains into several groups for their production of acetaldehyde, low, medium and high. The low and high phenotypes also differed considerably in the production of acetic acid, acetoin and higher alcohols. Wines obtained with the low acetaldehyde producers had traces of acetoin, lower amounts of acetic acid (<500 mg/L) and a higher total content of higher alcohols (>300 mg/L).

Wines obtained with the high producers showed a different pattern, containing detectable amounts of acetoin, elevated amounts of acetic acid (528 to 1185 mg/L), and a lower content of higher alcohols (256 to 270 mg/L). Longo *et al*. (1992) also found variations in the production of acetaldehyde from 13.1 to 24.3 mg/L among 14 strains of *S. cerevisiae*.

Diacetyl is then formed via the oxidative decarboxylation of α-keto-acetolactate and the final concentration is due by the balance between the rate of formation and the rate of degradation. In the later stages of the fermentation, diacetyl can be metabolized by the yeast to acetoin and butane- 2,3-dione.

The production of H_2S varies with the strain of *S. cerevisiae*, with some strains producing amounts exceeding 1 mg/L (Eschenbruch *et al*, 1978). Some strains were responsible for sulfur off odours in wines correlating to high concentrations of S-compounds. Some strains showed a slight increase in the formation of S-compounds producing wines with no offflavour, but masking the typical aroma of the variety. Nearly all studies to date concern the biochemical activities of *S. cerevisiae*; virtually no attention has been given to the production of S-compounds by other species, some of which (e.g. *Kloeckera*, *Hanseniaspora* and *Candida* spp.) can make significant contributions to wine fermentations.

Another important characteristic involved in yeast impact on wine flavor and aroma are extracellular enzymes, such as esterases, glycosidases, lipases, b-glucosidases, proteases and cellulases (Dizy & Bisson, 2000; Manzanares *et al*., 1999, Strauss *et al*., 2001; Maturano *et al*., 2012) usually involved in hydrolysis of structural components.

Generally, *S. cerevisiae* strains are poor producers of these enzymes. Capece *et al*. (2012) analyzed 132 *S. cerevisiae* strains, isolated from spontaneous fermentation of grapes collected in "Cinque Terre National Park", no isolates showed b-xylosidase activity, as expected for *S. cerevisiae* species, whereas about 30% of isolates resulted positive for b-glucosidase.

1.6.2 Influence of non-*Saccharomyces* strains on wine

At the beginning of AF the microflora present arise derived from the grapes, mainly comprises species with very limited ethanol tolerance, such as *Hanseniaspora/Kloeckera*, *Hansenula*, *Metschnikowia*, and *Candida*, and few strains of *S. cerevisiae*, which are more ethanol tolerant but only represent a minor part of the microflora at this stage.

The variability of microflora present on grapes and grape juice depends from several factors, such as the harvesting method, fermentation temperature and quantity of sulfide added.

The ratio of non-*Saccharomyces*/*Saccharomyces* yeasts influences the organoleptic properties of wine, for these reason the study of the non-*Saccharomyces* flora is very important for wine quality, also in must inoculated with *Saccharomyces* yeast or new mixed starter cultures. Several studies on mixed starter

cultures are carried out and wines produced differ significantly, both for their chemical composition and sensory characteristics (Egli *et al*., 1998; Fleet & Heard, 1993).

One of the most important characteristics that affect the differences between non-*Saccharomyces* and *Saccharomyces* species is the production of several extracellular enzymes involved in hydrolysis of structural components, such as esterases, glycosidases, lipases, b-glucosidases, proteases and cellulases.

These enzymes can improve particular phases of the process, such as maceration, filtration, or clarification, increase yield and color extraction, and enhance the characteristics of the wine, especially the aroma (Charoenchai *et al*., 1997).

Several studies have shown that, unlike *S. cerevisiae*, non-*Saccharomyces* yeasts are notable producers of extracellular enzymes (Dizy & Bisson, 2000; Manzanares *et al*.1999, Lagace & Bisson, 1990; Strauss *et al*., 2001; Maturano *et al*., 2012), the action of grape and *Saccharomyces* enzymes isn't enough to completely liberate aromatic compounds, so it is of particular interest to control the development of non-*Saccharomyces* yeasts as sources of these enzymes.

Among extracellular enzymes involved in winemaking, there are **pectolytic enzymes** that are able to cleave long-chain pectins and generate shorter, more soluble chains. Pectolytic enzymes plays an important role during grape ripening and during winemaking, in fact they facilitate grape pressing and contribute to clarification of the must, they can also improve filtration of the wines and increase the extraction of several compounds that contribute to wine color and aroma.

Between pectolytic enzymes, pectinase play a fundamental rule on wine, of which polygalacturonases are the most important in winemaking process and can be distinguished in two types, **endo** and **esopolygalacturonases** that respectively act at random or hydrolyze the terminal groups and reduce the chain length only slightly.

The endogalacturonases influence the viscosity of the pulp improving some steps of the winemaking process, such as clarification.

Several studies demonstrate that non-*Saccharomyces* strains secrete various extracellular enzymes, mainly polygalacturonase and pectin methylesterase (Rosi *et al*., 1994; Saha and Bothast, 1996;

Charoenchai *et al*., 1997). Yeast belonging to the genera *Candida*, *Pichia* and *Kluyveromyces* produces polygalacturonase, while pectin methylesterase is produced by *Candida*, *Debaryomyces* and *Pichia* (Strauss *et al*., 2001; Dizy and Bisson, 2000; Fernández *et al.,* 2000; Masoud and Jespersen, 2006; Serrat *et al.,* 2004; Thongekkaew *et al., 2008*).

Usually yeast enzymatic activities are studied in vitro with synthetic media, however several components of the media might have an effect on the induction or inhibition of enzyme production, therefore a definitive relationship between the presence of the yeast and the secreted activity cannot be taken.

Another important group of extracellular enzymes is represented by **proteolytic enzymes** that hydrolyze protein, improve the clarity and stability of the wine. In addition they generated by hydrolysis peptides and amino acids that could prevent stuck or sluggish fermentation due to a lack of assimilable nitrogen in grape juice.

Yeast proteases play an important role for autolysis during the aging of wines and in the development of protein haze (protein degradation), mainly in white wines.

Nevertheless, not all proteases, usually detect in vitro with synthetic media or wine, are active under the stressful conditions found in wine or their activity are very weak.

The use of proteases from non-*Saccharomyces* yeasts in winemaking has been studied. Protease activity was observed in several strains of *Candida, Kloeckera/Hanseniaspora* and *Pichia,* Dizy and Bisson (2000) demonstrated that some species belonging to the genus *Kloeckera/Hanseniaspora* are the highest producers of proteases in the must and affect the protein profile of the finished wines.

Also **cellulolytic** and **hemicellulolytic enzymes** has a fundamental rule on organoleptic properties of wine, in fact their activity will allow extraction and release of pigments and aromas from the grape skins and reduces the maceration time.

To date, the only non-*Saccharomyces* yeasts that have been described as producers of cellulolytic or hemicellulolytic enzymes are *Candida* and *Cryptococcus* spp.

Difficulties in the clarification and filtration can be due to the presence of high-molecular-weight β-glucans, produced by *Botrytis cinerea* in infected grapes, that even if present in low concentrations can

affect filtration and are impossible to remove by conventional treatments (centrifugation or fining). These polysaccharides can be eliminated by enzymatic treatment, with b-glucanases that are specific for their hydrolysis.

B-glucosidases are a group of enzymes quite well characterized and many information are available.

Several glycosidases (β-glucosidase, β-xylosidase, β-apiosidase, α-rhamnosidase and α-arabinofuranosidase) are involved in flavor releasing processes. Nevertheless, the major part of the studies focused attention on β-glucosidases because of their wide diffusion (Leclerc *et al*., 1987). β-glucosidases are produced by several yeast genera, such as *Candida, Debaryomyces, Hanseniaspora, Kloeckera, Kluyveromyces, Metschnikowia, Pichia, Saccharomycodes, Schizosaccharomyces*, and *Zygosaccharomyces.* This enzyme influences the wine flavor and aroma, thanks to the hydrolysis of grape terpenyl-glycosides and the consequent liberation of terpenols during the fermentation process.

Suboptimal conditions, such as pH, temperature or inhibition by glucose and ethanol cause a reduction of activity of hydrolytic enzymes. Consequently, these precursors are hydrolyzed in very small rate during fermentation. The degree of inhibition depends mainly from the species or strains of organism involved (Aryan *et al*., 1987; Delcroix *et al*., 1994; LeClerc *et al*., 1987; Rosi *et al*., 1994).

Yeast glycosidases can influence the varietal aroma of the wine, especially when fermentation is carried out under natural conditions, where non-*Saccharomyces* yeasts predominate during the initial stages (Fugelsang, 1997), thanks to their marked hydrolytic activity, which is absent in most *Saccharomyces* strains (Charoenchai *et al*., 1997; Fernández *et al*., 2000; Manzanares *et al*., 1999, 2000; Mendes-Ferreira *et al*., 2001; Strauss *et al*., 2001; Zoecklein *et al*., 1997).

Several authors demonstrated that various non-*Saccharomyces* (*Candida*, *Debaryomyces*, *Hanseniaspora/Kloeckera*, *Kluyveromyces*, *Metschnikowia*, *Pichia, Saccharomycodes*, *Schizosaccharomyces*, and *Zygosaccharomyces*) can produce β-glucosidase (Charoenchai *et al*., 1997; Manzanares *et al*., 2000; McMahon *et al*., 1999; Strauss *et al*., 2001), however their potential is reduced because these enzymes are intracellular and released only in very small amounts into the medium (McMahon *et al*., 1999). In addition, generally β-glucosidases activity is very weak in the presence of

glucose in the must or wine. This behavior make necessary to analyze their inhibition by this sugar. Several strains of the genera *Candida*, *Debaryomyces*, *Kluyveromyces*, and *Pichia* are able to produce extracellular β-glucosidases that are not inhibited by glucose.

β-glucosidases produced by several *Debaryomyces* spp. maintain their activity in the presence of high ethanol (up to 15% vol/vol) and glucose (200mM) concentrations and releases terpenes, also when added to the must during fermentation, where it increases the concentrations of linalool and nerol by 90 and 116%, respectively (Yanai & Sato, 1999; Yanai & Sato, 1999; Belancic *et al*., 2003).

In addition, β-D-xylosidases are involved in releasing aromas, although data are limited on its hydrolytic capacity. Manzanares *et al*. (1999) shown that the genera *Hanseniaspora* and *Pichia* are the best producers of b- D-xylosidase. Between these two genera, only *H. osmophila*, *H. uvarum*, and *P. anomala* exhibited b-D-xylosidase activity. Another genus that is able to produce this enzyme is *Candida*.

The various enzymatic activities described above are responsible for the primary aroma of the wine, nevertheless yeast affect also the secondary or fermentation aroma.

Volatile fatty acids	Higher alcohols	Esters	Carbonyl compounds
Acetic acid	Propanol	Ethyl acetate	Acetaldehyde
Butyric acid	Butanol	2-Phenylethyl acetate	Benzaldehyde
Formic acid	Isobutyl alcohol	Isoamyl acetate	Butanal
Isobutyrric acid	Amyl alcohol	Isobutyl acetate	Diacetyl
Isovaleric acid	Isoamyl alcohol	Hexyl acetate	Propanal
Propionic acid	Hexanol	Ethyl butanoate	Isobutanal
Valeric acid	Phenylethanol	Ethyl caprate	Pentanal
Hexanoic acid		Ethyl caprylate	isovaleraldehyde
Heptanoic acid		Ethyl caproate	2 Acetyl tetrahydropyridine
Octanoic acid		Ethyl isovaleriate	
Nonanoic acid		Ethyl 2 methylbutanoate	

Table 2. Principal Volatile Fatty Acids, Higher Alcohols, Esters, and Carbonyl Compounds Produced During Alcoholic Fermentation

This aroma arises from wine yeast metabolism and compounds produced. Ethanol, glycerol, and carbon dioxide are quantitatively the most abundant of these compounds and play a fundamental role in wine aroma, however their influence on the secondary aroma is limited. In fact, volatile fatty acids, higher alcohols, esters and aldehydes have a greater contribution to secondary aroma.

These compounds, shown in **Table 2**, arise from the conversion of fermentable sugars, long-chain fatty acids, nitrogenated and sulfur compounds, among others.

Although *S. cerevisiae* has a great influence on the fermentation products, the contribute of non-*Saccharomyces* yeasts should not be forgotten, either in spontaneous or induced fermentation.

Generally in induced fermentation, where selected starter cultures of *S. cerevisiae* strains are inoculated in grape juice, the influence of non-*Saccharomyces* yeasts is reduced, although deveral studies shown that the use of starter cultures doesn't prevent the growth or metabolic activity of other natural strains of *S. cerevisiae* or non-*Saccharomyces* species, such as *K. apiculata*, *H. uvarum*, *C. stellata*, or *Torulaspora delbrueckii* (Egli *et al*., 1998; Heard & Fleet, 1986, 1985; Henick-Kling *et al*., 1998; Lema *et al*., 1996).

Volatile acidity of wines is mainly due to acetic acid that represents about the 90% of the volatile acidity, instead the remaining fatty acids, such as propanoic and butanoic acid, are present in small quantities as products of yeast metabolism.

Usually acetic and lactic acid bacteria generate high levels of acetic acid, yeasts are also involved in its production. *Saccharomyces* yeast strains are classified into three groups according to their production of acetic acid: low (0-0.3 g/L), intermediate (0.31-0.60 g/L), and high (>0.61 g/L).

Studies of acetic acid production in non-Saccharomyces yeasts shown highly variable results, the concentrations reached may be greater than or less than those produced by *S. cerevisiae*.

For example some strains of *K. apiculata* produces between 1 and 2.5 g/L, *Metschnikowia pulcherrima* between 0.1 and 0.15 g/L, *C. stellata* between 1 and 1.3 g/L,*Candida krusei* 1 g/L, *T. delbrueckii* between 0.01 and 1.07 g/L and *Hansenula anomala* between 1 and 2 g/L (Fleet & Heard, 1993; Renault *et al*., 2009).

Among fatty acids long-chain fatty acids, C16 and C18, are essential precursors for the synthesis of many lipid compounds in yeast, but they don't appear in wines. Intermediate-chain fatty acids, C8, C10, and C12 appear in wine together with their esters. It should be remembered that the production of these fatty acids is also associated with bacterial growth.

Usually among intermediate-chain fatty acids C8, C10 and their esters are produced in lower quantities by non-*Saccharomyces* yeasts, compared with those produced by *S. cerevisiae* (Renault *et al*., 2009; Rojas *et al*., 2001; Viana *et al*., 2008).

Short-chain fatty acids can inhibit the growth of *S. cerevisiae* and stop fermentation, but generally non-*Saccharomyces* yeast are low producer of short-chain fatty acids, substantially below the levels that can inhibit the growth of *S. cerevisiae* (Edwards *et al*., 1990).

Higher alcohols have a great impact on wine aroma and quality, normally they are present in wine in low concentration, about 300 mg/L, they contribute to the aromatic complexity of the product. However if their concentrations exceed 400 mg/L, they are considered to have a negative effect on aroma. The most predominant is isoamyl alcohol, although propanol, isobutyl alcohol, and amyl alcohol are also produced.

Generally production of higher alcohols is a strain specific characteristic and can be used as a parameter to select commercial starter culture for industrial applications. In other also the ratio between isoamyl and amyl alcohol, isobutanol, and propanol (Herraiz *et al*., 1990), and the production of dodecanol and tetradecanol (Longo *et al.,* 1992), are specific to each strain. Several studies on higher alcohol production in non- *Saccharomyces* yeasts shown the influence that these yeasts can have on the chemical composition and quality of the wine (Herraiz *et al*., 1990; Longo *et al*., 1992; Mateo *et al*., 1991). Nevertheless, the production of higher alcohols by pure cultures of non-*Saccharomyces* yeasts is lower than those produced by *S. cerevisiae* (Moreira *et al*., 2008; Rojas *et al*., 2003; Viana *et al*., 2008, 2009), this difference seems to disappeare in mixed cultures of *S. cerevisiae* and non-*Saccharomyces* yeast.

Esters are the most abundant compounds found in wine, their concentration in wine is above the perception threshold, and several of the sensory parameters used in wine evaluation correspond to ester aromas, for example, the fruity aroma of young wines is due to a blend of esters generated during fermentation, mainly acetate esters.

Several esters can be found in wine, however the most abundant in wine are those arise from acetic acid or higher alcohols and ethyl esters of saturated fatty acids, respectively such as ethyl acetate, isoamyl

acetate, isobutyl acetate, and 2-phenylethyl acetate, ethyl butanoate, ethyl caproate, ethyl caprilate, and ethyl caprate.

Among non-*Saccharomyces* yeasts, several genera have been described as good producers of esters, *Candida*, *Hansenula*, and *Pichia* species are greater producers of ethyl acetate, more than *S. cerevisiae* (Ough *et al*., 1968). The genus *Hanseniaspora*, mainly *H. uvarum*, is a good producer of esters in general (Mateo *et al*., 1991; Romano *et al*., 1997). Viana *et al*. (2008) demonstrated that *Hanseniaspora* strains produce acetate esters, particularly 2-phenylethyl acetate. Among the ethyl esters, the genus *Saccharomyces* was the best producer of ethyl caproate, while the genus *Torulaspora* produce more ethyl caprylate.

In addition, the levels of esters produced is specie and strain dependent (Lambrechts & Pretorius, 2000). For example several species of the genus *Hanseniaspora (H. guilliermondii, H. osmophila,* and *H. uvarum*) produce significant amount of 2-phenylethyl acetate and isoamyl acetate (Moreira *et al*., 2005, 2008; Rojas *et al*., 2001, 2003; Viana *et al*., 2008, 2009), with significant differences among different strains (Viana *et al*., 2008).

Among carbonyl compounds, aldehydes have a great enological value, mainly for characteristics that they confer on the wine (apple, lemon, and nutty aromas) also if present in small quantities. Acetaldehyde constitutes more than 90% of the total aldehyde content of wines. Other carbonyl compounds of interest include diacetyl and the tetrahydropyridines, responsible for the acetamide (mousy) aroma due to the growth of LAB and *Brettanomyces* (Heresztyn, 1986).

Data are available on the effect of non- *Saccharomyces* yeasts on the total concentration of aldehydes in wine. Several strains of non-*Saccharomyces* (*K. apiculata*, *C. krusei*, *C. stellata*, *H. anomala*, and *M. pulcherrima*) produce undetectable quantity of aldehydes, about 40 mg/L, while *S. cerevisiae* strains produce between 6 and 190 mg/mL (Fleet & Heard, 1993). In addition, Romano *et al*. (2003) found little differences in the production of acetaldehyde by 52 strains of *S. cerevisiae*, instead the differences among the 59 strains of *H. uvarum* studied were significant.

Volatile phenols and sulfur compounds make a lesser contribution to wine aroma than the compounds described above. Nevertheless, qualitatively they are very important, mainly for their low perception thresholds and for their negative contribution to wine aroma.

Volatile phenols arise from hydroxycinnamic acids (trans-ferulic, trans-r-coumaric, and caffeic acid) present in the grapes thanks to the sequential action of two enzymes. First, hydroxycinnamate decarboxylase converts hydroxycinnamic into vinylphenols (4-vinyl guaiacol and 4-vinylphenol), and these are then reduced to ethylphenols (4-ethylguaiacol and 4-ethylphenol) by vinylphenol reductase.

Volatile phenols in wine are generally present in a range between 0 and 6047 μg/L, when they exceed the perception threshold, about 400 μg/L they are responsible for the phenolic aroma of wines. Furthermore, their presence is always undesirable, because they mask the fruity notes of white wines (Chatonnet *et al*., 1992). Yeast belonging to the genera *Brettanomyces/Dekkera* are the most important volatile phenols producers, however also other non-*Saccharomyces* yeast strains shown this capacity, such as *Pichia guilliermondii* species (Dias *et al*., 2003; Renault *et al*., 2009; Shinohara *et al*., 2000). Nevertheless, the first step of volatile phenols production, i.e. the decarboxylation of hydroxycinnamic acids into vinylphenols, is much more common in several non-*Saccharomyces* wine yeasts (e.g., *Hanseniaspora*, *Pichia*, and *Zygosaccharomyces* species) and in strains of *S. cerevisiae* (Chatonnet *et al*., 1992).

Several sulfur compounds are present in wine and they can be divided into various groups according to their chemical structure: sulfides, heterocyclic polysulfide compounds, thioesters, and thiols.

Sulfur compounds have different sensory properties, although most of them have a negative impact on wine aroma, they can also give a positive contribute to wine aroma through the introduction of fruity notes (Swiegers *et al*., 2005).

Among sulfur compounds, hydrogen sulfide is the most studied, and the major part of studies focused on *S. cerevisiae* and very limited information is available on their production by non-*Saccharomyces* yeasts. Between non-*Saccharomyces* species, yeast belonging to the genera *Candida* shown the highest hydrogen sulfide production (Strauss *et al*., 2001). Nevertheless, *Hanseniaspora* spp. and *T. delbrueckii* have also been reported to produce hydrogen sulfide (Renault *et al*., 2009; Viana *et al*., 2008).

Moreira *et al*. (2008) evaluated the capacity of *H. uvarum* and *H. guilliermondii* to produce heavy sulfur compounds showing significant differences between the two species, however the concentrations produced were similar to those of *S. cerevisiae*.

1.7 Molecular identification and characterization of wine yeast

Traditional phenotypical techniques to identify yeast are questionable, because they depend on the physiological state of the yeasts (Golden *et al*., 1994). While molecular biological techniques circumvent these difficulties by allowing direct analysis of the genome, irrespective of the physiological state of the cell.

A lot of molecular techniques have now been developed and successfully applied to the identification and characterization of yeasts.

The major part of the studies on molecular identification focused on yeast of the genus *Saccharomyces*, mainly *S. cerevisiae*, due to their importance in the winemaking process. Nevertheless, several studies were also performed on non-*Saccharomyces* yeast. Some of these studies demonstrated that for a definitive characterization of individual strains a combination of several techniques is required (Baleiras Couto *et al*., 1996; Fernàndez- Espinar *et al*., 2001; Pramateftaki *et al*., 2000).

1.7.1 Methods for species identification

1.7.1.1 Methods Based on Analysis of Ribosomal DNA (rDNA)

Yeast ribosomal genes, 5.8S, 18S, and 26S, are organized in tandem and form "transcription units" that generally are repeated 100 to 200 times throughout the genome. Each transcription unit is constituted by other two regions (as reported in Fig.6), the **internal transcribed spacer** (ITS) and the **external transcribed spacer** (ETS).

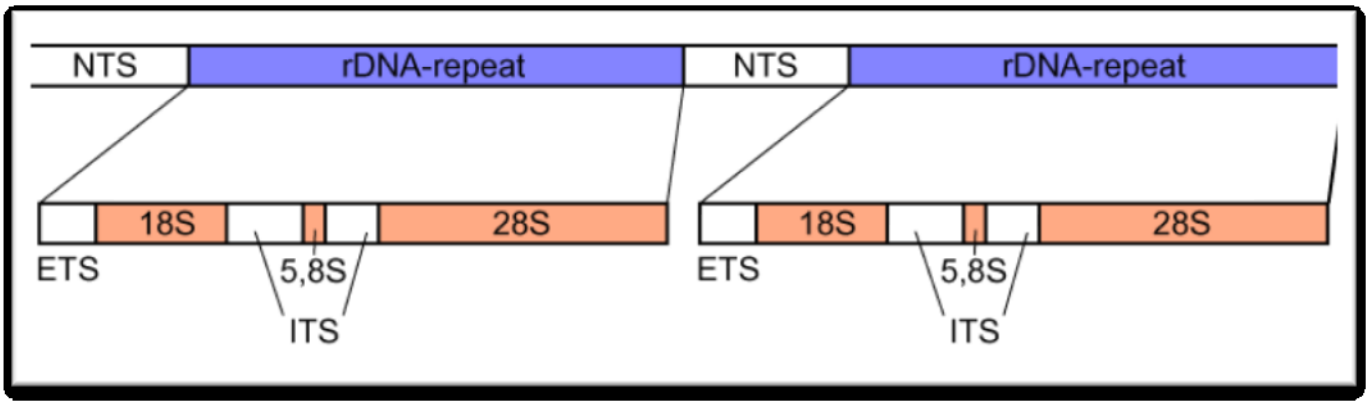


Figure 3. Yeast ribosomal genes organization

The coding regions divided by intergenic spacers (IGSs), usually known as non-transcribed spacers (NTSs). The sequences of the 5.8S, 18S, and 26S ribosomal genes and the ITS and NTS spacers are well conserved and similarities between repeated transcription units within a given species is greater than between units from different species. This sequence similarity within species makes these ribosomal DNA (rDNA) regions powerful tools to identify species and establish phylogenetic relationships between them.

Yeast species can be identified by comparison of nucleotide sequences from rDNA regions. The techniques most commonly used is based on the nucleotide sequences of the D1/D2 region of the 26S gene (Kurtzman & Robnett, 1998), which sequences are largely available in DNA databases and allows to assign unknown yeasts to a specific species when their homology of the sequences is greater than 99%.

Thanks to the technological advances that have been made and the widely availability of sequencing data on Web, sequencing has become a useful tool that complements the other molecular techniques.

Other techniques based on PCR amplification of rDNA region are been developed for use in industrial applications, these methods allows the direct amplification of target genes without further DNA purification. Different size of amplification products correspond to different species, however fragment of amplification of the same size, not always correspond to the same species, and digestion of these fragments with restriction enzymes is required for definitive identification. Digestion is performed directly on PCR product. Then the fragments obtained are separated by electrophoresis on 3% agarose gels and their size is established by comparison with DNA markers.

Different regions can be amplified with these techniques, Dlauchy *et al*. (1999) amplified the 18S ribosomal gene and the ITS1 intergenic region from several yeast associated with wine, beer and soft drinks using the primers NS1 (5'-GTAGTC ATA TGC TTG TCT C-3) and ITS2 (5- GCT GCG TTC TTC ATC GAT GC-3) and digesting the PCR products with 4 endonucleases (*AluI, HaeIII, MspI*, and *RsaI*). Another rDNA region generally used to distinguish different species is 5.8S gene and the adjacent intergenic regions ITS1and ITS2, which are amplified using the primers ITS1 (5-TCC GTA GGT GAA CCT GCG G-3) and ITS4 (5-TCC TCC GCT TAT TGA TAT GC-3), described by White *et al*. (1990). This technique was used by several authors (Guillamón *et al*.,1998; Granchi *et al*., 1999; Esteve-Zarzoso *et al*., 1999; Pham *et al*., 2011) for the rapid identification of wine yeasts. The amplification products obtained were digested with 4 different endonucleases (*HaeIII, HinfI, CfoI*, and *DdeI*), the size of the amplified fragments and restriction profiles are available online at http://yeast-id.com/.

Restriction analysis of other rDNA regions has also been used to identify other yeast species, mainly those belonging to the *Saccharomyces* sensu stricto complex, such as the NTS region (Baleiras Couto *et al*., 1996; Capece *et al*., 2003; Caruso *et al*., 2002; Nguyen & Gaillardin, 1997 Pulvirenti *et al*., 2000), the 18S gene (Capece *et al*., 2003) and various domains of the 26S gene (Baleiras Couto *et al*., 1996, 2005; Romancino *et al*., 2008; Smole-Mozina *et al*., 1997; van Keulen *et al*., 2003).

1.7.1.2 Real-time Polymerase Chain Reaction (PCR)

Real-time PCR was developed in 1996 and since then its use has increased almost exponentially across a range of applications (Wilhelm & Pingoud, 2003).

This technique is based on the direct monitoring of the amplification products during each PCR cycle, due to the detection and quantification of a signal generated by a fluorescent donor dye. This signal is directly correlated with the initial amount of DNA present in the reaction.

The data obtained are represented as an amplification curve with the point at which the intensity of the signal from the donor becomes greater than the background noise indicated. This is known as the threshold cycle (Ct) and it is inversely proportional to the number of copies of the target sequence in the

sample (DNA or cells). Consequently, it can be used to assess the starting quantity of target DNA with a high degree of accuracy over a wide range of concentrations.

The fluorescent signal may be derived from intercalating agents or probes. The intercalating agent SYBR green binds to double-stranded DNA, leading to an increase in fluorescence with increasing amounts of PCR product.

While probes can be distinguished into 3 different type:

- hydrolysis probes,
- hairpin probes,
- hybridization probes.

The most largely diffused probes are hydrolysis probe, mainly Taqman probe, which has both donor and acceptor fluorochromes. When both fluorochromes are bound to the probe, the donor does not emit a signal. When the probe is bound to a sequence of interest during the PCR reaction, the exonuclease activity of Taq polymerase activates the donor fluorochrome in the rest of the probe, leading to emission of a fluorescent signal. This signal is monitored as it accumulates during successive PCR cycles.

The choice of the fluorescence systems to use is influenced by their advantages and disadvantages. For example, SYBR green is the most appropriate for a simple, cheap, and easy system. However, during the PCR reaction it can bind primer dimers and other nonspecific products and lead to overestimation of the concentration of target DNA. The need for greater specificity calls for the use of a system involving probes.

Real-time PCR has a number of advantages over other identification techniques. It is highly specific and sensitive, quantitative, and does not require additional analyses, such as electrophoresis following PCR. The lack of requirement for additional procedures and the shorter reaction times and amplification cycles make real-time PCR a very rapid technique. This is particularly useful for routine analysis and applications requiring corrective measures. The most problematic steps of real-time PCR is the choice of primers set, generally are widely diffused free software to design primers that must be specific and sensible.

Generally, primers used in real-time PCR for yeast identification are based on sequence genes or genomic regions that allows to establish a phylogenetic relationships between yeast species. These sequences also have the advantage of being easily available via the Internet. They correspond to the ITS (James *et al*., 1996) and D1/D2 (Kurztman & Robnett, 1998) rDNA regions, the mitochondrial gene COX2 (Belloch *et al*., 2000; Kurztman & Robnett, 2003), and the nuclear gene actin (Daniel & Meyer, 2003). These have been applied in real-time PCR systems developed for the rapid detection and quantification of total yeasts in wine, and also to monitoring populations of *Saccharomyces* and *Hanseniaspora* species during alcoholic fermentation.

1.7.1.3 Polymerase Chain Reaction (PCR)- denaturing Gradient Gel Electrophoresis (DGGE)

PCR amplification and denaturing gradient gel electrophoresis (DGGE) allows to separate DNA fragments of the same length on the basis of sequence differences, due to the decreased electrophoretic mobility of a partially melted double stranded DNA molecule in polyacrylamide gels containing a linear gradient of denaturing agents (a mixture of urea and formamide). DNA migration is retarded when the DNA strands dissociate at a specific concentration of denaturing agent. However, a complete strand separation is prevented by the presence of a high-melting-point domain, thanks to DNA amplification using particular groups of universal primers, generally by adding a sequence containing guanines (G) and cytosines (C) to the 5 end of one of the PCR primers, co-amplified, and thus introduced into the amplified DNA fragments.

A similar technique is the “temperature gradient gel electrophoresis” (TGGE), based on a linear temperature gradient for separation of DNA molecules. DNA bands in DGGE or TGGE can be visualized using ethidium bromide or SYBR Green I. In other PCR fragments can be extracted from the gel and used in sequencing reactions for species identification.

1.7.2 Methods to differentiate between *Saccharomyces cerevisiae* strains

1.7.2.1 Hybridization Techniques

A considerable part of the *S. cerevisiae* genome is not transcribed or translated and does not affect the phenotype of the yeast. However, mutations in these noncoding DNA regions can eliminate or create restriction sites that can be found by hybridization of DNA probes corresponding to the target regions.

The restriction pattern obtained by digestion of the DNA is separated on agarose gels, transferred to nylon or nitrocellulose membranes by Southern blotting and finally hybridizated with specific probes.

The probes can be labeled radioactively or non-radioactively, respectively with ^{32}P or digoxigenin/biotin. These technique is very useful to distinguish *S. cerevisiae* strains or other yeast, several probes have been tested, in particular those against genes PFK2, PY30, and PDC1, which code for glycolytic enzymes; TRP1 and TRP3, which code for enzymes involved in amino acid synthesis and repetitive DNA regions, such as the retrotransposons Ty1 and Ty2. Nevertheless only few studies have applied the technique in wine yeasts.

Another similar technique used to separate chromosomes is the pulsed-field gel electrophoresis (PFGE).

In PFGE an alternative application of two transverse electric fields forced the chromosomes to change the direction of their migration. Therefore, large fragments of DNA are no longer detained in the agarose gel matrix and can be separated.

The yeasts are grown in liquid medium, then combined with molten agarose and placed in small molds. The cells are then lysed in situ and the released DNA is immobilized in the agarose matrix. The blocks are inserted into agarose gels, which are then exposed to electric fields.

Several parameters influence the resolution of the bands, such as the electric-field switching intervals, the agarose concentration, the temperature and the angle between the electric fields.

This method, also known as karyotype analysis, is highly efficient for the differentiation of *S. cerevisiae* strains, revealing a high polymorphism, due to the addition or elimination of long fragments of DNA in homologous chromosomes during the evolution of the yeast genome (Casaregola *et al*., 1998; Keogh *et al*., 1998; Wolfe & Shields, 1997).

Several studies based on karyotype analysis allow to characterize wine strains of *S. cerevisiae* (Schütz and Gafner, 1994; Martínez *et al*., 1995; Versavaud *et al*., 1995; Briones *et al*., 1996; Nadal *et al*., 1996; Egli *et al*., 1998).

1.7.2.2 Restriction Analysis of Mitochondrial DNA

S. cerevisiae strains have a small and highly variable mitochondrial DNA (mtDNA), generally its size is between 60 and 80 kb. mtDNA show an high degree of polymorphism that can be revealed by restriction analysis and allow to use it for the characterization of wine yeast strains.

Several authors investigated on yeast mtDNA (Aiglé *et al*., 1984; Gargouri, 1989; Querol & Barrio,1990). Restriction analysis of mtDNA used GCATtype enzymes that do not recognize GC- or AT- rich regions in digestions of total DNA, while recognized mtDNA, generally rich in AT (about 75%) or GT. So, given the small number of restriction sites in the mtDNA and the large number of sites in the nuclear DNA, led to digest total DNA into small fragments, while mtDNA forms large bands that can be clearly visualized over the background shadow of the digested nuclear DNA.

Nevertheless not all enzymes are able to reveal the degree of polymorphism; in other digestion patterns are species-dependent. For example, the most appropriate enzymes to differentiate between *S. cerevisiae* strains are *HinfI* and *HaeIII* (Guillamón *et al*., 1994).

1.7.2.3 Polymerase chain reaction (PCR)-based methods

Several methods based on PCR allow to differentiate wine yeast, in other some PCR-based techniques can be used to detect DNA polymorphisms without the use of restriction enzymes. Among these techniques, the most used to distinguish *S. cerevisiae* strains are RAPD, microsatellite analysis, amplification of δ sequences and intron splice sites.

1.7.2.3.1 Random amplification of polymorphic DNA (RAPD)

The random amplification of polymorphic DNA (RAPD) is a PCR-based method use short primer (around 10 nucleotides) that has a random sequence and a low annealing temperature, about 40°C, generally this technique use only one primer.

The short oligonucleotide used and the low annealing temperature lead to the amplification of a range of DNA fragments distributed throughout the genome. Therefore, the result is a pattern of amplified products of different molecular weight, like a fingerprint, that can be characteristic of the species or strains (Paffetti *et al*., 1995).

The main advantage of RAPD is that no prior sequence information is required in order to design a primer. Nevertheless, this technique reveals more polymorphism than techniques that analyze specific regions, allowing to analyze the variability throughout the entire genome. In addition, the low annealing temperature results in amplification profiles unstable and difficult to reproduce, furthermore multiple reactions are required for each sample using DNA from different extractions as the template. Only the bands present in all of the reactions can be considered.

Several results obtained with different oligonucleotides must be combined to achieve good resolution, the technique is not appropriate for routine industrial application. Consequently, it has not been used extensively for the characterization of *S. cerevisiae* strains and is more widely applied in taxonomic studies (Molnar *et al*., 1995).

1.7.2.3.2 Analysis of repetitive genomic DNA (microsatellites and minisatellites)

The high variability of repetitive regions of genomic DNA allow to identify several yeast species.

These repetitive regions, known as microsatellites and minisatellites, have different length and are present as tandem repeats distributed randomly throughout the genome.

Microsatellites are usually shorter than 10 base pairs, while minisatellites are between 10 and 100 base pairs in length. The variability of these regions can be shown by PCR amplification with specific oligonucleotides, such as (GTG)5, (GAG)5 and (GACA)4.

Several authors used microsatellites and minisatellites to distinguish *S. cerevisiae* strains, obtaining generally PCR products 700 to 3500 base pairs long, that can be visualized on agarose gels.

Sequence data from *S. cerevisiae* databases is assessed to identify repetitive regions and then used to design primers, although only four strains were analyzed. In addition, Gonzàlez Techera *et al*. (2001) and Pérez *et al*. (2001) designed new primers to differentiate *S. cerevisiae* wine strains.

Schuller *et al*. (2004) showed that the resolution of microsatellites is comparable to that obtained with δ elements and restriction analysis of mtDNA.

More recently, several authors proposed a method for the identification of *S. cerevisiae* based on PCR amplification of polymorphic regions of the genome using combinations of more than two primers in a single PCR reaction (Richards *et al*., 2009; Vaudano & García-Moruno, 2008).

Richards *et al*. (2009) generated a database containing 246 genotypes, including 78 commercial wine strains along with other natural isolates from various different regions of the world.

Amplification products are usually visualized in acrylamide gels, although automatic sequencers can also be used.

Marinangeli *et al*. (2004) found a variable numbers of microsatellites in some genes that code for cell-wall proteins from *S. cerevisiae* that allow to characterize several *S. cerevisiae* wine. The results of these technique are very stable and reproducible, thanks to the high annealing temperatures used (60-65°C) in the PCR reaction.

1.7.2.4.2 Amplification of δ sequences

Delta sequences are 0.3 kb elements that flank Ty1 retrotransposons, normally present in several copies, about 100, in the yeast genome, as part of Ty1 retrotransposons or as isolated elements. Usually these δ elements are localized in genomic regions adjacent to the transfer RNA genes (Eigel & Feldmann, 1982).

The number and localization of these elements allows to show a degree of intraspecific variability, used by Ness et al. (1993) to characterize *S. cerevisiae* strains using two specific primers, named d1 and d2. In

other they demonstrated the stability of δ elements, also confirmed by other groups (Legras and Karst, 2003; Capello *et al*., 2004; Ciani *et al*., 2004; Le Jeune *et al*., 2006).

Comparison with other high-resolution techniques, such as karyotyping or restriction analysis of mtDNA, has shown that δ elements can reveal a high variability within *S. cerevisiae* strains (Fernàndez-Espinar *et al*., 2001; Pramateftaki *et al*., 2000).

Legras and Karst (2003) optimized these technique by designing two new primers, named d12 and d22, localized very close to old primers d1 and d2. New primers allow to reveal greater polymorphism, which is reflected by the appearance of a larger number of bands. As a result, the new primers are able to differentiate more strains. Schuller *et al.* (2004) tested the primers designed by Ness *et al*. (1993) and Legras *et al*. (2003) demonstrating that the association of d2 and d12 allow to identify twice as many strains when compared to d1 and d2.

The two major problematic of these technique are the impact of the concentration of DNA and the appearance of "ghost" bands due to the low annealing temperature (42°C) used during the amplification reaction.

However, studies on delta elements are controversely, in fact, Ciani *et al*. (2004) used an annealing temperature of 55°C to characterize wine strains of *S. cerevisiae*, with a much more stable amplification profiles, but fewer bands were obtained. While Capece *et al*. (2012) used a new colony protocol, increasing the time and the temperature of initial denaturation (97°C for 10 minutes) and an annealing temperature of 45°C, and confirmed that these technique is the most discriminative method for *S. cerevisiae* characterization, as reported by other authors (Legras and Karst, 2003; Schuller *et al*., 2005; Xufre *et al*., 2011).

1.7.2.4.3 Amplified Fragment Length Polymorphism (AFLP)

Amplified fragment length polymorphism (AFLP) is another PCR-based technique; however, its methodology is very complex because involving the use of other techniques. In fact, AFLP involves first

the restriction of genomic DNA, then the binding of adapters to the fragments obtained and finally their amplification by PCR using the adapter sequence and restriction sites as the targets for the primers.

Also for AFLP, such as RAPD analysis, no prior sequence information is required to design primers. Furthermore, the technique is easily reproduced and yields extensive information, however it is very laborious and requires automatic sequencers, which are not appropriate for routine industrial applications, and the data produced are difficult to interpret.

Only few studies on yeasts used AFLP (Dassanayake & Samaranayake, 2003; Theelen *et al*., 2001), nevertheless it has been used for the characterization of different species of wine yeast by several authors (Azumi & Goto- Yamamoto, 2001; Curtin *et al*., 2007; de Barros Lopes *et al*., 1999; Flores Berrios *et al*., 2005; Lopandic *et al*., 2008).

1.8 Malolactic fermentation (MLF)

Malolactic fermentation (MLF) is the secondary process involved in winemaking, it consists in the bacterial driven conversion of L-malic acid to L-lactic acid and CO_2. The principal microorganisms responsible to MLF are lactic acid bacteria (LAB), which possess three possible enzymatic pathways for the conversion of L-malic acid to L-lactic acid and CO_2:

1) Direct conversion of malic acid to lactic acid via malate decarboxylase, generally known as malolactic enzyme (MLE),
2) Conversion of malic acid to pyruvic acid by malic enzyme, followed by reduction to lactic acid by L-lactate dehydrogenase,
3) Reduction of malate by malate dehydrogenase to oxaloacetate, followed by decarboxylation to pyruvate and reduction to lactic acid (Lonvaud-Funel, 1999).

The first pathway does not produce free intermediates during the decarboxylation process and requires NAD+ and Mn2+ as. Is generally recognized that the specific malolactic activity influence the rate of malate decarboxylation by LAB (Bartowsky, 2005). Lonvaud-Funel (1995) studied the malate decarboxylase (*mleA*) gene, purifying enzymes from several LAB species, isolated from wines and

grapes, including from *Lactobacillus* and *Leuconostoc* species. The majority of wine LAB utilise the first pathway to generate lactic acid.

The principal physiological function of the MLF is to generate a proton motive force (PMF) to obtain energy essential to drive cellular processes (Konings, 2002).

The MLF reaction catalyzed by the MLE enzyme can be divided into three stages: i) uptake of L-malic acid by wine LAB, ii) decarboxylation of L-malic acid to L-lactic acid and CO2 and iii) excretion of L-lactic acid together with a proton.

The decarboxylation reaction generate an electrical potential ($\Delta\psi$), the proton released during the decarboxylation reaction cause an increase in the internal pH of the bacterial cell which lead a pH gradient (ΔpH) across the membrane. These two components make up the PMF, which then generate ATP via membrane ATPases. The PMF is sufficient to drive energy-consuming reactions e.g. the transport of metabolites (Henick-Kling, 1993).

1.8.1 Lactic Acid Bacteria involved in malolactic fermentation

Lactic acid bacteria are gram-positive, non-sporing and non-respiring bacteria, usually coccoid, elongated cocci or rod-shaped bacilli. The principal product of carbohydrates fermentation is lactic acid. Several LAB, such as *Leuconostoc*, *Pediococcus*, *Lactobacillus* and Oenococcus *oeni*, are responsible for the changes of organoleptic and chemical properties of wine during fermentation process (Wibowo *et al*., 1985).

O. oeni has best adapted to the wine environment and concomitantly the majority of LAB present in wine belong to this species. *O. oeni* strains are also the selected bacteria used for commercial starter cultures (Wibowo *et al*., 1985; Davis *et al*., 1988; Drici-Cachon *et al*., 1996; Lonvaud-Funel, 1999).

1.8.1.1 Evolution of Lactic Acid Bacteria during malolactic fermentation

Several authors studied the evolution of LAB, from the vineyard to the final steps of winemaking, demonstrating an high degree of variability, due to various factors, such as the region, cultivar and

vinification procedures. Is usually recognized a successional growth of several species of LAB during the several vinification steps, in other *O. oeni* is the principal actor of MLF (Wibowo *et al*., 1985; Boulton *et al*., 1996; Fugelsang & Edwards, 1997). Nevertheless also other LAB occur during MLF, such as *Pediococcus damnosus*, *Pediococcus parvulus* and *Pediococcus pentosaceus*. In higher pH wines, several *Lactobacillus* species can be involved in MLF (Wibowo *et al*., 1985).

Furthermore the diversity and population density of LAB in the vineyard are very limited, especially when compared to the indigenous yeast population found on grapes (Fugelsang & Edwards, 1997).

Organisms are commonly present on both grapes and leaf surfaces (Wibowo *et al*., 1985), however on undamaged grapes and grape must LAB are rarely higher than 10^3 CFU/g (Lafon-Lafourcade *et al*., 1983).

The population size on grape surfaces is strictly correlated with the maturity and sanitary state of the grapes (Jackson, 2008), generally *Pediococcus* and *Leuconostoc* species can be found on grapes more frequently than *O. oeni*. In addition, several LAB species can be also isolated from the cellar environment, mainly barrels and poorly sanitized winery equipment (Donnelly, 1977; Boulton *et al*., 1996; Jackson, 2008).

Immediately after crushing at the beginning of AF, the LAB population on the grape juice generally range between 10^3 to 10^4 CFU/mL, at this steps mainly occur *Lactobacillus plantarum*, *Lactobacillus casei*, *Leuconostoc mesenteroides*, and *Pediococcus damnosus*, although it is possible to isolate few *O. oeni* strains (Wibowo *et al*., 1985; Lonvaud-Funel *et al*., 1991; Boulton *et al*., 1996; Powell *et al*., 2006).

The major part of LAB species generally do not multiply and decline quickly the end of AF, as a result of the increased ethanol concentrations, high SO_2 concentrations, low pH, low temperatures, the nutritional status and competitive interactions with the yeast culture, at the end of AF only *O. oeni* survive in the wine stressful ernvironment (Wibowo *et al*., 1985; Lonvaud-Funel *et al*., 1991; Fugelsang & Edwards, 1997).

After the completion of AF and the bacterial lag phase, the surviving bacterial cells, major representing by *O. oeni* strains, start to multiply. This step is characterized by vigorous bacterial growth, MLF started when bacterial populations reach 10^6 CFU/mL (Wibowo *et al*., 1985; Lonvaud-Funel, 1999).

The principal factor correlated with the dominant species of LAB is wine pH, usually pH about 3.5 favours the growth of *Lactobacillus* and *Pediococcus* species, while *O. oeni* strains dominate at lower pH values (Henick-Kling, 1993).

At the end of MLF, the remaining viable LAB are able to metabolize residual sugar, resulting in a spoilage, mainly due to an increased volatile acidity (Fugelsang & Edwards, 1997). This phenomenon occurs principally in high pH wines, driven by *Lactobacillus* and *Pediococcus* species that can contribute to wine spoilage (Wibowo *et al*., 1985).

By understanding the evolution of LAB from the vineyard/ grape surfaces, through the different vinification procedures, as well as their metabolic requirements, it is possible to control which species of LAB occur at a particular stage and to ensure that they make a positive contribution during MLF (Lerm & Du Toit, 2010).

1.8.2.1 O*enococcus oeni*

Oenococcus oeni is a gram-positive, non-sporing and non-respiring bacteria bacteria, ellipsoidal to spherical cocci, often in pairs or chains, 0,5-1,0×0,7-1,5µm identified by Garvie, (1967) and known as *Leuconostoc oenos* until 1995 (Dicks *et al*., 1995), in fact modern molecular techniques shown several phylogenic differences among rRNA sequences of *O. oeni* and *Leuconostoc* in "sensu stricto" species.

O. oeni usually growth in a range of temperature between 10 and 35°C, with an optimum of temperature of 18-24°C, they are able to tholerate low pH, generally about 3-3,2.

O. oeni metabolizes either pentose or esose carbohydrates, through eterofermentative pathway. Glucose and fructose represent about 99% of grape juice sugar (Coombe & Dry, 1992), all *O. oeni* strains are able to ferment both sugar (Garvie, 1967; Van Vuuren & Dicks, 1993).

O. oeni PSU1 is also able to ferment ribulose and cellobiose (Beelman *et al*., 1977) thanks to several pathway specific for these sugars.

Beelman *et al*. (1977) demonstrated that *O.oeni* PSU1 convert fructose into mannithol, a precursor of acetic acid and a common spoilage compound of wine if present in high amount (Boulton *et al*., 1996).

O. oeni is the principal responsible of MLF, due to its ability to decarboxylate malic acid into lactic acid and CO_2 (Lolkema *et al*., 1996).

Malic acid is a fundamental component of grape juice, it reach high level especially in warm region (about 5-1 g/l). MLF caused an increase in wine pH, from 0.2 to 0.5 units, a decrease in titratable acidity, which translates into a decrease in wine sourness, an increase of microbial stability, thanks to the removal of potential carbon sources which could be used by wine spoilage yeasts and bacteria, and the bacterial production of various secondary metabolites, which can improve the organoleptic properties of wine.

Several studies focused attention on citric acid metabolism by *O. oeni* (Lonvaud-Funel, 1999; Bartowsky *et al*., 2004), due to their fundamental rule on the production of several aromatic compounds.

Liu (2002) demonstrated that a co-fermentation of glucose and citric acid causes in an icreased production of acetic acid, with consequent negative impact on wine flavor and aroma. Nevertheless the level of acetic acid is a strain-dependent characteristic, influenced also from the chemical composition of grape juice (Maicas *et al*.,1999; Davis *et al*.,1985).

O. oeni strains are able to survive and growth in a stressful environment such as the wine, mainly thanks to the syntethys of several compounds fundamental for their response to stressful conditions. However these stress resistence is a characteristic strain-dependent, so not all the *O. oeni* strains are able to tholerate extreme environmental conditions (Drici-Cachon *et al*.,1996; Fortier *et al*., 2003).

1.8.3 Induction of malolactic fermentation

1.8.3.1 Use of starter culture to induce malolactic fermentation

Traditionally winemakers ground on spontaneous MLF, however after 1970's they are started to recognise the advantages of induced MLF, by inoculating grape juice or wine with commercial starter

cultures of LAB to ensure a complete and successful MLF (Davis *et al*., 1985; Fugelsang & Zoecklein, 1993; Henick-Kling, 1995; Krieger-Weber, 2009). Induced MLF allows also to reduce the risks associated with spontaneous MLF, such as the presence of unidentified/spoilage bacteria that can produce undesirable off-flavours or toxic compunds, such as biogenic amines (Davis *et al*., 1985), a delay in the onset or completion of MLF (Nielsen *et al*., 1996) and the development of bacteriophages (Bauer & Dicks, 2004). All these parameters resulted in a decrease in the quality of the wine (Bartowsky & Henschke, 1995; Fugelsang & Edwards, 1997).

Instead induced MLF, obtained by inoculating grape juice with a selected starter culture of *O. oeni,* allows to reduce the risk of potential spoilage bacteria, promote a rapid and complete MLF and also influence positively wine aroma and flavor (Krieger-Weber, 2009).

Nevertheless, during the last years also other LAB species, such as *L. plantarum*, has been used for application in a commercial starter culture (Bou & Krieger, 2004).

The first prototypes of commercial starter culture of LAB was developed in the 1960's to 1970's thanks to the concept of inoculating grape juice or wine for MLF with a single strain. MLF starter cultures were available in liquid form and used for decades until the early 1980's. Then, frozen and freeze-dried LAB starter cultures were developed and since the 1990's became available freeze-dried starter cultures that can be direct inoculated, "Viniflora oenos" being the first (Nielsen *et al*., 1996).

Commercial cultures are also easy to ship, store and use, which adds to their increasing popularity.

Usually they contains a very high population of viable bacteria, about ± 10^{11} CFU/g, so to have a LAB population enough to ensure the completion of MLF, also after loss in viability due to the wine conditions (Henick-Kling, 1993, 1995).

There are several types of LAB starter cultures still available, such as liquid, frozen or freeze-dried cultures. The liquid culture has only a shelf life of 2-20 days and requires a preparation time of 3 to 7 days, the frozen one need to be inoculated immediately after thawing and the pellets are directly added to the wine. In addition, the freeze-dried culture needs to be rehydrated in a wine/water mixture and then added to the wine and usually need a period of 3-14 days to complete MLF.

The selection and characterization of commercial culture strains is fundamental, mainly for the several characteristic LAB strains, such as the fermentation capabilities and growth characteristics (Britz & Tracey, 1990; Henick-Kling, 1993). Several criteria are used in the selection of starter culture, the principal criteria are : tolerance to low pH, high ethanol and SO_2 concentrations, good growth characteristics under winemaking conditions, compatibility with *S. cerevisia*e, ability to survive the production process, the inability to produce biogenic amines, the lack of off-flavor or off-odour production as well as the production of aroma compounds that could potentially contribute to a favourable wine aroma profile (Wibowo *et al*., 1985; Kunkee, 1991; Fugelsang & Zoecklein, 1993; Henick-Kling, 1993; Le Jeune *et al*., 1995; Drici-Cachon *et al*., 1996; Lonvaud-Funel, 2001; Marcobal *et al*., 2004).

The procedure of strain selection is a complex and laborious process that involve various screening procedures and trial vinifications (Lerm & Du Toit, 2010). Usually LAB are isolated from wine undergoing to spontaneous fermentations, with low pH, low temperature, high alcohol and high SO_2 levels. Then individual colonies are genetically identified and. So the strains are then evaluated for their resistance to the physiochemical properties in wine, metabolic properties, nutritional requirements and their ability to survive and retain viability after the drying process. One of the final steps is microvinifications to evaluate the strains under actual winemaking conditions (Bou & Powell, 2006; Capozzi *et al*., 2010; Ruiz *et al*., 2010).

Nevertheless also with the use of commercial starter culture a complete and efficient MLF is not always guaranteed, in particular with hostile wine conditions (i.e. low pH, high ethanol) (Guerzoni *et al*., 1995). The first step to have an efficient and complete MLF is to follow the directions for the reactivation of freeze-dried starter cultures as recommended by the manufacturer, in fact these directions allow to minimise the potential loss in viability due to direct inoculation in the wine (Davis *et al*., 1985; Nault *et al*., 1995; Nielsen *et al*., 1996). Another important parameter to initiate and successfully complete MLF is the timing of inoculation.

1.8.3.2 Timing of inoculation

Several studies on best time of inoculation for a rapid and complete MLF has been carried out, authors have still controversely opinions, generally time of inoculation for MLF can be distinguished into three main type of inoculum:

- simultaneous inoculation for AF and MLF (co inoculation),
- inoculation during AF,
- inoculation after the completion of AF (sequential inoculation).

Alexandre *et al*. (2004) affirmed that simultaneous inoculation might result a stuck AF and the production of off-falvors, mainly by undesirable/antagonistic interactions between yeast and/or bacteria.

Controversely, Jussier *et al*. (2006) shown that simultaneous inoculation doesn't affect negatively fermentation success or its kinetics, compared to traditional post AF inoculation. Moreover, they do not found significative difference in the final wine. They propose that simultaneous inoculation can be used as a tool to overcome high ethanol levels and reduced nitrogen content at the end of AF.

Zapparoli *et al*. (2009) studied acclimatised bacterial cells in co-inoculation and sequential inoculation to induce MLF in high alcohol wines, they demonstrated that simultaneous inoculation result in a complete and fast MLF, compared to the sequential inoculation.

Several authors affirmed that during co-inoculation *O. oeni* strains, a heterofermentative LAB, can produce more acetic acid due to the simultaneous metabolism of citric acid and (Liu, 2002; Costello, 2006). However other authors assert that wines that have undergone simultaneous AF/MLF usually are less buttery, retain more fruitiness and are more complex and better structured, levels of acetic acid are higher but sensorial insignificant (Henick- Kling, 1993; Bartowsky *et al*., 2002a; Jussier *et al*., 2006; Krieger, 2006).

Generally co-inoculation reduce overall fermentation duration, Jussier *et al*. (2006) demonstrated that in simultaneous inoculation levels of acetic acids are slightly higher, however the sensory panel could not differentiate co-inoculation from sequential inoculation, the differences were not statistically relevant and within the range of concentrations normally found in wine.

Other benefits of simultaneous inoculation is a more efficient MLF in 'difficult' wines (e.g. low pH) due to low levels of ethanol and higher nutrient concentrations. Wines are also immediately available for racking, fining and SO2 additions (Davis *et al*., 1985; Jussier *et al*., 2006).

Another type of inoculation is those during AF but some studies reported a strongest antagonism between yeast and bacteria (Rosi *et al*., 2003), in fact bacterial populations showed drastic decreases with this type of inoculation, this could be referred to several parameters, such as the removal of nutrients, accumulation of SO_2, ethanol production, toxic metabolite production and acid production by the yeast that decrease the pH. In addition the same study shown that at the end of AF, yeast presence promote the growth and malolactic activity of LAB, principally to yeast autolysis that release vitamins, amino acids, proteins and polysaccharides (Henick-Kling, 1993).

Sequential inoculation has some advantages, such as the positive interaction between yeast and bacteria and the minor amount of acetic acid produced, due to the smaller sugar concentration after AF (Costello, 2006). However, there are still risks related with sequential inoculation mainly due to a loss in viability may caused by high ethanol concentrations, low pH, high SO_2 concentrations, nutrient depletion and other antimicrobial compounds produced by the yeast (Larsen *et al*., 2003).

The timing of inoculation therefore merits careful consideration and will ultimately affect the style and quality of the wine. It is clear that the timing of inoculation for MLF and the concomitant interaction between the yeast and bacterial cultures play an important role in the success of MLF (Lerm & Du Toit, 2010).

1.8.4 Factor influencing malolactic fermentation

Several factors can influence LAB and MLF. These factors may directly influence the growth or affect the metabolic properties of LAB.

These factors include pH, temperature, ethanol, SO_2 and other products related to yeast metabolism.

In addition, it is not only the individual effects of the different factors that have to be taken into account, but the interactive and synergistic effects are to be considered. These influencing factors do not only

affect the growth and the malolactic activity of LAB, but also influence the effect that the LAB will have on wine aroma.

Several parameters are to be considered, including their interactions and the effect of the wine matrix. The following factors will be discussed in more detail: the yeast-related metabolic products (ethanol, medium chain fatty acids), physiochemical wine parameters (pH, temperature and SO_2), the presence of phenolic compounds, the addition of lysozyme the effect that different vinification procedures have on LAB and the interaction between yeast (*S. cerevisiae*) and bacteria.

1.8.4.1 Interaction between yeast and Lactic Acid Bacteria

The principal parameters that winemakers must control are 1st the selection of yeast and bacteria respectively for AF and MLF, and second the interaction between them. Infact interaction between yeast and bacteria during both AF and/or MLF can have a direct impact on LAB growth and malolactic activity. Several studies on interaction between yeast and bacteria are carried out (Henick-Kling & Park, 1994; Rosi *et al*., 2003; Arnink & Henick- Kling, 2005; Guilloux-Benatier *et al*., 2006; Jussier *et al*., 2006; Osborne & Edwards, 2006; Alexandre *et al*., 2004).

Alexandre *et al*. (2004) suggested that three fundamental parameters influence the degree and complexity of yeast-bacteria interactions.

The first factor to investigate is the combination of yeast and bacteria strain. Nehme *et al*. (2008) studied the interactions between *S. cerevisiae* and *O. oeni* during the winemaking process, demonstrating that the inhibition between these microorganisms is higly dependent on the selected strains of yeast and bacteria and it cauded mainly a decrease in bacterial growth, and also a little decline in their malolactic activity.

Controversely, Arnink & Henick-Kling (2005) studied several pairs of *O. oeni* and *S. cerevisiae* and demonstrated that vintages and grape varieties hve a major impact on LAB and MLF than the yeast/bacteria strain combination.

Costello *et al*. (2003) proposed a simple in vitro method for testing the compatibility between yeast and bacteria, without the effect of wine, such as high SO_2 concentration, low pH, high sugar concentration and the presence of pesticide residues. In this study a chemically defined medium was used to

characterize the metabolic interactions between the yeast and bacteria, also when the sinthetyc media was replaced with grape juice results obtained were similar. The results obtained allow to use this in vitro protocol in the screening yeast/LAB combinations to ensure their compatibility and lack of antagonistic or inhibitory effects. Winemaker must control also the vinification practices applied during the winemaking process, which can influence the interaction between the bacteria and yeast culture.

The second factor that must be considered is the uptake and release of several nutrients by the yeast, which can affect the nutrients available for the LAB. Is generally recognized that at the beginning of AF, *O. oeni* is inhibited by *S. cerevisiae*, this behavior is mainly due to the rapid uptake of grape metabolites, such as amino acids and vitamins (Larsen *et al*., 2003), with a consequent decrease of available nutrients for the bacteria. The amino acids and vitamins used during yeast metabolism are essential for bacterial proliferation and bacterial growth is delayed until yeast cells lyse (Nygaard & Prahl, 1997; Alexandre *et al*., 2004; Arnink & Henick-Kling, 2005). Yeast autolysis cause the release of several nutrients that are essential for LAB proliferation and survival (Alexandre *et al*., 2004), such as amino acids, peptides, proteins, glucans and mannoproteins. The release of these nutrients are yeast strain-dependant (Alexandre *et al*., 2001, 2004). Mannoproteins release is very important, in fact, it can stimulate bacterial growth by adsorbing medium chain fatty acids and consequent detoxifying the wine medium.

Yeast can influence the amount of nitrogen sources available for LAB consumption. This was also confirmed by Guilloux-Benatier *et al*. (2006) that demonstrated that proteolytic activity by yeast could affect the nitrogen composition of wine after AF, with a negative impact on the ability of *O. oeni* to grow and complete MLF.

Yeast can also have an inhibitory effect on LAB, mainly due to the production of extracellular compounds, rather than a competition for nutrients (Comitini *et al*., 2005). Thus, the third factor to investigate is the ability of the yeast to produce several metabolites that can have both a stimulatory or inhibitory/toxic effect on LAB.

Among yeast-derived inhibitory compounds, there are ethanol, SO_2, medium chain fatty acids and proteins. The first three are the compounds most commonly studied about LAB growth inhibition

(Alexandre *et al*., 2004). Osborne & Edwards (2006) discovered a peptide produced by *S. cerevisiae* that inhibited *O. oeni,* more in the presence of SO_2, probably for the disruption of the cell membrane. Furthermore, Comitini *et al*. (2005) affirmed that LAB inhibitory compound produced by yeast could have a proteinaceous nature and heat and protease sensitive. More recently, Nehme *et al*. (2010) suggested that the inhibition of *O. oeni* strains by *S. cerevisiae* is strightly correlated with a decrease in the malic acid consumption by the LAB strain.

Nevertheless yeast and bacteria interactions are quite complex, in fact some yeast strains can be both stimulatory and inhibitory, in other certain LAB strains are capable of inhibiting wine yeast and the composition of the must, as well as vinification practices, influence the interaction.

O. oeni and increasing ethanol concentrations (Davis *et al*., 1988; Henick-Kling, 1993; Alexandre *et al*., 2004; Bauer & Dicks, 2004). It is generally recognized that *O. oeni* strains are able to survive and proliferate in 10% (v/v) ethanol at pH 4.7 (Britz & Tracey, 1990). In adition, Galegría *et al*. (2004) demonstrated that *O. oeni* and *L. plantarum* strains are able to grow at 13% (v/v) ethanol, while Henick-Kling (1993) suggested that ethanol concentrations exceeding 14% (v/v) inhibit the growth of *O. oeni*. The degree to which LAB are able to tolerate ethanol concentrations are strain dependant, as well as being contingent upon the activation steps before inoculation in the wine (Britz & Tracey, 1990).

In a study on the effects of combined cold, acid and ethanol shock on the physical state of the cell membrane and survival of *O. oeni* has been demonstrated that ethanol shocks (10 to 14% v/v) resulted in instantaneous membrane fluidisation followed by rigidification and a decrease in cell viability, whereas the combined ethanol and acid shock of 10% (v/v) and pH 3.5, respectively, resulted in total cell death (Chu-Ky *et al*., 2005).

Zapparoli *et al*. (2009) studied wines with high ethanol concentrations that usually do not support MLF, using an alternative strategy to conduct MLF. The study was performed in “Amarone” wines with an alcohol content of up to 16% (v/v) and both co-inoculation and sequential inoculation were investigated. Complete degradation of L-malic acid was observed with a starter preparation of bacterial cells pre acclimatized in a wine/water mixture for 48 hours prior to inoculation in the wine. Complete MLF

occurred under both inoculation scenarios, the sequential inoculated wine took 112 days to complete MLF, compared to 70 days for co-inoculation.

The ability of LAB to tolerate elevated concentrations of ethanol is correlated to several factors, including temperature and strain selection.

Sulphur dioxide is usually used to inhibite and control microbial population, it can exits in several forms in equilibrium in the wine environment (Fugelsang & Edwards, 1997), such as:

- bound SO_2,
- molecular or free SO_2,
- bisulphite (HSO_3^{-1}) and sulphite (SO_3^{-2}) ions.

The equilibrium of the SO_2 is correlated to the pH of the medium. Several studies suggested that at low pH free SO_2 predominates, consisting mainly of bisulphite and a small fraction of molecular SO_2 and sulphite anions (Usseglio-Tomasset, 1992; Bauer & Dicks, 2004).

Molecular SO_2 has the major inhibitory effect, especially at lower pH values; in other is the only form of SO_2 that can cross bacterial cell walls via diffusion. Then, inside the cells, the molecular SO_2 is transformed into bisulphite that may react with several cell components, like proteins and as result affect the growth of LAB (Carreté *et al*., 2002; Bauer & Dicks, 2004). Low pH and high SO_2 concentrations, respectively 3.2 and 26 mg/l as reported by Nielsen *et al*. (1996), have a sinerginc inhibitory effect on freeze-dried *O. oeni* starter cultures.

Carreté *et al*. (2002) suggested that SO_2 inhibitory action on LAB is mainly due to rupturing of disulphide bridges in proteins as well as reacting with cofactors like NAD+ and FAD, thereby affecting the growth of LAB. The antimicrobial activity of SO_2 can also influence the malolactic activity (Lonvaud-Funel, 1999). Also low amount of molecular SO_2, such as as 0.1-0.15 mg/L, may be inhibitory to the growth of some strains, a concentration of total SO_2 and bound SO_2 of less than 100 mg/L and 50 mg/L respectively are recommended to ensure successful MLF (Powell *et al*., 2006).

In addition several compounds, primarily carbonyl compounds, such as acetaldehyde, α-ketoglutaric acid and pyruvic acid, are able to bind SO_2 resulting in the bound form with weaker antimicrobial activity (Henick-Kling, 1993).

Tolerance to SO_2 is a characteristic species-dependent; in fact, several authors demonstrated that *O. oeni* strains were less tolerant to high total SO_2 concentrations than strains of Pediococcus (Larsen *et al*., 2003).

SO_2 is added during the vinification process, nevertheless yeasts are also able to produce significant amounts of SO_2 (King & Beelman, 1986). This ability is strain-dependent, as well influenced by the media composition (Romano & Suzzi, 1993). Generally, yeast strains produce less than 30 mg/L, although some strains are able to produce more than 100 mg/L. Larsen *et al*. (2003) studied several wine yeast strains for their ability to inhibit *O. oeni* strains. They suggested that yeast strains produced SO_2 concentrations ranging from 15 mg/L to 75 mg/L of total SO_2. The yeast also produced very little or no free SO_2, this suggests that the remaining fraction of bound SO_2 may be inhibitorier.

The type of SO_2 present (free or bound) also influences the effect on LAB, impact both their malolactic activity and their growth.

García-Ruiz *et al*. (2008) suggested the concentrations of free SO2 to inhibit LAB: 10-30 mg/l for pH from 3.2 to 3.6, 30-50 mg/l for pH from 3.5 to 3.7 and 100 mg/l for wines with a pH of over 3.7.

It is essential for the winemaker to not only take the SO_2 added at different stages of the winemaking process into consideration, but also the possible levels of SO_2 produced by the yeast, particularly if MLF is required. It is important to choose a yeast strain that does not produce significant amounts of SO_2, and if sulphur is required, then only make small additions at crushing. If larger amounts (>30 mg/L) of sulphur is required (e.g. damaged grapes), then MLF inoculation should take place after AF has been completed (Henick-Kling & Park, 1994).

Medium chain fatty acids (hexanoic, octanoic, decanoic, dodecanoic acid) are one of the main inhibitory products to bacterial growth and MLF formed by yeast metabolism. Their inhibitory effects are highly correlated to the concentration and type of fatty acid (Capucho & San Ramao, 1994; Carreté *et al*., 2002),

the choice of both the yeast and bacteria strains (Nygaard & Prahl, 1997) and the wine pH. In fact, the inhibitory effect of medium chain fatty acids is higher at lower pH values (Alexandre *et al*., 2004).

Medium chain fatty acids influence the cell growth of LAB and thus their metabolic activity on malic acid, as result increasing the duration of MLF.

The fatty acids inhibit the ATPase activity of LAB and thereby reduce the ability of the bacteria to maintain the intracellular pH and transmembrane proton gradient, which is essential for the transport of metabolites across the cell membrane (Capucho & San Ramao, 1994; Carreté *et al*., 2002).

Selection of the most suitable yeast strain is imperative to the eventual success of MLF in wine. Care should be taken to choose a yeast strain that is compatible with the strain of LAB, resulting in no or very little antagonistic effects between the yeast/bacteria pairing. This includes a yeast strain that produces very low levels of SO_2 and medium chain fatty acids (Lerm & Du Toit, 2010).

The pH of the wine has a fundamental rule on the efficiency of MLF. Is generally accepted that wines with a pH of 3.3 or higher tend to be less problematic for MLF efficiency and LAB growth and survival, compared to wines with a lower pH.

The tolerance to pH is species and strain dependent, for example pH of 3.5 or lower promotes the growth of *O. oeni* species, while wines with pH levels higher than 3.5, generally favour the growth of *Lactobacillus* and *Pediococcus* species. Nevertheless, pH lower than than 3.2 has been shown to be inhibitory also to the survival of *O. oeni* (Henick-Kling, 1993).

As a result MLF in cooler climate regions where the pH can vary between 2.8 and 3.2 is very problematic (Liu, 2002).

The pH is a critical parameter to the commencement of MLF and to the time taken to complete MLF, Rosi *et al*. (2003) investigated the effect of pH on *O. oeni* and found the time it took to complete MLF increased with a decrease in pH, with MLF at pH 3.2 and 3.4 taking 15 to 20 days to complete compared to 10 days at pH 3.6.

pH has a synergic effect on LAB growth and MLF, in fact its relationship between pH and SO_2 affect the survival of LAB in wine.

Temperature is another fundamental parameter for MLF and LAB growth, it is easy to monitor and control, while having a distinct effect on the ability of LAB to survive in wine as well as to initiate and complete MLF. In fact, temperature affects both the growth rate, length of the lag phase and population numbers of LAB (Bauer & Dicks, 2004). The optimum growth temperature for *O. oeni* is about 27-30°C, but due to the presence of alcohol in wine, the optimum growth temperature in wine decreases to between 20 and 23°C (Britz & Tracey, 1990; Henick-Kling, 1993; Bauer & Dicks, 2004; Ribérau-Gayon *et al*., 2006).

The optimum temperature for both *O. oeni* growth and malic acid metabolism in wine is 20°C (Ribérau-Gayon *et al*., 2006). Other authors suggested that both *O. oeni* and *L. plantarum* are able to survive at 18°C, but temperatures below 18°C delay the onset of MLF and increase the duration of MLF, whereas temperatures below 16°C inhibit the growth of *O. oeni* as well as leading to a decrease in cellular activity (Galegría *et al*., 2004; Henick-Kling, 1993; Ribérau-Gayon *et al*., 2006). Controversely Chu- Ky *et al*. (2005) shown that cold shocks (8 and 14°C) affected the plasma membrane, however it did not effect cell survival. To ensure the rapid initiation and completion of MLF, it is essential to control the fermentation temperature. The fermentation temperature during MLF should be kept at 18 to 22°C to ensure optimum malolactic activity of the LAB.

In addition, the nutritional status of the wine is crucial to have an efficient and complete. LAB have been described as 'fastidious' with regards to their nutritional requirements as a result of their limited biosynthetic capabilities (Fugelsang & Edwards, 1997; Théodore *et al*., 2005; Terrade *et al*., 2009). A fundamental compound for LAB survival are amino acids, due to the incomplete amino acid biosynthetic ability in LAB, while the systems that are responsible for amino acid release, via protein hydrolysis, is well developed.

Several essential amino acids, different for each LAB species, have been identified, including glutamic acid, valine, arginine, leucine, isoleucine, as well as cysteine and tyrosine. Terrade & Mira de Orduña (2009) investigated the essential nutrient requirements of LAB strains from the *Oenococcus* and *Lactobacillus* genera. It was found that 10 compounds were essential for the growth of all the tested

strains and that the essential nutrient requirements are strain specific. These 10 compounds include the carbon and phosphate source, manganese and several amino acids and vitamins.

This situation can be aggravated by the addition of a yeast strain with a high nutrient demand and by higher SO2 production from some yeast strains in a nutrient deficient environment (Théodore *et al*., 2005). It has been proposed that co-inoculation of a malolactic starter culture or the addition of a bacterial nutrient could potentially overcome these difficulties. Strain selection of both the yeast and bacterial culture could be an essential tool to ward of future problems concerning the nutritional status of the grape must or wine (Jussier *et al*., 2006).

The major phenolic compounds present in grapes and wine include the non-flavonoids and flavonoids. The non-flavonoids compounds are benzoic- and cinnamic acids and their esters, while the flavonoids include the anthocyanins, flavanols, flavan-diols and flavonols (Cheynier *et al*., 2006).

The amount of phenolic compunds present in wine is a characteristic cultivar-dependent; however, it is also influenced by the vinification procedures (Rozès *et al*., 2003). The type and concentration of phenolic compounds present in the wine influenced their interaction with LAB, however their effect is also strain-dependent (Reguant *et al*., 2000; García-Ruiz *et al*., 2008). Phenolic compounds can affect also the rate of MLF.

Polyphenolic compounds can be transformed by LAB and clear differences in the phenolic content as a result of MLF have been reported (Hernández *et al*., 2007). The main compounds that can be transformed by different LAB include hydroxycinnamic acids and their derivatives, flavonols and their glycosides, flavanol monomers and oligomers, as well as trans-resveratrol and its glucoside (Hernández *et al*., 2006, 2007).

Phenolic compounds can influence bacterial metabolism, however their effect is higly different, in fact Reguant *et al*. (2000) reported that some phenolic acids inhibit the growth of LAB, while others stimulate *O. oeni*. García-Ruiz *et al*. (2008) reported that phenolic compounds concentrations exceeding 500 mg/L inhibit LAB. Reguant *et al*. (2000) found hydroxycinnamic acids to be inhibitory at high concentrations causing MLF to be delayed by ρ-coumaric acid at concentrations of more than 100 mg/L and ferulic acid

at concentrations of more than 500 mg/L. the mechanisms of inhibition of phenolics compound is still not entirely clear, there has been some speculation. Possible mechanisms are based on the interactions of phenolic compounds with cellular enzymes (Campos *et al*., 2003; García- Ruiz *et al*., 2008) and the adsorption of phenols to cell walls (Campos *et al*., 2003).

Certain characteristics of wine LAB, like the production of volatile acids and the malolactic activity, are differently affected by the presence of phenolics, and this is dependent on the bacterial strain (Campos *et al*., 2009).

Some phenolic compounds can also have a stimulatory effect on LAB, such as free anthocyanins and gallic acid are able to stimulate cell growth and malic acid degradation of LAB. In addition, phenol carboxylic acids and catechin seem to stimulate the growth of *O. oeni* by enhancing the metabolism of citric acid and reducing the initial lag phase of LAB (Rozès *et al*., 2003). Reguant *et al*. (2000) shown that the presence of catechin and quercitin stimulated the growth of *O. oeni*. Furthermore Rozès *et al*. (2003) studied the effect of several phenolic compounds in a synthetic medium on the growth of *O. oeni*. They suggested that a concentration of 50 mg/l of phenolic compounds has a stimulatory effect on *O. oeni* growth. This stimulatory effect probably is due to the protecting action of phenolic compounds to bacterial cells from ethanol and to the reduction of the redox potential of the wine, which promotes cell growth (Rozès *et al*., 2003).

The presence of phenolic compounds also has the potential to influence certain quality parameters in wine, in fact some LAB are able to metabolise hydroxycinnamic acids which result in the formation of volatile phenols with the potential to produce off-flavours (Cavin *et al*., 1993). In addition *O. oeni* by can produce higher concentrations of acetate in the presence of phenolic acids. This could be due to enhanced citric acid metabolism at the expense of sugar consumption (Campos *et al*., 2009; Rozès *et al*., 2003).

The effect that phenolic compounds have on LAB metabolic activity and growth, seem to be dependent on the type of compound and its concentration, as well as the strain of LAB.

1.8.5 Infuence of malolactic fermentation on wine flavor and healthiness

Several studies on MLF demonstrated that MLF could influence wine aroma and flavor, principally due to the modification and/or production of aromatic compounds (Davis *et al*., 1985; Lonvaud-Funel, 1999; Maicas *et al*., 1999; Nielsen & Richelieu, 1999; Gámbaro *et al*., 2001; Bartowsky *et al*., 2002b; Bartowsky & Henschke, 2004; D'Incecco *et al*., 2004; Swiegers *et al*., 2005).

Many authors suggested that MLF wines are to be preferred when compared with non-MLF wines, thanks to ther more full and round taste, also MLF impact minimal, but significant, wine aroma (Jeromel *et al*., 2008; Herjavec *et al*., 2001).

Generally wines undergone MLF are easily distinguishable to those that had not undergone MLF, there are several descriptors that allows to distinguish wines MLF, among these descriptors there are the buttery, nutty, vanilla, fruity, vegetative, toasty and wet leather flavor (Bartowsky *et al*., 2002b). The general consensus was that MLF resulted in a creamier palate, less fruit intensity and more butteriness. However, controverselyn, Henick-Kling (1993) found that MLF enhanced the fruity notes, as well as the buttery aroma, and reduced the vegetative, green and grassy aromas, probably for the catabolism of aldehydes (Liu, 2002).

Bartowsky & Henschke (1995) proposed three mechanisms by which LAB are able to modify wine aroma and flavour:

1) Bacterial production of volatile compounds by metabolizing grape constituents (e.g. sugars and nitrogen containing compounds like amino acids),
2) Modification of grape or yeast derived secondary metabolites by the bacteria,
3) Adsorption to the cell wall or metabolism of flavour compounds.

There are various important factors to consider when investigating the effect that MLF and LAB have on wine aroma.

The impact of MLF on wine flavor and aroma depends from several parameters, mainly the bacteria strain Responsible for MLF (Bartowsky & Henschke, 1995; Costello, 2006), also the grape cultivar and winemaking practices (Bartowsky *et al*., 2002b).

One of the most important factors is the matrix effect; in fact, the perception of wine aroma compounds can be affected by the chemical surroundings (Bartowsky *et al*., 2002b). Therefore, an odour-impact compound is defined not by its concentration, but rather its threshold value and the contribution that the specific compound makes to the aroma perception of the wine.

Other fundamental parameters are bacteria-yeast interactions, sterctly correlated to the timing of inoculation, precursor availability and enzymatic activity of the malolactic bacteria, and whether MLF is completed in a barrel and/or tank.

This section will focus on the main aroma compounds, as well as some of the key factors that influence their formation.

The main aroma compounds associated with MLF that contribute to the general aroma profile of the wine include carbonyl compounds, esters, sulphur- and nitrogen containing compounds, volatile phenols and volatile fatty acids. A number of these compounds are considered more important due to their larger contribution to the sensory profile.

Diacetyl (2,3-butanedione) influence the buttery, nutty and butterscotch flavor of the wine, also the yeasty character of sparkling wines, during MLF (Bartowsky & Henschke, 1995; Martineau *et al*., 1995; Bartowsky *et al*., 2002b; Bartowsky & Henschke, 2004). It is a very important aroma compounds produced during MLF (Bartowsky & Henschke, 1995; Lonvaud-Funel, 1999), it origins as an intermediate product of the metabolism of citric acid by the LAB during MLF (Bartowsky *et al*., 2002b; Bartowsky & Henschke, 2004). Usually during carbohydrate metabolism by LAB, pyruvate is reduced to lactate with the aim to maintain the redox balance of the bacterial cell. However when additional pyruvate is produced as a result of the citric acid metabolism, in the absence of sugar, pyruvate is metabolized for the production of acetoin and butanediol. In addition, pyruvic acid can be reductively decarboxylated to diacetyl via α-acetolactate (Lonvaud-Funel, 1999; Bartowsky et al., 2002b; Bartowsky & Henschke, 2004; Costello, 2006). Diacetyl is a compounds chemically very unstable, then it is further reduced to acetoin, that can be reduced to 2,3-butanediol (Bartowsky et al., 2002b; Costello, 2006).

The accumulation of diacetyl and/or acetoin is strigtly correlated to the rate of MLF, usually to an higher MLF rate correspond lower levels of diacetyl and acetoin. Maicas *et al*. (1999) shown decreased levels of diacetyl after MLF, instead increased levels of 2,3-butanediol, mainly due to the enzymatic reduction of diacetyl by LAB. This conversion has an high influence on wine aroma, probably for the higher threshold values of acetoin and 2,3-butanediol, respectively 150 mg/L (Francis & Newton, 2005) and 600 mg/L (Bartowsky & Henschke, 2004), as a result acetoin and 2,3-butanediol contribute to the buttery aroma to a lesser extent (Bartowsky *et al*., 2002b). Nevertheless, diacetyl has a lower odour threshold, in a range between 0.2-2.8 mg/l (Martineau *et al*., 1995).

Francis & Newton (2005) reported diacetyl levels of 0.2 to 1.84 mg/L generally found in young red wines and 1.25 to 3.39 mg/L in aged red wines. Higher amounts of diacetyl, when exceed 5-7 mg/l, are undesirable, due to a strong buttery attribute, while lower concentrations, between 1-4 mg/l, can contribute to the buttery and butterscotch aroma and result in a more complexity of the wine (Bartowsky & Henschke, 1995, 2004; Swiegers *et al*., 2005). The sensory perception of diacetyl is correlated to several factors, such as the style, age and type of wine (Swiegers *et al*., 2005; Costello, 2006), also the presence of other chemical compounds that can react with diacetyl, among these compounds the most important is SO_2 (Martineau *et al*., 1995; Bartowsky *et al*., 2002a; Bartowsky & Henschke, 2004; Swiegers *et al*., 2005).

Nielsen & Richelieu (1999) demonstrated a strong relationship between diacetyl and SO_2 concentrations in wine either during and after MLF.

The SO_2 added at the end of MLF binds to diacetyl with a consequent decrease of its concentration. However, during storage, the reaction is reversed with the resulting increase in diacetyl levels.

Winemakers can act on several parameters to manipulate the diacetyl content, according to the style of wine required. The most important parameter is the bacteria strain choosing, that can produce higher levels of diacetyl, in conjunction with manipulating the temperature, also the SO_2 content and lees contact during the vinification process. Some of these factors have a symbiotic effect. For example, a lower pH cause more SO_2 present in the active antimicrobial form, which will inhibit yeast and bacteria activity and

stabilise the diacetyl content. In addition, air contact during MLF has a result a higher wine redox potential that can promote the production of diacetyl from its precursor. This reaction is catalyzed by pyruvate decarboxylase, responsible for the decarboxylation of pyruvic acid and requires oxygen. Therefore, air exposure during MLF can directly affect the metabolic pathway.

In addition, esters are other important compounds for wine aroma; usually they are associated with fruity aromas in wine. Esters are fermentation-derived compounds, the most important esters associated with wine fruitness are gener acetate esters and ethyl fatty acid esters. Ethyl fatty acid arises from the enzymatic esterification of activated fatty acids formed during lipid biosynthesis. While acetate esters are formed through the condensation of higher alcohols with acetyl-CoA (Matthews *et al*., 2004). MLF and wine LAB can influence the ester content (Matthews *et al*., 2004). However, this behavior is not still very clear, in fact both increases and decreases in ester concentrations being observed in the literature. Furthermore, MLF is generally associated with increased concentrations of ethyl esters, such as ethyl lactate, ethyl acetate, ethyl hexanoate, ethyl octanoate and succinate (De Revel *et al*., 1999; Delaquis *et al*., 2000; Liu, 2002; Swiegers *et al*., 2005; Jeromel *et al*., 2008).

The most important esters produced during MLF are ethyl lactate and diethyl succinate (Maicas *et al*., 1999; Herjavec *et al*., 2001; Ugliano & Moio, 2005). The first arise from the esterification of lactate, produced by LAB during MLF, and ethanol produced through AF. Ethyl lactate has a positive impact on wine aroma, mainly for its fruity, buttery and creamy aromas, but also it can contribute to the mouthfeel of the wine (Ugliano & Moio, 2005).

The aroma threshold of (S)-ethyl lactate in wine is 110 mg/l (Lloret *et al*., 2002). MLF wines had levels of 90-150 mg/l, compared with those that had not been subjected to MLF (5 to 8 mg/l).

Succinic acid is a by-product arised from microbial α-ketoglutarate metabolism, which in turn is slowly and non-enzymatically esterified to form diethyl succinate (Ugliano & Moio, 2005).

This ester also contributes fruity and melon aromas to the wine and has an odour threshold of 1.2 mg/L (Peinado *et al*., 2004).

Gámbaro *et al*. (2001) suggested that ethyl- and acetate ester levels decreased during MLF, but these changes are strain-dependent. Ugliano & Moio (2005) studied the effect of four different malolactic starter cultures of *O. oeni* on the concentration of yeast-derived volatile compounds.

MLF affect also the levels of C4-C8 ethyl fatty acid esters and 3-methylbutyl acetate, this characteristic too is strain-dependent.

Usually several LAB exhibits esterase activities that contribute to the wine aroma, particularly to its fruitness. The changes in aroma are associated with the production and hydrolysis of esters. The majority of *O. oeni* and *Lactobacillus* strains showed esterase activity (Davis *et al*., 1988; Matthews *et al*., 2006). The most activity was found in *O. oeni* strains, followed by *Lactobacillus* and *Pediococcus* strains, respectively. Matthews *et al*. (2007) found that esterase showed greater activity towards short-chained esters (C2 to C8) in comparison to long chained esters (C10 to C18), furthermore they also demonstrated that esterase activity levels remained also under winelike conditions. Esterases arising from LAB could contribute to the wine aroma, because the enzymes are produced and active under wine conditions.

Many volatile aroma compounds are present in the grape bound to a sugar moiety (D'Incecco *et al*., 2004). These compounds are non-volatile in this glycosidic form and represent potential aroma compounds that could affect the overall perception of wine aroma (Bartowsky *et al*., 2004; D'Incecco *et al*., 2004; Swiegers *et al*., 2005). These potential aromatic volatiles compounds are monoterpenes, C13-norisoprenoids, benzene derivatives and aliphatic compounds (D'Incecco *et al*., 2004; Matthews *et al*., 2004).

LAB, such as *O. oeni* strains, can release these volatile compounds thanks to their esterase activity,so they become odour-active (Grimaldi *et al*., 2000; Boido *et al*., 2002; Liu, 2002; Barbagallo *et al*., 2004; D'Incecco *et al*., 2004; Matthews *et al*., 2004). Several authors demonstrated that *Lactobacillus* and *Pediococcus* species also possess glycosidase activity (Grimaldi *et al*., 2005a; Spano *et al*., 2005).

In other some *Oenococcus oeni* and *Lactobacillus* strains, studied by Hernandez-Orte *et al*. (2009), can release norisoprenoids, terpenes, phenols and vanillins from glycosidic precursors in a model wine solution.

Glycosidase activity is influenced by pH, temperature, sugars and ethanol (Grimaldi *et al*., 2000, 2005b). The wine ernvironment with its acidic conditions may denature or inhibit esterase. However, *O. oeni* β-glucosidase activity is still high, about 80%, at pH 3.5 (Grimaldi *et al*., 2000; Barbagallo *et al*., 2004; Mtshali *et al*., 2010).

In addition, other stress factors, like ethanol and SO_2, can impact enzymatic. Sulphur containing compounds associated with MLF as a result of LAB metabolism have been studied by Pripis-Nicolau *et al*. (2004) that demonstrate the ability of wine LAB to metabolise methionine to produce volatile sulphur compounds during MLF.

The metabolism of methionine by LAB leads to several compounds, such as methanethiol, dimethyl disulphide, 3-(methylsulphanyl) propan-1-ol (also known as methionol) and 3-(methylsulphanyl)propionic acid. These compounds plays an important role in the complexity of wine aroma because of their characteristic and powerful odours. Higher amounts of sulphur compounds impart negative aromas to the wine, but lower concentrations, below or close to the odour threshold, will add complexity to the wine. The production of these volatile sulphur compounds are specie and strain-dependant, usually *O. oeni* having a higher capacity for producing these compounds, compared to *Lactobacillus* spp. (Pripis-Nicolau *et al*., 2004).

Several parameters influence the production of these volatile sulphur compounds, very important are both the presence of methionine as precursor and the growth phase of the bacteria. For example, Vallet et al. (2008) suggested that the production of methionol occurred during the exponential growth phase of the LAB, while 3-(methylthio) propionic acid is produced during both the exponential and stationary growth phase. Inother methionol and 3-(methylthio) propionic acid production occur only in the presence of methional, an important precursor in their production.

Other compounds present in wine can have a significant effect on the perceived aroma of 3-(methylsulphanyl) propionic acid. In a synthetic media, the perception threshold of 3-(methylsulphanyl) propionic acid is 50 μg/L and impart chocolate and roasted aromas. Controversely, the perception threshold in wine is five times higher, about 244 μg/L, generally is correlated with ‘earthy’ and ‘red fruit’

aromas. Other sulphur compounds can affect wine aroma. Principally for several reactions between the sulphur-containing cysteine and α-dicarbonyl compounds like diacetyl. These are non-enzymatic reactions that usually take place after MLF and produce some compounds, such as tetramethylpyrazine and trimethyloxazole, associated with 'toasted', 'sulphur' and 'cabbage' aromas (Pripis-Nicolau *et al*., 2000; Swiegers *et al*., 2005; Landaud *et al*., 2008).

Wine LAB can produce heterocyclic volatile nitrogen bases responsible for the 'mousy' off-flavours of spoilage wines. The most important of these compounds are 2-acetyl-1-pyrroline (ACPY), 2-ethyltetrahydropyridine (ETPY) and 2-acetyltetrahydropyridine (ACTPY) (Lonvaud-Funel, 1999; Costello *et al*., 2001). These compounds are associated with heterofermentative LAB, such as *O. oeni*, also some *Lactobacillus* species and *Leuc. mesenteroides*, due to the metabolism of certain amino acids, mainly ornithine and lysine (Costello *et al*., 2001; Swiegers *et al*., 2005).

ACTPY and ACPY have a threshold in water of 1.6 μg/L and 0.1 μg/L, respectively. Usually spoiled wines with the 'mousy' off-flavours contain about 2.7-18.7 μg/l of ATPY, up to 7.8 μg/l ACPY and 4.8 to 106 μg/l of ACTPY. These compounds are either present in combination or individually. The availability of the precursor's lysine and ornithine has a significant impact on the ability of LAB to produce these compounds (Costello & Henschke, 2002).

LAB differ in their preference for the formation of the different nitrogen heterocyclic compounds. *O. oeni* promotes the production of ETPY, the least flavour active, the heterofermentative lactobacilli promote those of ACTPY and the homofermentative pediococci the formation of ACPY, the most flavour active. In general, the heterofermentative LAB show the highest ability to produce nitrogen-heterocycles and mousy off-flavours (Swiegers *et al*., 2005).

Wine contain also several phenolic compounds, such as phenolic acids, (ρ-coumaric acid and ferulic acid), that can be used as substrates in the formation of volatile phenol aroma compounds (Cavin *et al*., 1993; Lonvaud-Funel, 1999). LAB can use phenolic acids thanks to an active transport mechanism that allows to transfer the phenolic acids into the cell, then hydroxycinnamic acid decarboxylases convertedhe phenolic acids to their vinyl derivatives (4-vinylguaiacol and 4-vinylphenol). The vinyl derivatives can be

enzymatically reduced to the corresponding ethyl derivatives (4-ethylguaiacol and 4-ethylphenol) (Cavin *et al*., 1993; Swiegers *et al*., 2005). The vinyl derivatives can impart negative aromas, such as pharmaceutical odours, to the wine and their products, 4-ethylphenol and 4-ethylguaiacol, caused several off-odours, such as 'animal', 'medicinal' aromas, horse sweat, horse stable, barnyard and elastoplast aromas (Lonvaud-Funel, 1999). These aroma descriptors are generally associated with the presence of the spoilage yeast Brettanomyces (Chatonnet *et al*., 1992). In addition, LAB are able to produce volatile phenols (Nelson, 2008). However, it is still unclear if strains of *O. oeni* are able to produce levels of 4-vinylguaiacol and 4-vinylphenol that could be of sensorial significance (Swiegers *et al*., 2005). In addition, usually spontaneous MLF caused higher levels of volatile phenols.

Acetic acid is the most important volatile acid produced during fermentation, both quantitatively and sensorially. It has a sour, pungent and vinegar aroma in wine (Francis & Newton, 2005) with high concentrations, exceeding 0.7 g/L (Swiegers *et al*., 2005), while lower concentrations (0.2-0.6 g/l) can contribute to the complexity of wine aroma. The flavour threshold for acetic acid is influenced by both the type and style of wine (Bartowsky & Henschke, 1995; Lonvaud-Funel, 1999).

Generally, MLF is associated with an increase in acetic acid of 0.1 to 0.2 g/L (Bartowsky & Henschke, 1995). These increasing of acetic acid levels is correlated to two mechanisms. If MLF start before the completion of AF, the LAB are able to ferment hexoses that have not been completely fermented by the yeast, whit the conseguent production of acetic acid and D-lactic acid, via the 6-PG/ PK pathway and a slightly increasing of volatile acidity (Lonvaud-Funel, 1999; Swiegers *et al*., 2005).

In other during the formation of diacetyl, LAB can produce acetic acid during the citric acid metabolic pathway catalyzed by the citrate lyase enzyme (Bartowsky et al., 2002b; Bartowsky & Henschke, 2004). The rate of acetic acid accumulation is correlated to the rate of MLF, in fact higher concentrations of acetic acid being formed with a higher MLF rate (Lonvaud-Funel, 1999).

In addition, other studies demonstrated that the co-inoculation do not lead to higher acetic acid concentrations.

Volatile fatty acids arised from the hydrolysis of tri-, di- and monoacylglycerols (lipids) (Liu, 2002). Usually wine consists of a blend of straight chain fatty acids and branched chain fatty acids. Among the straight chain fatty acids the major part are short chain (C2-C4), but also medium chain (C6-C10) or long chain (C12-C18) fatty acids. As the chain length of fatty acids increase, the volatility decreases and the odour changes from sour to rancid and cheesy (Francis & Newton, 2005).

Maicas et al. (1999) found no significant increase in isovaleric, isobutyric and hexanoic acids after MLF, although capric acid and caprylic acid levels were higher. This behavior is positive for wine aroma because isobyturic and isovaleric acids are generally associated with rancid, butter, cheese and sweaty aromas (Francis & Newton, 2005).

Higher alcohols arised from the decarboxylation and reduction of α-keto acids that are produced as intermediates during amino acid biosynthesis and catabolism. Amino acid biosynthesis is responsible for most of the higher alcohols formed during fermentation.

Jeromel et al. (2008) found that MLF had an insignificant effect on the higher alcohol concentration of wine, only levels of isobutanol and 2-phenylethanol incresead. Furthermore, several authors suggested that higher alcohols production is strain-dependent (Herjavec *et al*., 2001; Maicas *et al*., 1999).

Pozo-Bayón *et al.* (2005) saw increased levels of higher alcohols after MLF, but none of the increases were significant. The concentration of higher alcohols can have both a positive or negative impact on the wine aroma, both for the aroma intensity of the respective alcohols or the style of wine.

Based on the available literature, it is clear that MLF has an effect on the sensory character of wine. These effects are diverse and sometimes contradicting and may be due to the following factors: the influence of the different bacteria strains, the presence and availability of precursors, LAB associated enzymatic activity, the wine type, the intensity of the inherent wine flavor and cultivar character, the vinification conditions under which the wine was produced and the training and skills of the sensory panel that evaluate the wine.

Malolactic fermentation in generally correlated to an increase in the buttery attribute reduced vegetative character, modification in the fruitiness and improved mouthfeel and flavour persistence. In other wine aroma can be also influenced by the type of LAB and wood interactions.

Future studied on the investigation, identification and quantification of relevant aroma precursors will be carried out; in fact, aroma precursors are very important for wine quality, and also the vineyard practices can influence their occurrence and concentration and also their derivative aroma active compounds (Swiegers *et al*., 2005). Furthermore also are significant the mechanisms of how LAB contribute to this process. The choice of bacterial strain seems to be one of the most influential factors on the production of odour-impact compounds associated with MLF.

Another important compound associated to wine healthiness and quality are biogenic amines that are a group of organic nitrogen-containing compounds. The most important wine biogenic amines are putrescine, histamine, tyramine and cadaverine, followed by phenylethylamine, spermidine, spermine, agmatine and tryptamine (Ten Brink *et al*., 1990; Lonvaud-Funel, 2001).

Several LAB are able to produce biogenic amines by enzymatic decarboxylation of amino acids (Ten Brink *et al*., 1990; Lonvaud-Funel, 2001). These compounds are of importance in wine due to their potential toxicological effects in sensitive humans. These include symptoms like headaches, hypo- or hypertension, cardiac palpitations and in extreme cases even anaphylactic shock (Shalaby, 1996). Phenylethylamine and tyramine can caused high blood pressure and migraines, while putrescine and cadaverine can enhance the toxicity of histamine, tyramine and phenylethylamine, also can have a detrimental effect on wine quality due to their off-flavours of putrefaction and rotten meat, respectively (Shalaby, 1996). In addition, high concentrations of alcohol, SO_2 and other amines could potentially amplify the toxic effect of certain biogenic amines (Fernandes & Ferreira, 2000). Therefore, the production of biogenic amines is another important criteria for the selection of LAB starter cultures.

Several parameters can affect the biogenic amine content. First the amino acid composition, but also the microflora present in the wine and the ability of these microflora to decarboxylate amino acids.

The essential role of LAB and MLF in the formation of biogenic amines has been confirmed by various authors (Lonvaud-Funel & Joyeux, 1994; Moreno-Arribas *et al*., 2000; Marcobal *et al*., 2006; Landete *et al*., 2007a).

It is generally recognized that spoilage LAB are responsible for the formation of biogenic amines, mainly species of *Pediococcus* and *Lactobacillus* (Moreno-Arribas & Polo, 2008).

Several authors that suggested other LAB species, such as *L. brevis*, *L. hilgardii* or *L. plantarum* are able to produce various biogenic amines, respectively tyramine, phenylethylamine and putrescine (Landete *et al*., 2007; Arena & Manca de Nadra, 2001; Manfroi *et al*., 2009).

Moreno- Arribas *et al*. (2000) identified *O. oeni* as the main LAB responsible for histamine formation and lactobacilli for tyramine formation.

Also Lucas *et al*. (2008) identified several O. oeni histamine producer, but they also demonstrated that LAB might lose this ability due to instability of the phenotype. Generally histamine producing LAB carry an hdcA gene coding for a histidine decarboxylase (HDC) that converts histidine to histamine. This hdcA gene was detected on a large and unstable plasmid, which could result in a loss of histamine producing ability.

The concentration of histamine and tyramine produced by *O. oeni* is strain-dependant, in other other factors can affect its concentrations, such as the effect of the yeast strain on the wine composition, the length of bacteria-yeast contact time after MLF completion and the screening method used for biogenic amine determination.

Several oenological parameters can influence the decarboxylase enzyme activity and the biogenic amine producing ability of LAB (Landete *et al*. 2008). Usually HDC activity is promoted at pH 3.5 and has an optimum pH of 4.8 (Lonvaud-Funel & Joyeux, 1994), while tyrosine decarboxylase (TDC) is active in a pH range between 3 to 7, but exhibits optimum activity at pH 5 (Moreno-Arribas & Lonvaud-Funel, 1999). In wines with higher pH values, decarboxylase positive bacteria are more likely to survive. So an higher pH will concomitantly lead to higher biogenic amine concentrations (Wibowo *et al*., 1985; Lonvaud-Funel & Joyeux, 1994; Gardini *et al*., 2005; Landete *et al*., 2005b; Martin-Álvarez *et al*., 2006).

In addition at a higher pH, the SO2 fraction will be less effective and these can result in higher concentration of biogenic amines (Gerbaux & Monamy, 2000), principally for an high viability of LAB population (Marcobal *et al*., 2006). Another important factor is the ethanol content of the wine. Usually, to higher ethanol concentrations correspond a decrease in the formation of biogenic amines (Gardini *et al*., 2005).

In a study investigating the potential of commercial cultures to produce tyramine, histamine and putrescine, it was found that none of the commercial starter cultures produced biogenic amines (Moreno-Arribas *et al*., 2003). In a study comparing spontaneous and inoculated MLF in Spanish red wine, the incidence of biogenic amines was reduced in the inoculated MLF (Martín-Álvarez *et al*., 2006).

Ethylcarbamate (EC) is a suspected carcinogen (Fugelsang & Edwards, 1997). LAB, also commercial strains of *O. oeni*, are able to degrade arginine via the arginine pathway. This pathway involved three enzymes. First arginine deiminase is responsible for the production of L-citrulline from L-arginine, then ornithine transcarbamylase converts L-citrulline to L-ornithine and carbamyl phosphate and finally carbamate kinase generated ATP from carbamyl phosphate. The catabolism of arginine contributes to LAB growth due to the generation of ATP, but two of the intermediates formed, citrulline and carbamyl phosphate, are able to react with ethanol to form EC (Liu *et al*., 1994, 1995; Arena & Manca de Nadra, 2002; Araque *et al*., 2009).

Several authors demonstrated that strains of *O. oeni* and *Lactobacillus buchneri* are able to excrete citrulline and carbamyl phosphate (Liu *et al*., 1994; Mira de Orduña *et al*., 2000, 2001).

Inhibition of the LAB population immediately after the completion of MLF could avoid the formation of citrulline from arginine and concomitant EC formation (Terrade & Mira de Orduña, 2006).

1.8.6 Monitoring of malolactic fermentation

The production and consumption of lactic acid and malic acid is generally used to monitor the progression of MLF.

Several analythic methods are used for monitoring the malic acid concentration, such as chromatography, reflectance and enzymatic assays, also analytical techniques like Fourier-transform infrared (FT-IR) spectroscopy and capillary electrophoresis (CE) or the use of high-performance liquid chromatography (HPLC). These techniques differ in their accuracy, time needed for analysis as well as the cost involved.

Among these techinques, the chromatography, like paper chromatography (PC) and thin layer chromatography (TLC), is the method most often implemented in wineries due to the low cost involved. However, these methods are not very accurate if compared to the analytical techniques.

The more accurate methods usually involve the acquirement of expensive equipment like a CE or HPLC. Furthermore, in order to accurately monitor the progression of MLF, fast and accurate results are required.

Enzymatic kit can be a compromess between sensibility and rapidity, although the cost involved is still relatively high, it is less expensive than acquiring instruments like an HPLC or a FT-IR spectrometer.

Also the monitoring of the microbial population is another important parameters to monitoring MLF, There are two established microbiological techniques that are generally used, including microbial plate counts and microscopy, the 1st method allows to isolate the viable LAB in the wine by the growth of the bacterial cells on a nutrient medium.

Spoilage LAB, such as *Pediococcus* and *Lactobacillus* can grow in 2-4 days and can be quickly obtained. Contrewise, the slow growth of *O. oeni*, requires up to 7 days.

Microscopy is an alternative technique for monitoring the microbial population with the direct observation of a wine sample using a microscope and the fast evaluation of its microflora. With the consequent identification of the bacterial population due to the distinct morphologies which allow for discrimination of wine LAB (Kollar & Brown, 2006).

O. oeni are the smallest cells with their round or slightly elongated usually form distinct chains of individually linked cells.

While *Pediococcus* cells are almost completely round and do not form chains. They appear singly, in pairs, tetrads or small bunches and appear bright white under the microscope. *Lactobacillus* is rod shaped

and appears as single cells or pairs in wine and appears bright white under the microscope (Dicks & Endo, 2009). This method is also not quantitative without specific tools (Kollar & Brown, 2006).

Other important techiques that allows the identification of wine LAB are molecular-bbased methods (Lonvaud-Funel, 1995). These techniques allows to identify and differentiate LAB, in other alsco several strains within the same species can be distinguished (Bartowsky *et al*., 2003).

Among these techniques, there are several techniques used to differenziate LAB strains, such as DNA-DNA hybridisation, 16S and 23S rRNA sequence analysis, DNA-fingerprinting and pulsefield gel electrophoresis (PFGE) as well as PCR-based DNA fingerprinting known as randomly amplified polymorphic DNA (RAPD) analysis (Bartowsky *et al*., 2003; Zapparoli *et al*., 1998; Bartowsky & Henschke, 1999). Future techniques that require further development and need to be improved, include DNA sequencing, amplified fragment length polymorphism (AFLP), ribotyping as well as species-specific and multiplex-PCR.

However, traditional PCR-based methods did not give quantitative results, while real-time PCR allows to quantify diagnostic amplicons on-line with a fluorescence detection system.

Recently real-time PCR assays have been successfully applied for the detection and quantification of microorganisms in food (Kimura *et al*. 2001), also several authors developed rapid protocols for a direct quantification of viable population of *O. oeni* strains in wine by real-time PCR (Pinzani *et al*., 2004; Solieri & Giudici, 2010).

1.8.7 Molecular methods for the identitication and characterization Lactic Acid Bacteria

Traditional methods used to distinguish several LAB species, usually based on physiological and biochemical criteria, are quite difficult to interpretate because LAB are very similar for their nutritional and growth requirements in environmental conditions. However during the recent years, molecular biology became more popular and allow to circumvent these difficulties.

Several molecular techniques have been used to identify and characterize LAB isolated from wine, such as techniques based on restriction fragment length polymorphisms (RFLPs) (Zapparoli *et al*., 2000),

pulsed-field gel electrophoresis (PFGE) (Gindreau et al., 1997), DNAeDNA hybridization (Dicks et al., 1995; Lonvaud-Funel *et al*., 1991; Sato *et al*., 2001), specific DNA hybridization probes (Lonvaud-Funel *et al*., 1991; Sohier *et al*., 1999), polymerase chain reaction (PCR) (Groisillier & Lonvaud-Funel, 1999), randomly amplified polymorphic DNA (RAPD) (Zavaleta *et al*., 1997), amplified fragment length polymorphisms (AFLPs) (Cappello *et al*., 2008), and the study of genes encoding 16S ribosomal RNA (rRNA) (Guerrini *et al*., 2003; Sato *et al*., 2001).

Amplified rDNA restriction analysis (ARDRA) has been used as a quick tool for identify the main LAB involved in winemaking (Rodas *et al*., 2003). Nevertheless, this method has several limitation, for example it does not allows to distigush strains of *L. plantarum* and *L. pentosus* to their high level of similarity in 16S rDNA sequence (Collins *et al*., 1991; Quere *et al*., 1997). Amplified rDNA fragment analysis via PCR followed by denaturing gradient gel electrophoresis (DGGE) has also been used to compare diversity and monitor changes in populations of lactic acid bacteria during the winemaking process (López *et al*., 2003).

RAPD and ribotyping are useful for identifying and classifying these bacteria, while ARDRA is useful only for identification purposes and PFGE-RFLP is useful for distinguishing between different strains of the same species (Rodas *et al*., 2003, 2005).

O. oeni is the most important LAB associated with MLF in wines, real-time quantitative PCR methods are been developed with the aim of a rapid detection and quantification of these bacteria in wine samples obtained during fermentation. Real time PCR is a uselful tool for a rapid quantification of viable cells of LAB that allows rapid corrective action to be taken in order to control bacterial growth (Pinzani *et al*., 2004). A molecular typing method that combines RAPD and multiplex PCR has been described for characterizing different strains of *O. oeni* during winemaking and evaluating the impact of malolactic starter cultures (Reguant & Bordons, 2003).

Several studies on the population structures of *O. Oeni* has led to contradictory results. In fact, molecular methods based on DNA-DNA hybridization, sequencing of the genes encoding 16S and 23S rRNA and the intergenic region between 16S and 23S rDNA suggested a high homogeneity between *O. oeni* strains.

While analysis of metabolic and physiological characteristics, such as fatty acid profile and sugar fermentation patterns, led to opposite results. Tenreiro *et al*, 1994 on the basis of these resuts proposed to divid *O. oeni* species in two separate species or subspecies. These result was confirmed recently by several authors that using the multilocus sequence typing (MLST) showed that *O. oeni* strains can be classified into well-differentiated groups and that recombination events play an important role in the genetic heterogeneity of this species (Bilhère *et al*., 2009; de las Rivas *et al*., 2004).

MLST is a molecular technique based on the sequence polymorphism of a set of housekeeping genes, usually 7 to10, which has the advantages of being robust (based on genetic data) and electronically portable, to generate data that can be used not only for strain differentiation but also for evolutionary and population studies (Maiden *et al*., 1998). Although it was originally developed for pathogenic bacteria, MLST became the gold standard for studying lineages and population structures of all kinds of microorganisms (Maiden *et al*., 2006).

Analysis of *O. oeni* strains by multilocus sequence typing (MLST) has provided a new picture of the diversity and population structure of the species, De Las Rivas et al, 2004 demonstrated that the *O. oeni* population is panmictic, i.e. no line of clonal descent is easily discernible, in other they proposed that frequent recombination events and horizontal gene transfers (HGTs) occurred between several strains. This hypothesis was also supported by several studies on LAB genomes that suggested substantial number of gene losses and acquisitions during the evolution of LAB (Makarova *et al*., 2006-2007).

In addition, HGTs may be particularly favored in *O. oeni* that lacks the *mutS* and *mutL* genes. The lack of the these genes causes an high variability, largely due to the loss of the DNA mismatch, consequently these lack contributed to this species' greater adaptation to the conditions found in winemaking (Marcobal *et al*., 2008).

However, the hypothesis of a panmictic population contrasted with both the genetic homogeneity and the existence of subspecies suggested by other typing methods, such as MLST results reported both by Bilhère *et al*., 2009 and Bridier *et al*., 2010, that suggested the esistence of two distint subpopulation among *O. oeni* strains. The detection of these two subpopulations was not the result of an evolutionary

distortion due to one or a few genes given that it was supported by the topologies of six independent trees constructed from *gyrB*, *g6pd*, *ddl*, *dnaE*, *purK*, and *rpoB* sequences.

Recently Claisse et al., 2012 developed a new PCR-based techinique that allows to distinguish several O. oeni strains, these techinique is based on analysis of tandem repeat sequences.

Variable number of tandem repeat (VNTR) analysis is a method that can be used to discriminate between different strains of a bacterial species and can therefore infer genetic relationships between them.

This approach is based on the presence of a variable number of tandem repeats (TR) at a specific locus in the genome of a microorganism, due to DNA polymerase enzyme slippage during replication.

The VNTR method is highly discriminating, easy to interpret and facilitates the rapid and reliable typing of *O. oeni* strains, using only five tandem repeat regions. In other Claisse *et al*., 2012 also demonstrated that the VNTR technique is the most discriminanting method used for *O. oeni* typing, better both than PFGE and MLST techniques.

2. AIM OF THE STUDY

The principal aim of this study is to enhance quality of the finished product via an improve management of microbial resources:

1) Design of microbial starter for the production of typical Apulian wines, coherent with the status of 'Geographical Indication' (GI), i.e. research and development of starter culture, tailored for specific Apulian production IGT and DOC, focusing on typical production of "Capitanata", able to innovate this typical production in consumer-oriented manner.

"GI is a sign used on goods that have a specific geographical origin and possess qualities, reputation, or charactteristics that are essentially atributable to that place of origin" (World Intellectual Property Organization (WIPO), 2011).

The study of microbial biodiversity related to food GIs has been receiving growing scientific attention and world interest (e..g. Benito *et al*., 2007; De Angelis *et al*.,2008; Ercolini *et al*., 2008; Gala *et al*., 2008; Gullo and Giudici, 2008; Capozzi *et al*., 2010; Csoma *et al.*,2010; Valmorri *et al.*, 2010; Cocolin *et al*., 2011; Cordero-Bueso *et al.*, 2011;Tristezza *et al*., 2011).

2) Determine the best time for inoculation of starter culture for alcoholic and malolactic fermentation, with the aim to making processes more efficient and reduce production time.

3) Evaluated the use of multi-strain starter culture to improve specific quality of Apulian wines.

Our specific objective was to isolate microbial strains (yeast and bacteria) representing the "virtuous" microbial biodiversity of several typical wine productions. The autochthonous strains were genotypically/phenotypically characterized and deposited in the laboratory microbial collection.

3. MATERIALS AND METHODS

3.1 Wine fermentation and strains isolation

1.00 Kg of grapes berries of white and red grapes were taken during the 2011-12 vintage in the cellars of several wine-producing localities (see **Fig.4**).

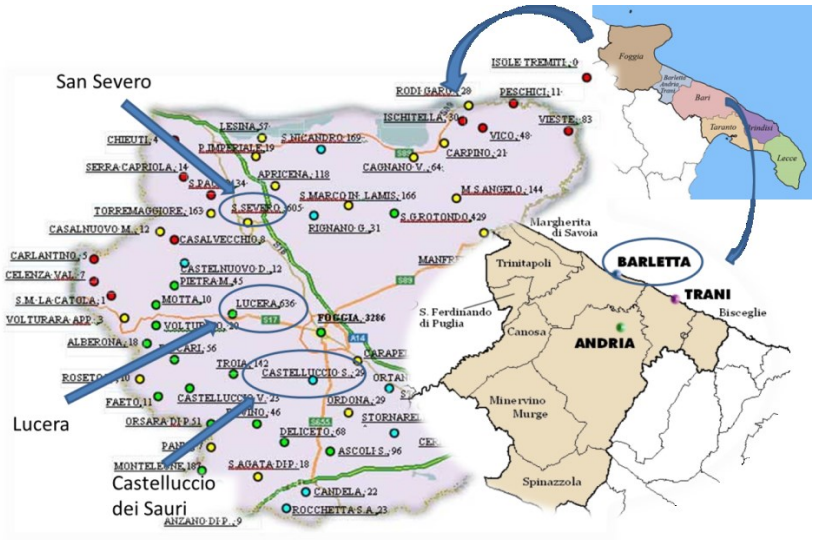


Figure 4. Localization of sampling grape localities

Several grape cutivars and two different wines were analysed: Nero di Troia and Rosso Barletta DOC. Rosso Barletta DOC wines are constituted by various grape cultivars, i.e. Nero di Troia, Sangiovese, Montepulciano and Malbec. For the list of grape cultivars used in this study see Table 3.

Grape cultivar	Date of sampling	Locality
Uva di Troia	29 09 2011, 26 09 2011	San Severo, Italy
Uva di Troia	06 10 2011	Barletta, Italy
Uva di Troia	08 09 2011	Castelluccio dei Sauri, Italy
Uva di Troia	26 08 2011	Lucera, Italy
Sangiovese	25 08 2011	Barletta, Italy
Merlot	25 08 2011, 23 08 2011	Barletta, Italy
Bombino bianco	09 09 2011, 12 09 2011, 22 09 2011	San Severo, Italy
Bombino bianco	12 09 2011	Lucera, Italy
Montepulciano	12 09 2011, 13 09 2011	Lucera, Italy
Montepulciano	29 09 2011	San Severo, Italy
Syrah	12 09 2011	Lucera, Italy
Falanghina	14 09 2011	Lucera, Italy
San Severo bianco	26 09 2011	San Severo, Italy

Table 3. List of grape cultivars used in this study, with date of sampling and locality

3.2 Yeast isolation from grape berries surface

Yeasts were isolated from grapes surface according to Prakitchaiwattana *et al*. (2004). Grapes were aseptically harvested from vineyards in the "Capitanata" area, collepted from two different winemakers. Samples consisted of healthy, undamaged grape bunches taken from different locations within the vineyard. Duplicate samples were collected. In some cases, bunches of grapes that were visibly damaged were also collected.

Individual grape berries were randomly and aseptically removed from the bunches, and combined to give 50 g samples. Then these samples were rinsed in 450 ml of 0.1% peptone water with 0.01% Tween 80 by orbital shaking in a flask at 150 rpm for 30 min. The rinse was poured from the grapes and were serially diluted in 0.1% peptone water. Aliquots of 0.1 ml from serially diluted samples were plated on Wallerstein Laboratory (WL) nutrient agar (Oxoid) e Lysine Medium (Oxoid). Plates were incubated at 25 °C for 4 days, media were supplemented with 10 mg l-1chloramphenicol to inhibit the growth of bacteria. 35 colonies representatives of the different yeast types were isolated and purified by streaking on WL nutrient agar, then inoculated on YPD broth and stored at -80°C in YPD broth supplemented with glycerol (20% vol/vol).

3.3 Yeast isolation from grape musts and wines

For the yeast isolation 1.00 Kg of each grape berries were collected aseptically, pressed for 20 minutes using a Bag Mixer® (Interscience), then spontaneous fermentation of grape juices obtained were carried out in laboratory at 28 °C temperature without further inoculation of starter culture and monitored for 1 month. Grape berries were directly collected in the vineyard with the aim to avoid yeast contamination due to wine cellar, due to the use of several commercial culture strains from the winemakers.

Yeast sampling were accomplished at two stages, at the beginning and at the end of fermentation, which were determined on the basis of alcohol content, about 1% at the beginning and 9% at the end of fermentation. Aliquots of 0.1mL from serially diluted samples in physiological solution were plated on

different media, Wallerstein Laboratory (WL) nutrient agar (Oxoid) and Lysine medium (Oxoid). Both media were supplemented with 10 mg/L chloramphenicol to inhibit bacterial growth, in according with Lopandic *et al*. (2008). Selective Lysine medium was used for isolation of non-*Saccharomyces* yeasts, as *Saccharomyces* species are not capable of utilizing lysine and show no growth. Instead, WL supports the growth of the *Saccharomyces* and non-*Saccharomyces* species, *Saccharomyces* species can be distinguished from the other yeasts by the formation of pale green-to-cream colonies, while the major part of non-*Saccharomyces* species colonies growth on WL nutrient agar became green, more or less intense. After incubation (25°C, 3–5 days) 20 colonies from every fermentation stage were selected, streacked two or three times and the inoculated and stored at -80°C in YPD medium supplemented with glycerol (20%v/v). Selection of non-*Saccharomyces* yeasts was based on colony morphology, whereas the *Saccharomyces* strains were isolated randomly.

For composition media used in this study see tables 4, 5 and 6, respectively for WL nutrient agar, YPD and Lysine medium composition.

Ingredients	g/litre
Yeast extract	4.0
Tryptone	5.0
Glucose	50.0
Potassium dihydrogen phosphate	0.55
Potassium chloride	0.425
Calcium chloride	0.125
Magnesium sulphate	0.125
Ferric chloride	0.0025
Manganese sulphate	0.0025
Bromocresol green	0.022
Agar	15.0

Table 4. Composition of WL nutrient agar

Ingredients	g/litre
Glucose	20
Yeast extract	10
Bacteriological peptone	20
Agar	15

Table 5. Composition of YPD medium

Ingredients	g/litre	Ingredients	g/litre
Glucose	44.5	Ferrous sulphate	0.0002225
Potassium dihydrogen phosphate	1.78	Lysine	1.0
Magnesium sulphate	0.89	Inositol	0.02
Calcium chloride fused	0.178	Calcium pantothenate	0.002
Sodium chloride	0.089	Aneurine	0.0004
Adenine	0.00178	Pyridoxine	0.0004
DL-methionine	0.000891	p-aminobenzoic acid	0.0002
L-histidine	0.000891	Nicotinic acid	0.0004
DL-tryptophane	0.000891	Riboflavin	0.0002
Boric acid	0.0000089	Biotin	0.000002
Zinc sulphate	0.0000356	Folic acid	0.000001
Ammonium molybdate	0.0000178	Agar	17.8
Manganese sulphate	0.0000356		

Table 6. Composition of Lysine medium

3.4 LAB isolation from spontaneous MLF wines

LAB species were isolated from several wines undergoing spontaneous MLF during vintage 2011-2012, collected from different winemakers (see Tab. 7).

Wine	Date of sampling	Locality
Bombino base spumante	14 12 2011	Lucera, Italy
Bombino bianco	21 11 2011	San Severo, Italy
Bombino bianco	02 12 2011	San Severo, Italy
Bombino bianco	02 12 2011	San Severo, Italy
Cabernet	21 11 2011	San Severo, Italy
Cacc'e e mmitte	14 12 2011	Lucera, Italy
Cacc'e e mmitte	27 01 2012	Lucera, Italy
Merlot	12 03 2012	San Severo, Italy
Montepulciano	21 11 2011	San Severo, Italy
Montepulciano rosato	21 11 2011	San Severo, Italy
Nero di Troia	11 11 2011	Barletta, Italy
Nero di troia	14 12 2011	Lucera, Italy
Nero di Troia	02 12 2011	San Severo, Italy
Nero di Troia	02 12 2011	San Severo, Italy
Nero di Troia	17 01 2012	Barletta, Italy
Nero di Troia	27 01 2012	Lucera, Italy
Nero di Troia	16 02 2012	Castelluccio dei Sauri, Italy
Nero di Troia (bio SO_2 ROSATO)	16 02 2012	Castelluccio dei Sauri, Italy
Nero di troia base spumante	14 12 2011, 27 01 2012	Lucera, Italy
Nero di Troia biologico SO_2	16 02 2012	Castelluccio dei Sauri, Italy
Nero di Troia SO_2 free	16 02 2012	Castelluccio dei Sauri, Italy
San Severo rosso DOC	21 11 2011	San Severo, Italy
San Severo rosso DOC	21 11 2011	San Severo, Italy
Syrah	14 12 2011, 27 01 2012	Lucera, Italy
Syrah	14 05 2012	Lucera, Italy
Trebbiano	12 03 2012	San Severo, Italy

Table 7. List of spontaneous MLF wines used in this study

Sample were collected from spontaneous MLF wines, recovered directly from wine cellar, because winemakers do not use commercial starter culture for MLF.

Wine samples were diluted with sterile physiological solution (NaCl 8.5 g/l) and plated either onto MRS (pH 5.5) (De Man *et al*. 1960) or onto FT80 (pH 5.3) (Cavin *et al*. 1989). Both media used were supplemented with 100 mg/l cycloheximide (Sigma) to prevent the growth of yeasts and other fungi, and then plates were incubated anaerobically at 30°C.

Isolates were identified as putative LAB by positive Gram staining and negative catalase assay. All strains were stored at -80°C in MRS supplemented with glycerol (20% v/v).

For media composition see Tables 8 and 9, respectively for MRS and FT80.

Ingredients	g/litre
Peptone	10.0
Lab-Lemco powder	8.0
Yeast extract	4.0
Glucose	20.0
Sorbitan mono-oleate	1ml
Dipotassium hydrogen phosphate	2.0
Sodium acetate $3H_2O$	5.0
Triammonium citrate	2.0
Magnesium sulphate $7H_2O$	0.2
Manganese sulphate $4H_2O$	0.05

Table 8. MRS composition (De Man *et al*. 1960)

Ingredients	g/litre
Casamino acids	5.00
Yeast extract	4.00
KH_2PO_4	0.6
KCl	0.45
$CaCl_2$ $2H_2O$	0.13
$MgSO_4$ $7H_2O$	0.13
$MnSO_4$ H_2O	0.003
Tween 80	1 ml
L-(-) Malic acid	10.00
D-(+) Fructose	35.00
D-(+) Glucose	5.00
Agar	15.00

Table 9. FT80 composition (Cavin *et al*., 1989)

3.5 Growth conditions and media

Yeast strains used in this study were cultivated on YPD, agar or broth, and incubated at room temperature, about 25°C, for 24-48 h. While LAB strains were cultivated on MRS, broth or agar and incubated at 30°C for 24-72h. In particular, to cultivate *O. oeni* strains was used MRS broth supplemented with 5 g/l of L- malic acid and incubated anaerobically at 30°C.

3.6 Yeast molecular identification and chacterization internal transcribed spacers

Yeast identification was performed with several molecular methods, such as the RFLP analysis of 5.8S rRNA gene and the two ribosomal internal transcribed spacer, its sequencing and a specie-specific PCR for *S. cerevisiae*.

All the amplifications for yeast identification and characterization were performed in colony, without further DNA extraction.

3.6.1 RFLP analysis and sequencing of 5.8 S rRNA gene and the two ribosomal internal transcribed region

The RFLP analysis of 5.8S rRNA gene and the two ribosomal internal transcribed spacer was performed in according with Esteve-Zarzoso *et al*. (1999), with some modifications.

The Amplification reaction were performed using PCR reaction mix containing 0,5 µM of each primer (ITS1 and ITS4), 200 µM dNTP, buffer 10X, solution Q and 1,25 unit of QIAGEN Taq DNA Polymerase (Taq PCR Core, Qiagen).

PCR was performed in a thermocycler (I-Cycler, Bio-Rad), using the following program: initial denaturation at 95 °C for 10 min, followed by 35 cycles of denaturing at 94 °Cfor 1 min, annealing at 55.5 °C for 2 min and extension at 72 °C for 2 min; and a final extension at 72 °C for 10 min, then samples were conserved at 4°C. Products of amplification obtained were previously verificated on 2% agarose gels, with 1X TBE buffer and stained with ethidium bromide. After electrophoresis, gels were visualized under UV light and photographed (Versa Doc, BIO-RAD). Sizes were estimated by comparison against a DNA length standard (50 bp ladder, Promega) with Quantity One Software (BIO-RAD). Then PCR products were digested without further purification with the fast restriction endonucleases *HaeIII*, *HhaI* and *HinfI* (Fermentas, M-MEDICAL), although additionally in some particular cases was used endonuclease *DdeI*.

Restriction analysis was performed following the manufacture's instruction, using a mix containing 10 µl (about 0.2 µg) of PCR, 2 µl of 10X Fast Digest® Green buffer, 1 µl of each endonucleases and 17 µl

of bidistillated water. The mix was then incubated at 37°C for 20 minutes using a thermocycler (I-Cycler, Bio-Rad).

The restriction fragments were separated on 3% agarose gel with 1X TBE buffer and stained with ethidium bromide.

Yeast analyzed were identified with the YEAST-ID database (CECT-IATA, Spanish Type Culture Collection, Universitat de València).

The PCR products obtained with primers ITS1-ITS4 (White *et al*., 1990) were sequenced at PRIMM srl (Milan, Italy) to confirm the specie assignment.

3.6.2 Specie-specific PCR of Saccharomyces cerevisiae

Yeast strains identified as *S. cerevisiae* by ITS PCR/RFLP analysis were confirmed by PCR specie-specific for *S. cerevisiae,* with primer SC1 (5'-AACGGTGAGAGATTTCTGTGC-3') and SC2 (5'-AGCTGGCAGTATTCCCACAG-3') (Josepa *et al*., 2000), designed on ITS-1 region and LSU gene of *S.cerevisiae* (see Fig.5).

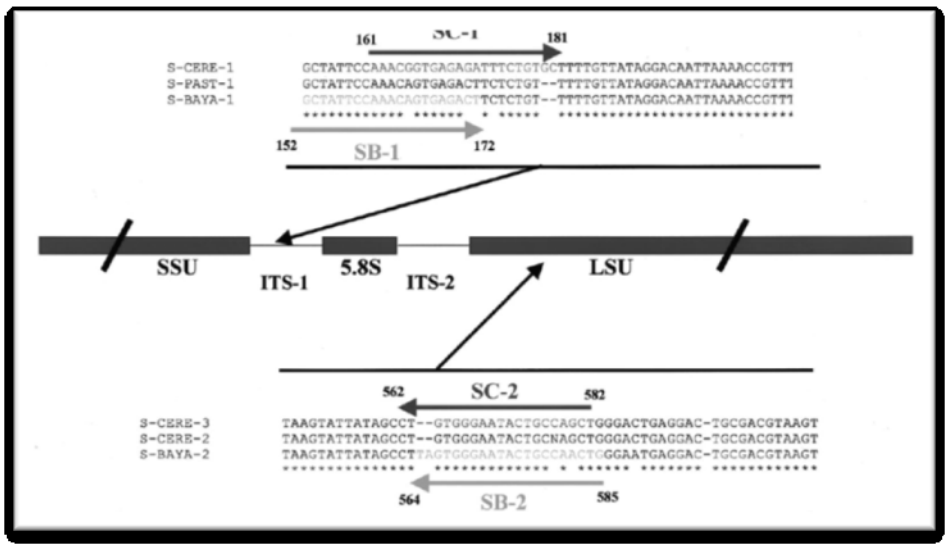


Figure 5. Diagrammatic representation of position of the rDNA primers used for specie-specific amplification of S. cerevisiae strains

PCR reaction was performed using a PCR master mix containing 0,5 µM primers (SC1 and SC2), 200µM dNTPs, buffer 10X, solution Q and 1,25 unit of QIAGEN Taq DNA Polymerase (Taq PCR Core, Qiagen). PCR conditions were as follows in a thermocycler (I-Cycler, Bio-Rad): initial denaturation at 94 °C for 5 min; 35 cycles of denaturing at 94 °C for 1 min, annealing at 55°C for 1 min and extension at 72 °C for 1 min; and a final extension at 72 °C for 10 min, then samples were conserved at 4°C. The PCR products were separated on 1,8% agarose gel, with 1X TBE buffer and stained with ethidium bromide. After electrophoresis gel were visualized under UV light and photographed (Versa Doc, BIO-RAD).

Sizes of PCR products obtained were estimated by comparison against a DNA length standard (1 Kb ladder, Promega). The aspected size of the fragment of amplification for *S. cerevisiae* strains is about 1000 bp.

3.6.3 Genotipic characterization of *Saccharomyces cerevisiae* strains: analysis of δ sequences

Yeast strains identified by RFLP analysis, ITS sequencing and specie-specific PCR as *S. cerevisiae* were subjected to further characterization. The genetic variability was evaluated by amplification of δ region, with the primers proposed by Legras and Krast (2003), δ12 (5'TCAACAATGGAATCCCAAC3') and δ21 (5'-CATCTTAACACCGTATATGA-3'), following the protocol described by Capece *et al*. (2012), modified in some steps.

The amplification of δ region was performed directly from the colony, without previous DNA extraction, by increasing the time and the temperature of initial denaturation. The amplification was performed in a thermocycler (I-cycler, BioRad) using a reaction mix containing 1 µM primers (δ12 and δ21), 200µM dNTPs, buffer 10X, solution Q, 2 mM $MgCl_2$ and 1, 5 unit of QIAGEN Taq DNA Polymerase (Taq PCR Core, Qiagen). The protocol implemented is the following: initial denaturation at 97°C for 10 min, then reaction mixture was cycled 35 times with 30 s denaturation at 94°C, 1 min primer annealing at 42°C and 2 min primer extension at 72°C, followed by a 10-min final extension step at 72°C.

The PCR products were separated on 3% agarose gel, with 1X TBE buffer and stained with ethidium bromide. After electrophoresis gel were visualized under UV light and photographed (Versa Doc, BIO-RAD). Sizes of PCR products obtained were estimated by comparison against a DNA length standard (1 Kb ladder, Promega).

Electrophoresis gels, scanned with Versadoc system (Bio-Rad, Richmond, CA, USA), were converted to TIFF images, compared and analysed with FP Quest TM software (BioRad Laboratories, Richmond, USA); then, the electrophoretic patterns were grouped, and analysed for the similarity and cophenetic correlations through the Dice coefficient. Cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA). Cophenetic correlation is a measure of how faithfully the tree represents the dissimilarities among observations.

3.7 Technological characterization of yeast strains

3.7.1 Determination of hydrogen sulphide production

Production of hydrogen sulphide was estimated by the blackening of a yeast culture on BIGGY agar (Difco) after three days of culture as described by Mortimer (1994). Five levels of color were used: 1 – white, 2 – light brown, 3 – brown, 4 – dark brown, 5 – black (Marullo *et al*., 2004). The determination was done twice.

3.7.2 Fermentation medium

A model synthetic medium (MSM) was used to simulate standard grape juice. This medium was buffered to pH 3.3 and contained (grams per liter): glucose (120); fructose (120); L+ tartaric acid (3); citric acid (0.3); L-malic acid (0.3); mineral salts (milligrams per liter): KH_2PO_4 (2,000), $MgSO_4 \cdot 7H_2O$ (200), $MnSO_4 \cdot H_2O$ (4), $ZnSO_4 \cdot 7H_2O$ (4), $CuSO_4 \cdot 5H_2O$ (1), KI (1), $CoCl_2 \cdot 6H_2O$ (0.4), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (1), and H_3BO_3 (1); vitamins (micrograms): myoinositol (300), biotin (0.04), thiamine hydrochloride (1), pyridoxine hydrochloride (1), nicotinic acid (1), calcium panthothenate acid (1), and para-amino benzoic acid (1); anaerobic growth factors: ergosterol (1.5 mg/L), sodium oleate (0.5 mg/L), and 0.05 mL Tween 80/ethanol (1:1, *v*/*v*); and nitrogen source: 190 mg/L available nitrogen provided by 300 mg/L $(NH_4)_2SO_4$ (corresponding to 63.6 mg/L available nitrogen) and a mixture of 18 amino acids corresponding to 126.4 mg/L available nitrogen (Marullo *et al*., 2006). Note that although no iron was added, its concentration in MSM was 0.15 mg/L, provided by impurity of chemical compounds (purity $\geq$ 98 %). Before yeast inoculation, the MSM was sterilized by filtration (0.45 µm nitrate cellulose membrane, Millipore, Molsheim, France) and supplemented with sulfur dioxide (20 mg/L), according to usual oenological practice.

3.7.3 Fermentation conditions

Fermentation kinetics were monitored by CO_2 release (Bely *et al*., 1990a; b). The amount of CO_2 release (grams per liter) was determined by automatic measurement of fermentor weight loss every 20 min. The CO_2 production rate (grams per liter per hour) was obtained by polynomial smoothing of the last 11 CO_2measurements. The large number of CO_2 acquisitions combined with precision weighing (0.01 g) gave six kinetic parameters with good accuracy: (1) lag phase (hours) was the time between inoculation and the beginning of CO_2 release, (2) Vmax (grams per liter per hour) was the maximum CO_2 production rate, (3) TVmax (hours) was the time necessary to reach the maximum CO_2 production rate, (4) FD (hours) was the time required to ferment all the sugars in the medium, (5) CO_2 max (grams per liter) was the total amount of CO_2 released, and (6) T50 (hours) was the time between the beginning of CO_2 release and 50 % sugar consumption (i.e., the time required to release 50 % of total expected CO_2, excluding the lag phase). Weight loss due to evaporation was under 2 %.

Yeasts were precultured in Erlenmeyer flasks filled with MSM without sulfur dioxide at 24 °C for 24 h. Fermentations were carried out in little glass fermentors (about 100 mL) and in glass 1.2-L fermentors. These two types of fermentors were locked to maintain anaerobiosis throughout alcoholic fermentation, and CO_2 was released through a sterile air outlet condenser. All experiments were performed in triplicate.

3.7.4 Population dynamics

In pure cultures, the cell concentration was determined using a Beckman Coulter (Villepinte, France) Z2 electronic particle counter. Cell viability rate was estimated using methylene blue staining. The combination of these two techniques produced concentrations expressed in viable cells per milliliter. However, it was not possible to count the two yeast populations separately in a mixed culture but only the total population.

3.7.5 Fermentation product analysis

Ethanol concentration (volume percent) was measured by infrared refractance (Infra Analyser 450, Technicon, Plaisir, France). Sugar (gram per liter) and volatile acidity (expressed in grams per liter of acetic acid) were determined chemically by colorimetry (460 nm) in continuous flux (Sanimat, Montauban, France). Ammonia was assayed by enzymatic methods (Biosystems, Evry, France), while primary amino acids were evaluated using an OPA/NAC spectrophotometric assay, as described by Dukes and Butzke (*1998*).

3.7.6 Plate assay for killer activity/sensitivity

In order to test killer activity, plates were seeded with 48-h cultures of sensitive strains and strains to be tested were loaded onto the seeded agar (4 µl of 48-h cultures to produce patches of 5 mm diameter). To assess killer sensitivity, plates were seeded with 48-h cultures of strains to be tested and killer strains were loaded onto the seeded agar (4 µl of 48-h cultures to produce patches of 5 mm diameter). The diameter of the growth inhibition zone was measured to quantify the killer activity/sensitivity.

3.8 Molecular identification and characterization of wine Lactic Acid Bacteria

LAB isolated from several MLF wines were identified with specie- specific PCR for the identification of *Oenococcus oeni* species. Then the strains identified as *O. oeni* were characterized with two molecular techniques, the Variable Number of Tandem Repeats (VNTR) and Multi Locus Sequence typing analysis.

3.8.1 *Oenococcus oeni* specie-specific PCR

Genomic DNA of putative *O. oeni* strains was isolated using the Ultra Clean Microbial DNA Isolation Kit (Cabru) the following protocol, as described by manufacturer's procedure.

The DNA obtained with the UltraClean Microbial DNA Isolation Kit (Cabru) was quantified by absorbance spectroscopy at 260, 280 and 320 nm utilizing theTake3™ Multi-Volume Spectrophotometer System (BioTek Instruments, Inc.). All measurements were accomplished using 2 μ l sample volumes. All concentrations depicted are based on a 1 cm pathlength and 50 ng/ μ L/OD.

Two species-specific PCR, the first PCR of the malolactic enzyme (MLE) using primers On1 and On2 according to Zapparoli et al. (1998) performed the identification of the isolated strains. The size of specific PCR products is about 1000bp.

The amplification reaction was carried out in a thermocycler (I-cycler, BiorRad) using a PCR master mix containing 0.3 µM primers (On1 and On2), 200µM dNTPs, buffer 10X, solution Q, 2 mM $MgCl_2$ and 1, 5 unit of QIAGEN Taq DNA Polymerase (Taq PCR Core, Qiagen) and 1 ml of sample.

The amplification profile was: initial denaturation at 94°C for 2 minutes, then 94 °C for 45 s, 64 °C for 2 min and 72 °C for 2 min, which was repeated for 30 cycles. A final extension of 72 °C for 10 min were also included. The amplification products were separated on 1.2% agarose gels with 1X TAE buffer, then visualized by ethidium bromide staining after gel electrophoresis and visualized under UV light and photographed (Versa Doc, BIO-RAD).

Sizes of PCR products obtained were estimated by comparison against a DNA length standard (1 Kb ladder, Promega).

The 2nd specie-specific PCR amplified the internal transcribed region of *O. oeni* strains, using primers Oo_smISRf and Oo_smISRr developed by Hirschhaüser *et al*. (2005), the estimated size of PCR product is 125 bp.

Each reaction mixture (50 µl) contained 0.3 µM primers (Oo_smISRf and Oo_smISRr), 200µM dNTPs, buffer 10X, solution Q, 2 mM $MgCl_2$ and 1, 5 unit of QIAGEN Taq DNA Polymerase (Taq PCR Core, Qiagen) and 2 ml of isolated DNA.

The amplification was conducted in a thermocycler (I-cycler, BioRad), using the following program: initial denaturation at 94°C for 5 minutes, followed by 35 cycles of DNA denaturation at 94°C for 30

s, primer annealing for 30 s at 52°C and an elongation step at 72°C for 20 s. In the last cycle, the elongation step at 72°C was extended to 7 minutes.

The amplification products were separated on 2% agarose gels with 1X TAE buffer, then visualized by ethidium bromide staining after gel electrophoresis and visualized under UV light and photographed (Versa Doc, BIO-RAD). Sizes of PCR products obtained were estimated by comparison against a DNA lenght standard (1 Kb ladder, Promega).

3.8.2 Genotipic characterization of *Oenococcus oeni* strains

3.8.2.1 Variable number of tandem repeat (VNTR) analysis

Suitable TR sequence loci used were those identified by Claisse *et al*. (2012) on the genome sequence of *O. oeni* PSU-1 (GenBank accession No. NC_008528) (Makarova *et al*., 2006), using the Tandem Repeat Finder program (http://tandem.bu.edu/trf/trf.html) (Benson, 1999). All primers used to amplify the selected TR regions (reported on **Table 10.**) were designed in the flanking regions using the primer3 software V. 0.4.0 (Rozen and Skaletsky, 2000) and synthesized by Eurofins MWG operon (Ebersberg, Germany).

Locus	Product[a]	Lenght[a] (bp)	Period (bp)	No of repeats[a]	Primers (5'-3')	
TR1	Plpe like lipoprotein	268	9	25	TR 1 f	GGTAAGGGAAAAGTTATCCTCG
					TR 1 r	GTTTTACCTTCGGTCGAGC
TR2	Protein kinase	563	60	08	TR 2 f	CATAATAGAATTCACTTCGCTTACC
					TR 2 r	GTAGCTGGTACGAGCTCTTC
TR3	LysM domain containing protein	673	147	04	TR 3 f	CTAATTCTTCCTCGCCCTTTG
					TR 3 r	GGACTGACTGTACTTATTTGAGG
TR4	Peptide ABC transporter ATPase	150	8	04	TR 4 f	GTGACCGACCAAAGCATAAC
					TR 4 r	AAAAACGCTCCAAGAAAGGT
TR5	Membrane carboxypeptidase	95	6	03	TR 5 f	AAATCCTGGTTTTGTCCGTA
					TR 5 r	GGCTTCCTATCCATTTTGGT

Table 10. Characteristic of TR loci and primers used for VNTR analysis of *O. oeni* strains (modified from Claisse *et al*., 2012)**.**

The amplifications of the 5 TR loci choose (TR2, TR2, TR3, TR4 and TR5) were performed in a 20 µl reaction volume of PCR mix containing the DyNAzyme TM II PCR master mix (ThermoScientific, France), 10 ng of template DNA, and 0.25 µM of each primer.

The programs of amplification was the same for all TR loci and included an initial denaturation step (95 °C for 5 min), followed by 30 cycles at 95 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, and a final extension of 72 °C for 7 min. The amplifications were performed using a thermal cycler PTC-200 DNA Engine (Bio-Rad, France). The amplified PCR products were analyzed by capillary electrophoresis on a MultiNA MCE 202 (Shimadzu, France) at 37°C during 75 s using the kit DNA-1000 (Shimadzu, France) containing the separation buffer with SyBr Gold (Invitrogen, France) and an internal size calibrator. They were automatically injected onto chips with a maximum rate voltage of 1.5 kV and a maximum current of 250 µA; the peaks were identified using a LED-excited (470 nm excitation wavelength) fluorescence detector. The size of the amplified DNA fragments was calculated on the MultiNA using the phi-X174 HaeIII DNA ladder (New England Biolabs, USA) as a reference, and then the number of tandem repeat units was calculated for each TR locus using its respective period size.

3.5.2.2 Multi locus sequence typing (MLST) analysis

The seven housekeeping targeted genes used (*gyrB*, *g6pd*, *pgm*, *dnaE*, *purK*, *rpoB*, and *recP)* were amplified according to Bridier *et al.* (2010), with minor modification. Primers used for the amplification of the seven housekeeping genes are listed on **Table 11**.

Gene	Gene product function		Primers (5'-3')	Amplicon Size (bp)
gyrB	Gyrase, β subunit	*gyrB-1*	CTTCGGTTGTTAATGCTTTGTC	674
		gyrB-2	CAACTTGGTTTTTGTCTGCC	
g6pd	Glucose-6-phosphate dehydrogenase	*g6pd-1*	TTATATGTCTGTTGCTCCTCGT	669
		g6pd-2	CCGGTTCTGATGTAAAAAGG	
Pgm	Phosphoglucomutase	*recP-1*	AGCGACAAACCATCCTTTATC	654
		recP-2	CGACAGCTAAGGAATCATGAG	
dnaE	DNA polymerase III, α subunit	*rpoB-1*	CGATATTCTCCTTTCTCCAATG	714
		rpoB-2	CTTTAGCGATCTGTTCCAATG	
purK	Phosphorybosylaminoimidazole carboxylase	*pgm-1*	ATATCTGCCGAAGTGCTAAGAG	597
		pgm-2	AGCAGCAATTTGATTTCCAG	
rpoB	RNA polymerase, β subunit	*dnaE-1*	CGTATATAGAGCGCTTTGCC	665
		dnaE-2	CGTTCTTATCGCGAGTTGTAC	
recP	Transketolase	*purK-1*	TGGTTATCATGTTGGTATTTTGG	676
		purK-2	GAAGCAGGAGCATAGGAAAGA	

Table 11. Gene name, function, amplicon size and primers sequence of housekeeping genes selected for MLST analysis of *O.oeni* strains.

The PCR was carried out in a thermal cycler PTC-200 DNA Engine (Bio-Rad, France), using a master mix containing the DyNAzyme TM II PCR master mix (ThermoScientific, France), 10 ng of template DNA, and 10 pmol of each primer. The PCR program was as follows: 95°C for 5 min, 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. For some loci, and in order to improve the amplification process, the annealing temperature was lowered to 45°C and the number of cycles were increased to 35. For the amplification of the *purK* locus, the elongation time was increased to 1 min.

PCR fragment amplification was verified by electrophoresis of the obtained PCR products in 1% agarose gels containing 10 µl GelRed (Biotium) for 100 ml agarose, and then sequenced by the Genotyping and Sequencing Laboratory of Bordeaux 2 University.

The nucleic acid sequences generated by the MLST analysis were analysed with the Bionumerics 5.1 software (Applied Maths, Kortrijk, Belgium).

Phylogenetic and molecular evolutionary analyses were carried out with MEGA software, version 5.2 (Tamura *et al*., 2011), building a phylogenetic tree by the neighbor-joining method with a Kimura two-parameter distance model. Bootstrap values were obtained after 1,000 iterations.

3.9 Microvinification assays

In order to evaluate the malolactic actitude of the *O. oeni* strains and their interaction with autochthonous *Saccharomices cerevisiae* strains preliminary microvinification assays were performed in grape must of "Nero di Troia".

For preliminary microvinification assays alcoholic fermentation was carried out on magnetic stirrers at 25 °C for 45 days.

Figure .6 Example of micorvinification assays

To induce simultaneous AF/MLF, bacteria were co-inoculated with yeast, while to induce sequential AF/MLF, bacteria were inoculated at the end of alcoholic fermentation.

Bacteria were inoculated, in both, sequential or co-inoculation approaches, in 50 ml of grape must to a final concentration of $2x10^6$ CFU/ml. For simultaneous inoculation bacteria were cultivated on MRS

broth for 48-72 h, while for sequential inoculation bacteria were cultivated on MRS broth for 24 h, then a pre-acidic stress treatment was realized, by inoculation on MRS broth at pH 3.5, for 16 h at 30 °C.

MLF was monitored by measuring the consumption of malic acid and the production of lactic acid, with enzymatic kit for L-lactic and L-malic acid (BioGamma).

L-Malic acid is oxidized by nicotinamide adenine dinucleotide (NAD) to oxaloacetate using L-Malate dehydrogenase (L-MDH) as a catalyst:

$$\text{L-Malate} + NAD^{+} \xrightarrow{\text{L-MDH}} \text{oxaloacetate} + NADH$$

The formation of oxaloacetate is removed using a second reaction catalyzed by glutamate oxaloacetate transaminase enzyme (GOT) that converts the oxaloacetate to L-aspartate in the presence of L-glutamate. The amount of NADH formed and measured stoichiometrically related to the amount of L-Malic acid (L-Malate) present in the sample. The method is specific fot the L-Malic acid and it is linear until 1.2 g/l.

The enzymatic determination of L-malic acid present in grape juice/wine has been carried out in a mix containing 2 ml of working reagent (1 ml of chromogen and 1 ml of chromogen diluent), 50 µl of enzyme (L-MDH, GOT) and 50 µl of sample (diluted 1:5)/standard. The adsorbance of the samples and the stnadards has been measured at 340 nm against the blank, i.e. distillated water (ABS1), then the enzyme has been added to the mix, mix thoroughly and incubate for 10 min at 37°C and finally was measured the adsorbance of the samples and the standard (ABS2).

The amount of L-Malic acid present in wine can be calculated with the following formula:

$$L - malic\ acid\ (gl^{-1}) = \frac{(ABS2 - ABS1 * 0{,}976)Sample}{(ABS2 - ABS1 * 0{,}976)Standard}\ [Standard]$$

Where ABS2 is the adsorbance both of the sample or the standard after 10 min at 37°C, ABS1 is the adsorbance before add the enzyme and [Standard] is the concentration of the standard solution (1 g/l).

L-Lactic acid is oxidized by nicotinamide adenine dinucleotide (NAD) to pyruvate using L-Lactate dehydrogenase (L-LDH) as a catalyst:

$$\text{L-Lactate} + NAD^{+} \xrightarrow{\text{L-LDH}} \text{pyruvate} + NADH$$

The formation of pyruvate is removed using a second reaction catalyzed by glutamate pyruvate transaminase enzyme (GTP) that converts the pyruvate to L-alanine in the presence of L-glutamate. The amount of NADH formed and measured is stoichiometrically related to the amount of L-lactic acid (L-Lactate) present in the sample. The method is specific fot the L-Lactic acid and it is linear until 0.6 g/l.

The enzymatic determination of L-Lactic acid present in grape juice/wine has been carried out in a mix containing 2 ml of working reagent (1 ml of chromogen and 1 ml of chromogen diluent), 100 µl of enzyme (L-MDH, GOT) and 40 µl of sample (diluited 1:3)/standard. The adsorbance of the samples and the stnadards has been measured at 340 nm against the blank, i.e. distillated water (ABS1), then the enzyme has been added to the mix, mix thoroughly and incubate for 10 min at 37°C and finally was measured the adsorbance of the samples and the standard (ABS2).

The amount of L-Malic acid present in wine can be calculated with the following formula:

$$L - Lactic\ acid\ (gl^{-1}) = \frac{(ABS2 - ABS1 * 0{,}976)Sample}{(ABS2 - ABS1 * 0{,}976)Standard}\ [Standard]$$

Where ABS2 is the adsorbance both of the sample or the standard after 10 min at 37°C, ABS1 is the adsorbance before add the enzyme and [Standard] is the concentration of the standard solution (0.6 g/l).

From the microvinification analysis, six different *O. oeni* strains were selected on the base of their fermentation attitude and used for sequential or co-inoculation approaches in industrial scale-up.

For the industrial scale up microvinification assays were performed in flasks of 1.00 L. Alcoholic fermentation was carried out on magnetic stirrers at 25 °C for 40 days. To induce simultaneous

AF/MLF, bacteria were co-inoculated with yeast, while to induce sequential AF/MLF, bacteria were inoculated at the end of alcoholic fermentation. Bacteria were inoculated, in both, sequential or co-inoculation approaches, in 1.00 L of grape must to a final concentration of $2x10^6$ CFU/ml. For simultaneous inoculation bacteria were pre-cultivated on MRS broth for 48-72 h, while for sequential inoculation bacteria were cultivated on MRS broth for 24 h, then a pre-acidic stress treatment was realized, by inoculation on MRS broth at pH 3.5, for 16 h at 30 °C.

During fermentation were monitoraded several parameters, such as pH, MLF trend (by measuring the production of L-Lactic acid and the consumption of L-Malic acid), AF trend (by measuring the release of CO_2) and growth of yeast and bacteria trought all the assays.

MLF trend was monitored by measuring the production/ consumption of L-Lactic/L- Malic acid, respectively, through the use of enzymatic kit for L-lactic and L-malic acid (BioGamma), as described above.

Population of yeast and bacteria were monitorated by plate count during all the assays. Grape juice/wines were serially diluited and 0.1 ml of diluited samples were plated on two different media, YPD agar and MRS agar, respectively for yeast and bacteria isolation. Both the media were supplemented with two different antibiotic, 10 mg/l of chloramphenicol and 10 mg/l cicloeximide, respectively to inhibit bacteria and yeast, mold and fungi.

Plates were incubated at 30°C for 24-48 h for yeast isolation, while LAB were cultivated anaerobically at 30°C for 5 days.

4. RESULTS AND DISCUSSION

4.1 Yeast isolation from grape berries surface

The grape samples were collected aseptically from two different vineyards, both located in the "Capitanata" area. The grape berries were collected from several areas of the vineyards, with the aim to obtain a representative sample. Yeast from grape surface were isolated in according to Prakitchaiwattana *et al*. (2004), that suggest a more vigorous pre-isolation treatment than just a simple rinsing procedure to ensure complete dislodgement of yeasts from the grape surface. In fact, the grape berries surface is covered by a waxy layer that can influence the adhesion of microbial cells (Hardie et al. 1996), also this waxy layer result in an uncomplete extraction of microorganisms difficult when current methods of food microbiology are applied.

Several yeasts strains live on grape berry surfaces, the composition of yeast population that naturally occur on grape is quite different during the different steps of grape ripening. Many intrinsic and extrinsic factors could influence the presence and growth of microorganisms on the surfaces of grape berries, such as rainfall, temperature, berry maturity, physical damage and the application of agrochemicals (Longo *et al*. 1991; Dubois *et al*. 1996). Therefore, it is evident that each region has a characteristic microflora in accordance with the area in which the vineyards are located (Ribereau-Gayon *et al*. 2000).

The microbial species recovered from grapes can be distinguished into several groups according to their technological importance in grape and wine production. Among these microbial species the most important are the microorganisms of the wine microbial consortium (WMC: yeasts, acetic acid bacteria and lactic acid bacteria), that are able to survive or grow on wine, depending on the efficiency of adequate processing measures.

Several studies on sound grapes suggest that grape berries can be colonized by a wide variety of yeast species without any obvious explanation (Li *et al*., 2010; Combina *et al*., 2005; Barata *et al*., 2008; Barata *et al*., 2012).

The WMC of grape berries can be divided into three main yeast groups:

i) oligotrophic, oxidative basidiomycetous yeasts;

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.).

Code	CFU/ml	N° isolate	Cultivar	Sample
B06 12 240912 A	2,30E+05	35	Uva di Troia	Grape surface
B06 12 240912 B	1,50E+05	35	Uva di Troia	Grape surface
B05 12 240912 A	1,59E+06	35	Uva di Troia	Grape surface
B05 12 240912 B	3,45E+06	35	Uva di Troia	Grape surface

Table 12. Yeast population and number of yeast isolated from grape surfaces of Uva di Troia.

Yeast population of samples B06 12 240912 A and B06 12 240912 B is respectively $2.30*10^5$ and $1.5*10^5$, while samples B05 12 240912 A and B05 12 240912 B shown yeast population higher, respectively $1.59*10^6$ and $3,45*10^6$. The higher yeast population of samples B05 12 240912 A and B05 12 240912 B is probably due to the presence of sound and damaged grape berries, that induced at least an increase of one log cycle (Barata *et al*., 2012). The yeast populations of the grape berry surfaces studied ranged from 10^5 to 10^6 CFU/mL, which correspond to values generally reported for mature grapes (Combina *et al*., 2004; Fleet *et al*., 2002). Damaged grapes possess, besides much higher cell counts, wider species diversity than sound grapes.

4.2 Yeast isolation from grape musts and wines

The grape samples were collected from different vineyards, located in the "Capitanata" area. 1.00 Kg of grape berries were collected aseptically from several areas of the vineyards, with the aim to obtain a representative sample, then after pressing spontaneous fermentation of grape juices obtained were carried out in laboratory at 28°C temperature without further inoculation of starter culture and monitored for 1 month.

Yeast sampling were accomplished at two stages, at the beginning and at the end of fermentation, which were determined on the basis of alcohol content, about 1% at the beginning and 9% at the end of fermentation in according with Lopandic *et al*., 2008.

About 700 putative non-*Saccharomyces* strains were selected from several grape juice undergoing into spontaneous fermentation, collected at the beginning of AF (1% EtOH vol/vol), putative non-*Saccharomyces* strains were selected for their colony morphology.

While about 800 putative *Saccharomyces* spp. were randomly isolated at the end of AF (9% EtOH vol/vol).

The initial population of non-*Saccharomyces* yeasts at the beginning of AF, in all the samples studied, varied between 10^4 and 10^8 CFU/mL, depending on the winery and grape cultivar. While, at the end of AF, the population of putative *Saccharomyces* spp. ranged between 10^5-10^8 CFU/ml. Usually during the first stages of fermentation, non-*Saccharomyces* yeasts can reach populations of 10^6-10^7 CFU/ml, comparable to those reached by the *S. cerevisiae* yeast in full fermentation (Fleet, 2003; Ocòn *et al*., 2010).

Generally non-*Saccharomyces* yeasts (*Hanseniaspora, Candida, Metschnikowia, Torulaspora, Kluyveromyces*, and *Zygosaccharomyces* spp.) begin AF in wine must, when alcoholic content increased (exceeds 5–7% vol/vol), the *Saccharomyces* species became the predominant yeasts (Fleet and Heard 1993; Zambonelli 1998).

The activity of non-*Saccharomyces* yeasts in winemaking is very important for the aromatic profile of wines, because these yeasts developed a wide range of volatile and non-volatile products thanks to different enzymatic reactions (Romano *et al*. 2003; Ciani *et al*. 2006; Domizio *et al*. 2007).

Code	CFU/ml	N° isolate	Sample			Cultivar	Code	CFU/ml	N° isolate	Sample			Cultivar
03 24 250811A	1,98E+08	19	Grape juice	(1%	EtOH	Sangiovese	**03 24 250811A**	1,99E+08	25	Wine	(9%	EtOH	Sangiovese
			vol/vol)							vol/vol)			
03 25 250811	3,99E+05	25	Grape juice	(1%	EtOH	Merlot	**03 25 250811**	3,71E+05	33	Wine	(9%	EtOH	Merlot
			vol/vol)							vol/vol)			
03 24 250811B	1,08E+06	30	Grape juice	(1%	EtOH	Sangiovese	**03 24 250811B**	1,00E+06	24	Wine	(9%	EtOH	Sangiovese
			vol/vol)							vol/vol)			
05 12 260811A	3,15E+06	10	Grape juice	(1%	EtOH	Uva Di Troia	**05 12 260811A**	8,72E+05	45	Wine	(9%	EtOH	Uva Di Troia
			vol/vol)							vol/vol)			
05 12 260811B	6,38E+07	23	Grape juice	(1%	EtOH	Uva Di Troia	**05 12 260811B**	3,96E+05	28	Wine	(9%	EtOH	Uva Di Troia
			vol/vol)							vol/vol)			
03 25 230811	3,15E+06	20	Grape juice	(1%	EtOH	Merlot	**03 25 230811**	4,21E+07	16	Wine	(9%	EtOH	Merlot
			vol/vol)							vol/vol)			
06 12 080911B	5,40E+04	20	Grape juice	(1%	EtOH	Uva Di Troia	**06 12 080911B**	3,70E+06	21	Wine	(9%	EtOH	Uva Di Troia
			vol/vol)							vol/vol)			
09 04 090911A	3,93E+06	22	Grape juice	(1%	EtOH	Bombino Bianco	**09 04 090911A**	2,15E+06	34	Wine	(9%	EtOH	Bombino Bianco
			vol/vol)							vol/vol)			
09 04 090911B	1,00E+04	17	Grape juice	(1%	EtOH	Bombino Bianco	**09 04 090911B**	1,08E+06	21	Wine	(9%	EtOH	Bombino Bianco
			vol/vol)							vol/vol)			
05 12 120911A	1,45E+06	16	Grape juice	(1%	EtOH	Uva Di Troia	**05 12 120911A**	2,80E+06	22	Wine	(9%	EtOH	Uva Di Troia
			vol/vol)							vol/vol)			
05 04 120911	4,07E+05	11	Grape juice	(1%	EtOH	Bombino Bianco	**05 04 120911**	1,16E+08	2	Wine	(9%	EtOH	Bombino Bianco
			vol/vol)							vol/vol)			
01 04 120911A	1,16E+07	24	Grape juice	(1%	EtOH	Bombino Bianco	**01 04 120911A**	3,23E+06	15	Wine	(9%	EtOH	Bombino Bianco
			vol/vol)							vol/vol)			
05 27 120911B	4,79E+06	28	Grape juice	(1%	EtOH	Montepulciano	**05 27 120911B**	2,36E+07	18	Wine	(9%	EtOH	Montepulciano
			vol/vol)							vol/vol)			
05 28 120911A	9,22E+06	14	Grape juice	(1%	EtOH	Syrah	**05 28 120911A**	1,45E+06	27	Wine	(9%	EtOH	Syrah
			vol/vol)							vol/vol)			

05 28 120911B	1,03E+07	5	Grape juice	(1% vol/vol)	EtOH	Syrah	**05 28 120911B**	8,57E+06	20	Wine	(9% vol/vol)	EtOH	Syrah
05 15 140911A	7,58E+05	16	Grape juice	(1% vol/vol)	EtOH	Falanghina	**05 15 140911A**	1,75E+07	24	Wine	(9% vol/vol)	EtOH	Falanghina
05 15 140911B	3,94E+05	12	Grape juice	(1% vol/vol)	EtOH	Falanghina	**05 15 140911B**	4,02E+07	27	Wine	(9% vol/vol)	EtOH	Falanghina
06 12 140911	4,65E+04	11	Grape juice	(1% vol/vol)	EtOH	Uva Di Troia	**06 12 140911**	2,56E+07	28	Wine	(9% vol/vol)	EtOH	Uva Di Troia
05 27 130911	4,04E+07	25	Grape juice	(1% vol/vol)	EtOH	Montepulciano	**05 27 130911**	4,00E+07	17	Wine	(9% vol/vol)	EtOH	Montepulciano
02 04 220911A	2,08E+06	20	Grape juice	(1% vol/vol)	EtOH	Bombino Bianco	**02 04 220911A**	1,00E+08	20	Wine	(9% vol/vol)	EtOH	Bombino Bianco
02 04 220911B	6,46E+06	20	Grape juice	(1% vol/vol)	EtOH	Bombino Bianco	**02 04 220911B**	1,02E+08	20	Wine	(9% vol/vol)	EtOH	Bombino Bianco
02 28 260911A	2,85E+06	20	Grape juice	(1% vol/vol)	EtOH	San Severo Bianco	**02 28 260911A**	2,33E+08	20	Wine	(9% vol/vol)	EtOH	San Severo Bianco
02 28 260911B	2,19E+06	20	Grape juice	(1% vol/vol)	EtOH	San Severo Bianco	**02 28 260911B**	4,05E+08	20	Wine	(9% vol/vol)	EtOH	San Severo Bianco
01 27 290911	1,83E+07	20	Grape juice	(1% vol/vol)	EtOH	Montepulciano	**01 27 290911**	4,24E+06	20	Wine	(9% vol/vol)	EtOH	Montepulciano
01 12 290911A	3,48E+07	20	Grape juice	(1% vol/vol)	EtOH	Uva Di Troia	**01 12 290911A**	3,61E+07	20	Wine	(9% vol/vol)	EtOH	Uva Di Troia
01 12 290911B	1,53E+07	20	Grape juice	(1% vol/vol)	EtOH	Uva Di Troia	**01 12 290911B**	1,54E+08	20	Wine	(9% vol/vol)	EtOH	Uva Di Troia
02 12 290911A	2,38E+07	20	Grape juice	(1% vol/vol)	EtOH	Uva Di Troia	**02 12 290911A**	1,33E+07	20	Wine	(9% vol/vol)	EtOH	Uva Di Troia
02 12 290911B	5,13E+07	20	Grape juice	(1% vol/vol)	EtOH	Uva Di Troia	**02 12 290911B**	1,10E+08	20	Wine	(9% vol/vol)	EtOH	Uva Di Troia

03 12 061011A	7,00E+07	20	Grape juice (1% vol/vol)	EtOH	Uva Di Troia	**03 12 061011A**	9,40E+06	20	Wine (9% vol/vol)	EtOH	Uva Di Troia			
03 12 061011B	1,94E+07	20	Grape juice (1% vol/vol)	EtOH	Uva Di Troia	**03 12 061011B**	7,60E+07	20	Wine (9% vol/vol)	EtOH	Uva Di Troia			
03 12 061011C	1,75E+07	20	Grape juice (1% vol/vol)	EtOH	Uva Di Troia	**03 12 061011C**	1,64E+07	20	Wine (9% vol/vol)	EtOH	Uva Di Troia			
04 12 061011A	1,25E+06	20	Grape juice (1% vol/vol)	EtOH	Uva Di Troia	**04 12 061011A**	2,44E+07	20	Wine (9% vol/vol)	EtOH	Uva Di Troia			
04 12 061011B	7,07E+04	20	Grape juice (1% vol/vol)	EtOH	Uva Di Troia	**04 12 061011B**	1,56E+07	20	Wine (9% vol/vol)	EtOH	Uva Di Troia			
04 12 061011C	2,40E+06	20	Grape juice (1% vol/vol)	EtOH	Uva Di Troia	**04 12 061011C**	2,72E+07	20	Wine (9% vol/vol)	EtOH	Uva Di Troia			
04 12 061011D	1,76E+06	20	Grape juice (1% vol/vol)	EtOH	Uva Di Troia	**04 12 061011D**	1,50E+07	20	Wine (9% vol/vol)	EtOH	Uva Di Troia			
04 12 061011E	7,98E+06	20	Grape juice (1% vol/vol)	EtOH	Uva Di Troia	**04 12 061011E**	1,65E+07	20	Wine (9% vol/vol)	EtOH	Uva Di Troia			

Table 13. Yeast population isolated from grape juice/ wine of several grape cultivar

4.3 Yeast identification: RFLP analysis and sequencing of 5.8 S rRNA gene and the two ribosomal internal transcribed region

4.3.1 RFLP analysis and sequencing of 5.8 S rRNA gene and the two ribosomal internal transcribed region of reference strains

Various strains type were used as reference. Two *S. cerevisiae* strains, a commercial starter culture and a strain isolated, from grapes of Nero di Troia from wine during vintage 2010, four *Candida stellata* (*C. stellata* 1kut 15*, C. stellata* 3tor 18, *C. stellata* 3t 36 , *C. stellata* 3t 16), a *Issatchenkia terricola*, an *Metschnikowia pulcherrima* and an *Hanseniaspora uvarum*. All reference strains analyzed, unless *S. cerevisiae* y64 10-4 and *S. cerevisiae* EP2 Maurivin, were kindly provided ISPA Culture Collection (http://www.ispa.cnr.it/Collection).

Specie	**ITS**	***CfoI***	***HaeIII***	***HinfI***	***DdeI***
I.terricola	450	120-100-80-70-60	280-110	215-90-90	-
M.pulcherrima	400	213-93-84	280-90	185-170	-
C.stellata 1kut 15	450	200-100-50	450	230	-
C.stellata3 tor 18	450	200-100-50	450	230	-
C.stellata3 t 16	450	200-100-50	450	230	-
Z.hellenicus 2M2	650	300	600	320-160-110	-
C.stellata3 t 36	450	190-100-60	420	225-215	-
S.cerevisiae EP2 Maurivin	880	340-120	470-300-220-170-130	360-330-120	-
S.cerevisiae y65 10^-4	880	340-120	300-220-170-130	360-330-120	-
H.uvarum HTEM9785	750	310-100	750	340-180-160-60	360-160-90-50

Table 14. Size of amplified products of the 5.8S-ITS region and restriction fragment from strains type (non-*Saccharomyces* and *Saccharomyces cerevisiae* spp).

In **Table 14** a restriction pattern of all the type strains analysed is reported. Restriction pattern obtained for all the reference strains analyzed confirmed results previously reported on literature (Esteve-Zarzoso *et al*., 1999; Pham *et al*., 2011).

4.3.2 RFLP analysis and sequencing of 5.8 S rRNA gene and the two ribosomal internal transcribed region of non-*Saccharomyces* yeast isolated from grape berries surface

The identification of non-*Saccharomyces* yeasts in grape musts is the first significant step in understanding the enzymatic activities at the beginning of the fermentation process (Esteve-Zarzoso *et al*. 1998; Romancino *et al*., 2007).

35 colonies of putative non-*Saccharomyces* strains isolated from grape berries surface were selected from each samples, choosing different colony morphology.

Yeast were subjected to PCR–restriction-fragment length polymorphism (RFLP) analysis of the ITS regions of the rDNA gene. The ITS-PCR RFLP technique has a sufficient level of resolution to identify a number of yeasts associated with fermentation of wine and alcoholic beverages.

On the basis of the restriction patterns generated using the *CfoI*, *HaeIII* and *HinfI* restriction enzymes, the isolates generated a total of 20 different banding profiles. Among this 20 restriction pattern profiles has been identified 40 strains of putative *M. pulcherrima*, showing the typical restriction pattern of these strain (5.8-ITS amplicon 440 bp, restriction patterns obtained with *CfoI* 200-90-80, *HaeIII* 260-100 bp and *HinfI* 200-180 bp). 13 strains of putative *C. stellata*, showing two different restriction patterns, respectively the 5.8-ITS amplicon 450 bp, the restriction patterns obtained with *CfoI* 200-100, *HaeIII* 450 bp and *HinfI* 230-220 bp and the 5.8-ITS amplicon 450 bp, the restriction patterns obtained with *CfoI* 200-110-70, *HaeIII* 450 bp and *HinfI* 240 bp, both restriction patterns are similar to those obtained by Pham *et al*. (2011) for several strains of *C. stellata*, 5 strains were identified as putative *I. terricola* (5.8-ITS amplicon 450 bp, restriction patterns obtained with *CfoI* 150-100-80-70-60, *HaeIII* 360-80 bp and *HinfI* 240-200 bp). 2 strains were identified as putative *D. bruxellensis* (5.8-ITS amplicon 450 bp, restriction patterns obtained with *CfoI* 190-160-60-50, *HaeIII* 370-180-90 bp and *HinfI* 220-150-140 bp). 47 strains were identified as putative *H. uvarum* (5.8-ITS amplicon 720 bp, restriction patterns obtained with *CfoI*

300-110, *HaeIII* 720 bp and *HinfI* 340-190-160-70 bp). 7 strains were identified as putative *S. cerevisiae* (5.8-ITS amplicon 800 bp, restriction patterns obtained with *CfoI* 350-320-140, *HaeIII* 300-220-160-120 bp and *HinfI* 350-120 bp) and 1 strains was identified as putative *C. boidinii* (5.8-ITS amplicon 700 bp, restriction patterns obtained with *CfoI* 300-100-90, *HaeIII* 700 bp and *HinfI* 340-190-160-70 bp).

In **Tab. 15** is reported the size of the amplified products of the 5.8S-ITS region and relative restriction pattern of yeast isolated from grape surfaces of Uva di Troia, with putative specie assignment.

ITS	*CfoI*	*HaeIII*	*HinfI*	Species	N° isolate
380	200-90-80	250-100	200-180	*Metchinkowia pulcherrima*	40
450	200-110-70	450	230-220	*Candida stellata*	10
450	150-100-80-70-60	360-80	240-200	*Issatchenkia terricola*	5
450	200-100	450	240	*Candida stellata*	3
500	190-160-60-50	370-180-90	220-150-140	*Dekkera bruxellensis*	2
700	300-100-90	700	340-190-160-70	*Candida boidinii*	1
720	300-110	720	340-190-160-70	*Hanseniaspora huvarum*	47
800	350-320-140	300-220-160-120	350-120	*Saccharomyces cerevisiae*	7
640	300-280-70	640	320-300		1*
640	300-280-70	290-190-80	320-300		2*
400	170-100-80-70	350-80	250-210		1*
400	120-90-80-60	270-115	230-100		1*
450	170-100-90-80-70-65	350-200-150-90	250-215-170-90		1*
500	200-90	250-90	200-190-100		1*

Table 15. Size of amplified products of the 5.8S-ITS region, restriction fragment from yeast strains isolated from grape berries surface and yeast species identification. * Unidentified yeast species.

12 of restriction patterns obtained weren't identified, about 17% of the strains analyzed (see **Table 15**.), so to confirm the identification of yeast strains analyzed was sequenced the 5.8S rRNA gene and the two ribosomal internal transcribed region of two strains for each restriction patterns.

Combining RFLP analysis and sequencing of the 5.8S rRNA gene and the two ribosomal internal transcribed region were identified about 140 putative non-Saccharomyces strains isolated from grape surfaces of "Uva di Troia".

In **Tab. 16.** is reported the size of the amplified products of the 5.8S-ITS region and relative restriction pattern of yeast isolated from grape surfaces of Uva di Troia, with specie assignment after sequencing of the 5.8S-ITS region.

ITS	CfoI	HaeIII	HinfI	Species	N° isolate
380	200-90-80	250-100	200-180	*Metschnikowia pulcherrima*	40
450	200-110-70	450	230-220	*Candida zemplinina*	10
450	150-100-80-70-60	360-80	240-200	*Pichia fermentans*	5
450	200-100	450	240	*Candida zemplinina*	3
500	190-160-60-50	370-180-90	220-150-140	*Issatchenkia orientalis*	2
700	300-100-90	700	340-190-160-70	*Candida boidinii*	1
720	300-110	720	340-190-160-70	*Hanseniaspora huvarum*	27
720	720-670	340-185-155-70	570-300-100	*Hanseniaspora guilliermondii*	19
800	700	320-180-150-80	340-100	*Hanseniaspora opuntiae*	1
800	350-320-140	300-220-160-120	350-120	*Saccharomyes cerevisiae*	7
640	300-280-70	640	320-300	*Kluiveromyces thermotolerans*	1
640	300-280-70	290-190-80	320-300	*Kluiveromyces thermotolerans*	2
400	170-100-80-70	350-80	250-210	*Pichia fermentans*	1
400	120-90-80-60	270-115	230-100	*Issatchenkia terricola*	1
450	170-100-90-80-70-65	350-200-150-90	250-215-170-90	*Pichia fermentans*	1
500	200-90	250-90	200-190-100	*Metschnikowia pulcherrima*	1
710	320-210-130-100	280-100	380-360	*Torulaspora delbrueckii*	1
730	350-320-120	730	370-360	*Torulaspora delbrueckii*	1
760	300	760	340-190-160	*Kluiveromyces thermotolerans*	1
340	210-100	270-90	190-180-100	*Metschnikowia pulcherrima*	1
360	200-90-80	350-110	180-160	*Metschnikowia chrysoperlae*	1
700	300-280-80	290-200-90	330	*Kluiveromyces thermotolerans*	4

Table 16. Size of amplified products of the 5.8S-ITS region, restriction fragment from yeast strains isolated from grape berries surface and **yeast species identification after sequencing**.

Sequencing of the 5.8S-ITS region confirmed the identification of 40 strains of *M. pulcherrima* and allows to identified other 2 strains of *M. pulcherrima* that shown a restriction profiles different from those obtained for the strains yet identified by RFLP analysis of the 5.8S-ITS region, the patterns obtained were respectively 5.8-ITS amplicon 340 bp, restriction patterns obtained with *CfoI* 210-

100, *HaeIII* 270-90 bp and *HinfI* 190-180-100 bp and 5.8-ITS amplicon 500 bp, restriction patterns obtained with *CfoI* 200-90, *HaeIII* 250-90 bp and *HinfI* 200-190-100 bp.

All the strains previously identified as *C. stellata* were assigned to the *C. zemplina* specie, a new specie identified by Sipiczki (2003) that can easily be confused with *C. stellata* when conventional taxonomic tests and routine PCR restriction fragment length polymorphism (RFLP) analysis of the internal transcribed spacer (ITS) region are used for identification (Sipiczki, 2003; Sipiczki et al., 2005).

The 5 strains previously identified as *I. terricola* were assigned to *P. fermentans* specie after sequencing, also the 2 strains formerly identified as *D. bruxellensis* were designated as *I. orientalis*.

Among the 47 strains formerly identified as *H. uvarum* sequencing allows to distinguish 3 different strains, *H. uvarum*, *H. guilliermondii* and *H. opuntiae*.

Sequencing of the 5.8S-ITS region confirmed the identification of *S. cerevisiae* strains, in other allows to identify the 12 patterns previously unidentified, such as several strains of *K. thermotholerans*, *P. fermentans*, *I. terricola*, *M. chrysoperlae*, *M.pulcherrima* and *T. delbrueckii.*

In **Table 17.** is reported the size of amplified product of the 5.8S-ITS region and the restriction fragments obtained with three different endonucleases, respectively *HaeIII*, *HinfI* and *CfoI,* for each strains isolated, with the correct assignment of specie.

Sample	**ITS**	***HaeIII***	***HinfI***	***CfoI***	**Specie**
B0512240912A 19	450	450	230-220	200-110-70	*C. zemplinina*
B0512240912A 21	450	450	230-220	200-110-70	*C. zemplinina*
B0512240912A 24	450	450	240-230	200-100-70	*C. zemplinina*
B0512240912A 27	490	450	240-230	200-100-70	*C. zemplinina*
B0612240912A 35	450	450	240	200-100	*C. zemplinina*
B0612240912B 1	450	450	240	200-100	*C. zemplinina*
B0612240912B 16	450	450	230	200-100	*C. zemplinina*
B0612240912B 23	450	450	250-240	200-100-60	*C. zemplinina*
B0612240912B 31	450	450	250-240	200-100-60	*C. zemplinina*
B0612240912B 33	450	450	250-240	200-100-60	*C. zemplinina*

B0512240912B 6	460	460	230-220	200-100-60	*C. zemplinina*
B0512240912B 7	460	460	230-220	200-100-60	*C. zemplinina*
B0512240912B 22	400	400	240-230	200-100-70	*C. zemplinina*
B0612240912A 22	720	720-670	340-185-155-70	570-300-100	*H. guilliermondii*
B0612240912A 23	720	720-670	340-185-155-70	570-300-100	*H. guilliermondii*
B0612240912A 24	720	720-670	340-185-155-70	570-300-100	*H. guilliermondii*
B0612240912A 25	720	720-670	340-185-155-70	570-300-100	*H. guilliermondii*
B0612240912A 26	720	720-670	340-185-155-70	570-300-100	*H. guilliermondii*
B0612240912A 27	720	720-670	340-185-155-70	570-300-100	*H. guilliermondii*
B0612240912A 28	720	720-670	340-185-155-70	570-300-100	*H. guilliermondii*
B0612240912A 29	720	720-670	340-185-155-70	570-300-100	*H. guilliermondii*
B0612240912A 30	720	720-670	340-185-155-70	570-300-100	*H. guilliermondii*
B0612240912A 32	720	720-670	340-185-155-70	570-300-100	*H. guilliermondii*
B0612240912A 33	720	720-670	340-185-155-70	570-300-100	*H. guilliermondii*
B0612240912A 34	720	720-670	340-185-155-70	570-300-100	*H. guilliermondii*
B0612240912B 2	720	720-670	340-185-155-70	570-300-100	*H. guilliermondii*
B0612240912B 3	720	720-670	340-185-155-70	570-300-100	*H. guilliermondii*
B0612240912B 5	720	720-670	340-185-155-70	570-300-100	*H. guilliermondii*
B0612240912B 8	720	720-670	340-185-155-70	570-300-100	*H. guilliermondii*
B0612240912B 9	720	720-670	340-185-155-70	570-300-100	*H. guilliermondii*
B0612240912B 14	720	720-670	340-190-160-110-70	570-300-100	*H. guilliermondii*
B0612240912B 15	720	720-670	340-185-155-70	570-300-100	*H. guilliermondii*
B0512240912A 34	800	700	320-180-150-80	340-100	*H. opuntiae*
B0512240912A 1	720	720	340-190-160-70	300-110	*H. uvarum*
B0512240912A 13	800	800	300-140-60	300-100	*H. uvarum*
B0512240912A 17	720	720	310-170-140-60	310-100	*H. uvarum*
B0512240912B 5	700	700	340-190-160-70	300-100-90	*H. uvarum*
B0512240912B 28	700	700	340-190-160-70	300-100-90	*H. uvarum*
B0612240912A 1	760	760	340-190-160-50	300-100	*H. uvarum*
B0612240912A 2	760	760	340-190-160-50	300-100	*H. uvarum*
B0612240912A 3	760	760	340-190-160-50	300-100	*H. uvarum*
B0612240912A 4	760	760	340-190-160-50	300-100	*H. uvarum*
B0612240912A 5	760	760	340-190-160-50	300-100	*H. uvarum*
B0612240912A 9	760	760	340-190-160-50	300-100	*H. uvarum*
B0612240912A 10	760	760	340-190-160-50	300-100	*H. uvarum*
B0612240912A 16	760	760	340-190-160-50	300-100	*H. uvarum*
B0612240912A 19	760	760	340-190-160-50	300-100	*H. uvarum*
B0612240912A 20	760	760	340-190-160-50	300-100	*H. uvarum*
B0612240912A 21	760	760	340-190-160-50	300-100	*H. uvarum*
B0612240912B 18	720	720	340-190-160-70	300-100	*H. uvarum*
B0612240912B 19	720	720	340-190-160-70	300-100	*H. uvarum*
B0612240912B 20	720	720	340-190-160-70	300-100	*H. uvarum*
B0612240912B 21	720	720	340-190-160-70	300-100	*H. uvarum*
B0612240912B 24	720	720	340-190-160-70	300-100	*H. uvarum*
B0612240912B 25	720	720	340-190-160-70	300-100	*H. uvarum*
B0612240912B 26	720	720	340-190-160-70	300-100	*H. uvarum*
B0612240912B 27	720	720	340-190-160-70	300-100	*H. uvarum*

B0612240912B 28	720	720	340-190-160-70	300-100	*H. uvarum*
B0612240912B 29	720	720	340-190-160-70	300-100	*H. uvarum*
B0612240912B 30	720	720	340-190-160-70	300-100	*H. uvarum*
B0612240912B 32	720	720	340-190-160-70	300-100	*H. uvarum*
B0612240912B 34	720	720	340-190-160-70	300-100	*H. uvarum*
B0612240912B 35	720	720	340-190-160-70	300-100	*H. uvarum*
B0512240912B 2	500	370-180-90	220-150-140	190-160-60-50	*I. orientalis*
B0512240912A 25	420	280-115	230-100	120-90-80-70-60	*I. terricola*
B0512240912B 8	410	280-115	230-100	120-90-80-70-60	*I. terricola*
B0612240912B 17	400	270-115	230-100	120-90-80-70-60	*I. terricola*
B0512240912A 16	680	270-180-80	300-280	300-280-80	*K. thermotolerans*
B0512240912A 22	700	290-200-90	330	300-280-80	*K. thermotolerans*
B0512240912A 26	700	290-200-90	330	300-280-80	*K. thermotolerans*
B0512240912A 28	700	290-200-90	330	300-280-80	*K. thermotolerans*
B0512240912A 33	700	290-200-90	330	300-280-80	*K. thermotolerans*
B0512240912B 10	700	700	340-190-160-70	350-320-120	*K. thermotolerans*
B0512240912B 17	640	290-190-80	320-300	300-280-70	*K. thermotolerans*
B0512240912B 18	640	640	320-300	300-280-70	*K. thermotolerans*
B0512240912B 32	700	700	340-190-160-70	350-320-120	*K. thermotolerans*
B0512240912B 20	360	350-110	180-160	200-90-80	*M. chrysoperlae*
B0512240912A 10	380	250-100	200-180	200-90-80	*M. pulcherrima*
B0512240912A 11	380	250-100	200-180	200-90-80	*M. pulcherrima*
B0512240912A 12	380	250-100	200-180	200-90-80	*M. pulcherrima*
B0512240912A 14	380	250-100	200-180	200-90-80	*M. pulcherrima*
B0512240912A 15	380	250-100	200-180	200-90-80	*M. pulcherrima*
B0512240912A 18	380	250-100	200-180	200-90-80	*M. pulcherrima*
B0512240912A 30	420	280-100	200-190	210-100-90	*M. pulcherrima*
B0512240912A 32	450	280-100	200-190	210-100-90	*M. pulcherrima*
B0512240912A 35	480	280-100	200-190	210-100-90	*M. pulcherrima*
B0512240912A 4	380	250-100	200-180	200-90-80	*M. pulcherrima*
B0512240912A 5	380	250-100	200-180	200-90-80	*M. pulcherrima*
B0512240912A 6	380	250-100	200-180	200-90-80	*M. pulcherrima*
B0512240912A 8	380	250-100	200-180	200-90-80	*M. pulcherrima*
B0512240912A 9	380	250-100	200-180	200-90-80	*M. pulcherrima*
B0512240912B 3	370	280-100	200-180	200-90-80	*M. pulcherrima*
B0512240912B 4	370	280-100	200-180	200-90-80	*M. pulcherrima*
B0512240912B 15	370	280-100	200-180	200-90	*M. pulcherrima*
B0512240912B 21	360	280-100	200-180	200-100	*M. pulcherrima*
B0512240912B 24	340	270-90	200-190	210-100	*M. pulcherrima*
B0512240912B 25	340	270-90	200-190	210-100	*M. pulcherrima*
B0512240912B 26	340	270-90	190-180-100	210-100	*M. pulcherrima*
B0512240912B 27	340	270-90	190-180-100	210-100	*M. pulcherrima*
B0512240912B 29	360	270-90	190-180-100	210-100	*M. pulcherrima*
B0512240912B 30	360	270-90	190-180-100	210-100	*M. pulcherrima*
B0512240912B 31	360	270-90	190-180-100	210-100	*M. pulcherrima*
B0512240912B 33	360	270-90	190-180-100	210-100	*M. pulcherrima*
B0512240912B 34	360	270-90	190-180-100	210-100	*M. pulcherrima*

B0512240912B 35	360	270-90	190-180-100	250-230-120	*M. pulcherrima*
B0612240912A 6	380	280-100	200-180	200-90-80	*M. pulcherrima*
B0612240912A 7	380	280-100	200-180	200-90-80	*M. pulcherrima*
B0612240912A 8	380	280-100	200-180	200-90-80	*M. pulcherrima*
B0612240912A 11	380	280-100	200-180	200-90-80	*M. pulcherrima*
B0612240912A 12	380	280-100	200-180	200-90-80	*M. pulcherrima*
B0612240912A 13	380	280-100	200-180	200-90-80	*M. pulcherrima*
B0612240912A 14	380	280-100	200-180	200-90-80	*M. pulcherrima*
B0612240912A 15	380	280-100	200-180	200-90-80	*M. pulcherrima*
B0612240912A 17	380	280-100	200-180	200-90-80	*M. pulcherrima*
B0612240912A 18	380	280-100	200-180	200-90-80	*M. pulcherrima*
B0612240912A 31	380	280-100	200-180	200-90-80	*M. pulcherrima*
B0612240912B 4	380	280-100	200-180	200-90-80	*M. pulcherrima*
B0612240912B 6	380	280-100	200-180	200-90-80	*M. pulcherrima*
B0612240912B 7	380	280-100	200-180	200-90-80	*M. pulcherrima*
B0612240912B 10	380	280-100	200-180	200-90-80	*M. pulcherrima*
B0612240912B 11	380	280-100	200-180	200-90-80	*M. pulcherrima*
B0612240912B 12	380	280-100	200-180	200-90-80	*M. pulcherrima*
B0612240912B 13	380	280-100	200-180	200-90-80	*M. pulcherrima*
B0612240912B 22	380	250-90	200-190-100	200-90	*M. pulcherrima*
B0512240912A 23	450	360-80	240-200	150-100-80-70-60	*P. fermentans*
B0512240912A 29	490	350-80	240-200	150-100-80-70-60	*P. fermentans*
B0512240912A 3	450	350-200-150-90	250-215-170-90	170-100-90-80-70-65	*P. fermentans*
B0512240912A 36	550	360-80	240-200	150-100-80-70-60	*P. fermentans*
B0512240912B 23	400	350-80	250-210	170-100-80-70	*P. fermentans*
B0512240912A 2	800	300-220-160-120	350-120	350-320-140	*S. cerevisiae*
B0512240912A 20	800	300-220-160-120	340-110	350-320-140	*S. cerevisiae*
B0512240912A 31	800	300-220-160-120	340-100	380-340-140	*S. cerevisiae*
B0512240912A 7	800	300-220-160-120	340-100	350-320-140	*S. cerevisiae*
B0512240912B 1	800	300-220-160-120	350-120	340-320-120	*S. cerevisiae*
B0512240912B 9	800	300-220-170-130	350-120	320-300-120	*S. cerevisiae*
B0512240912B 11	800	300-220-160-120	340-100	300-100	*S. cerevisiae*
B0512240912B 12	730	730	370-360	350-320-120	*T. delbrueckii*
B0512240912B 19	710	280-100	380-360	320-210-130-100	*T. delbrueckii*

Table 17. Size of amplified products of the 5.8S-ITS region and restriction fragment from non-*Saccharomyces* spp. Isolated from grape surface of Nero di Troia (samples B0512240912A, B0512240912B, B0612240912A and B0612240912B).

A great biodiversity of yeast strains of oenological interest was observed. Indeed, strains belong to *H. uvarum, H. guilliermondii, C. zemplinina, S. cerevisiae* and *M. pulcherrima* were identified**.**

Usually during grape ripening oxidative or weakly fermentative ascomycetes species, such as *Hanseniaspora*, *Candida*, *Metschnikowia* and *Pichia* spp, increased, due to a major amount of nutrients available for juice release, even in visually intact berries.

In **Fig. 7** is reported the frequency of the predominant yeast isolated from grape surfaces of Uva di Troia. The majority of the strains isolated belong to *M. pulcherrima*, a species of oenological interest. *M. pulcherrima*, which represents 34% of strains isolated, is common on wine grapes at the time of harvest and in grape must during the early stages of wine fermentation; occurs more frequently on damaged berries, on berries used to produce ice wine, and in botrytized (noble-rotted) wines. Several authors have investigated the potentiality of *M. pulcherrima* for wine fermentation. However, results were discrepant and the absence of relevant changes in fermentation rate and chemical composition often observed (Jolly *et al*., 2003; Comitini *et al*., 2010). However, a significant decrease in volatile acidity and in total acidity of the final wines was noted (Comitini *et al*., 2010).

Moreover, *M. pulcherrima* influenced positively the content of medium-chain fatty acids, 2-phenyl ethanol, isoamyl acetate and polysaccharides (Comitini *et al*., 2010).

Other yeast of oenological interest isolated from grape surfaces of Uva di Troia belonged to *Hanseniaspora* spp., that represent about 35% of the strains identified. Among *Hanseniaspora* spp. identified the most important strains, from an oenological point of view, are *H. guilliermondii* and *H. uvarum*, that represent respectively the 14 and 21% of the strains analyzed.

Our results confirmed results previously reported on literature, in fact Čadež *et al*. (2010) demonstrated that the apiculate *H. uvarum/K. apiculata* appears to be the most common grape berry species worldwide, this results in other is consistent with its predominance in the beginning of spontaneous must fermentations.

In addition has been demonstrated that the apiculate yeasts *H. uvarum* and *H. guillermondi* enhance the production of desirable compounds, such as esters, without increasing the undesirable heavy sulphur compounds, either in pure or in mixed starter cultures with *S. cerevisiae* (Moreira *et al*. 2008). In particular, in mixed fermentation, *H. uvarum* increased the isoamyl acetate content in

wine, whereas *H. guilliermondii* resulted in an enhancement of 2-phenylethyl acetate (Moreira *et al*. 2008).

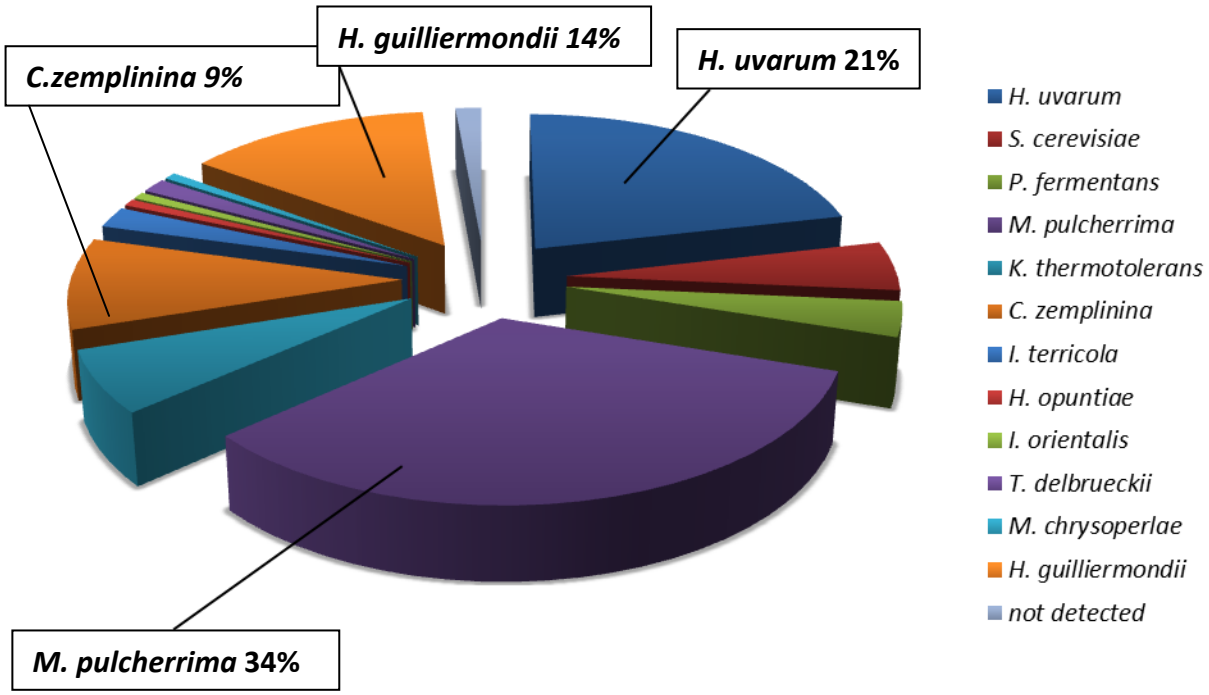


Figure 7. Identification of predominant yeast isolated from grape surface of Uva di Troia and its frequency.

In damaged grapes *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. (Barata *et al*., 2008).

The 9% of the yeast isolated and identified belonged to *C. zemplinina* species, a new osmotolerant and psycrotolerant yeast, formerly identified as *C. stellata*, identified by Sipiczki (2003), that can be used in sweet wine production, thanks to its properties. Several yeast ecology studies demonstrated the frequent presence of this species in wine fermentations (Brezna *et al*., 2010; Li *et al*. 2010; Magyar & Bene, 2006; Nisiotou & Nychas, 2007; Tofalo *et al*., 2009; Tofalo *et al*., 2012; Urso *et al*., 2008; Zott *et al*., 2008), is a typical contaminant of botrytised juice fermentations but its dissemination is also spread to sound grapes (Barata *et al*., 2008).

C. zemplinina is a osmotolerant and fructophylic yeast that produces low amounts of acetic acid, together with relevant quantities of glycerol (Mills *et al*., 2002; Tofalo *et al*., 2012).

Several studies focused on the potential application of *C. zemplinina* in wine fermentations (Andorrà *et al*., 2010; Magyar & Toth, 2011; Tofalo *et al*., 2011; Tofalo *et al*., 2012), mainly due to its ethanol and low temperature tolerance, osmotic resistance and fructophylic character. In addition other studies also suggest that *C. zemplina* strains are able to produce relevant quantities of glycerol and low amounts of acetic acid (Magyar & Toth, 2011; Tofalo *et al.,* 2012), in particular when used in multistarter mixtures with *S. cerevisiae* (Rantsiou *et al*., 2012).

Among the strain of oenological interest isolated from grape surfaces of Uva di Troia minor part (about 1%) is represented by *Torulaspora delbrueckii* strains, usually the genera *Zygosaccharomyces* 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; Nisiotou and Nychas, 2007), however they are rarely the dominant population because the aerobic conditions promote the fast growing populations of *Candida*, *Hanseniaspo*ra and *Pichia* spp.

T. delbrueckii is a strain yet used in winemaking, under standard condition. Bely *et al*. (2008) shown that *T. delbrueckii* is characterized by pure fermentation, with very low volatile acidity and acetaldehyde production. However *T. delbrueckii* is a low ethanol producer, but can be used in sweet wine fermentation, because it doesn't react to osmotic stress in the same way as *S. cerevisiae*, that produce high quantities of volatile acidity, mainly during the initial stage of fermentation (about the 35% of the total volatile acidity production), this overproduction of volatile acidity by S. cerevisiae is linked to increased glycerol production, induced by osmotic stress.

Several authors suggest to reduce the volatile acidity in high-sugar fermentation the use of mixed cultures of *T. delbrueckii* and *S. cerevisiae*, with a higher concentration of *T. delbrueckii* to promote its growth (Bely *et al*., 2008; Ciani *et al*., 2006, Ciani & Ferraro, 1998).

The *Saccharomyces* spp. are usually present in low numbers and in low frequencies, even in damaged grapes. (Davenport 1973, 1974). Results reported in literature suggested that the frequency of *S. cerevisiae* is about 0.05-0.1% in sound berries and 25% in damaged berries, usually with numbers of about 10^5–10^6/berry (Mortimer and Polsinelli, 1999), in our samples *S. cerevisiae* represent about 5% of the strains analyzed, these result is due to the presence of sound berries, but also damaged one.

The other strains identified from grape surfaces of Uva di Troia represent a minor part of the strains analyzed, for both frequency and oenological interest. These strains represent about 13% of the strains analyzed, among these strains there are oxidative or weakly fermentative ascomycetous species, such as *Pichia*, *Kluyveromyces* and *Issatchenkia* spp.

4.3.3 RFLP analysis and sequencing of 5.8 S rRNA gene and the two ribosomal internal transcribed region of non-*Saccharomyces* yeast isolated from grape juice at the beginning of AF

About 130 colonies of putative non-*Saccharomyces* strains were selected from those isolated at the beginning of AF, from several grape cultivar collected from various vineyard. Yeast were choose for their different colony morphology. Yeast were subjected to PCR–restriction-fragment length polymorphism (RFLP) analysis of the ITS regions of the rDNA gene. The ITS-PCR RFLP technique has a sufficient level of resolution to identify a number of yeasts associated with fermentation of wine and alcoholic beverages.

Based on the restriction patterns generated using the *CfoI*, *HaeIII* and *HinfI* restriction enzymes, the isolates from sample 03 12 061011A-B-C generated a total of 2 different banding profiles,

respectively 5.8-ITS amplicon 700 bp, restriction patterns obtained with *CfoI* 300-100, *HaeIII* 700 bp and *HinfI* 330-180-150-60 bp for the strains identified as *Candida boidinii,* and 5.8-ITS amplicon 700 bp, restriction patterns obtained with *CfoI* 340-320-120, *HaeIII* 300-220-160-120 bp and *HinfI* 350-120 for those identified as *S. cerevisiae*. Usually at the beginning of AF non-*Saccharomyces* strains are dominant and the frequency of *S. cerevisiae* is about 0.05-0.1% in grape juice obtained from sound berries, while its frequency increased to 25% in those obtained from damaged berries (Mortimer and Polsinelli, 1999), in our samples *S. cerevisiae* frequency is very high (about 58%), probably due to an high presence of grape berries damaged in the samples analyzed.

In **Tab. 18** is reported the size of amplified products of the 5.8S-ITS region and relative restriction patterns obtained with the endonucleases *CfoI*, *HaeIII* and *HinfI* of putative non-*Saccharomyces* strains isolated from samples 03 12 061011A-B-C (grape cultivar Uva di Troia) at the beginning of AF.

Sample		**ITS**	***CfoI***	***HaeIII***	***HinfI***	***Specie***
03 12 061011A	1	700	300-100	700	330-180-150-60	*Candida boidinii*
03 12 061011A	3	690	290-90	700	330-180-150-60	*Candida boidinii*
03 12 061011A	4	690	290-90	690	330-180-150-60	*Candida boidinii*
03 12 061011A	10	780	290-90	690	330-180-150-60	*Candida boidinii*
03 12 061011A	11	700	340-320-120	300-220-160-120	350-120	*S.cerevisiae*
03 12 061011A	16	760	290-90	720	330-180-150-60	*Candida boidinii*
03 12 061011A	7	780	370-360-140	300-220-160-120	350-120	*S.cerevisiae*
03 12 061011A	19	780	370-360-140	300-220-160-120	350-120	*S.cerevisiae*
03 12 061011B	2	760	340-320-120	300-220-160-120	350-120	*S.cerevisiae*
03 12 061011B	3	760	340-320-120	300-220-160-120	350-120	*S.cerevisiae*
03 12 061011B	10	800	340-320-120	300-220-160-120	350-120	*S.cerevisiae*
03 12 061011B	11	670	300-100	670	320-170-150-60	*Candida boidinii*
03 12 061011B	17	760	340-320-120	300-220-160-120	350-120	*S.cerevisiae*

03 12 061011B	20	790	340-320-120	300-220-160-120	350-120	*S.cerevisiae*
03 12 061011B	1	780	360-330-120	300-220-160-120	360-120	*S.cerevisiae*
03 12 061011B	14	800	360-330-120	300-220-160-120	360-120	*S.cerevisiae*
03 12 061011C	5	760	340-320-120	300-220-160-120	350-120	*S.cerevisiae*
03 12 061011C	6	670	300-100	670	320-170-150-60	*Candida boidinii*
03 12 061011C	11	670	300-100	670	320-170-150-60	*Candida boidinii*
03 12 061011C	12	670	300-100	670	320-170-150-60	*Candida boidinii*
03 12 061011C	13	770	340-320-120	300-220-160-120	350-120	*S.cerevisiae*
03 12 061011C	18	810	340-320-120	300-220-160-120	350-120	*S.cerevisiae*
03 12 061011C	2	700	315-290-110	300-210-160-120	310-100	*S.cerevisiae*
03 12 061011C	15	700	290-90	700	300-170-140	*Candida boidinii*

Table 18. Size of amplified products of the 5.8S-ITS region and restriction fragment from non-*Saccharomyces* spp. Isolated from wines at the beginning of AF (1% EtOH, Nero di Troia), samples 03 12 061011A- B- C.

The strains isolated from samples 04 12 061011A - B- C- D- E (grape cultivar Uva di Troia) on the basis of the restriction patterns obtained using the *CfoI*, *HaeIII* and *HinfI* restriction enzymes generated a total of 5 different banding profiles, respectively identified as *H. guilliermondii* (5.8-ITS amplicon 700 bp, restriction patterns obtained with *CfoI* 300-100, *HaeIII* 700 bp and *HinfI* 340-180-150-60 bp), *C. zemplinina* (5.8-ITS amplicon 450 bp, restriction patterns obtained with *CfoI* 200-100-50, *HaeIII* 450 bp and *HinfI* 230-220 bp), *I. terricola* (5.8-ITS amplicon 450 bp, restriction patterns obtained with *CfoI* 110-90-70-60-50, *HaeIII* 280-110 bp and *HinfI* 210-90 bp), *Zygosaccharomyces bailii* (5.8-ITS amplicon 700 bp, restriction patterns obtained with *CfoI* 300-100, *HaeIII* 700 bp and *HinfI* 320-180-150-60 bp) and *S. cerevisiae* (5.8-ITS amplicon 700 bp, restriction patterns obtained with *CfoI* 340-320-120, *HaeIII* 300-220-160-120 bp and *HinfI* 350-120).

The major part of the strains identified belonged to *H. guilliermondii* strains, a strain of oenological interest, in other our result confirmed those reported in literature (Zott *et al*., 2008; Urso *et al*., 2008) that suggested that non-*Saccharomyces* strains are predominant during the first steps of AF,

until ethanol level reach 5-7% (vol/vol). Among strains analyzed about 20% belonged to *S. cerevisiae* strains, also for the samples 04 12 061011A-B-C-D-E the frequency of *S. cerevisiae* strains is quite high, comparable to results reported in literature, that suggest that the frequency of *S. cerevisiae* strains increase from 0.1-0.5% of sound berries to 25% in damaged one.

In **Tab. 19** is reported the size of amplified products of the 5.8S-ITS region and relative restriction patterns obtained with the endonucleases *CfoI*, *HaeIII* and *HinfI* of putative non-*Saccharomyces* strains isolated from samples 04 12 061011A-B-C-D-E (grape cultivar Uva di Troia) at the beginning of AF.

Sample		**ITS**	***CfoI***	***HaeIII***	***HinfI***	**Specie**
04 12 061011A	8	760	350-325-120	300-220-160-120	340-100	*S.cerevisiae*
04 12 061011A	9	670	300-100	770	340-180-150-60	*H. guilliermondii*
04 12 061011A	5	425	200-100-50	460	230-220	*Candida zemplinina*
04 12 061011A	18	690	350-325-120	300-220-160-120	340-100	*S.cerevisiae*
04 12 061011A	14	690	300-100	780	340-180-150-60	*H. guilliermondii*
04 12 061011A	11	730	300-100	780	340-180-150-60	*H. guilliermondii*
04 12 061011A	1	450	110-90-70-60-50	280-110	210-90	Issatchenkia terricola
04 12 061011A	20	450	190-100-50	450	220-210	*Candida zemplinina*
04 12 061011B	1	690	300-100	780	340-180-150-60	*H. guilliermondii*
04 12 061011B	4	690	300-100	780	340-180-150-60	*H. guilliermondii*
04 12 061011B	5	690	350-325-120	300-220-160-120	350-100	*S.cerevisiae*
04 12 061011B	3	700	300-100	700	320-180-150-60	Zygosaccharomyces bailii
04 12 061011C	1	700	300-100	780	340-180-150-60	*H. guilliermondii*
04 12 061011C	4	425	180-100-50	460	230-220	*Candida zemplinina*
04 12 061011C	6	425	350-325-120	300-220-160-120	350-100	*S.cerevisiae*
04 12 061011C	14	740	300-100	780	340-180-150-60	*H. guilliermondii*
04 12 061011C	11	690	300-100	780	340-180-150-60	*H. guilliermondii*
04 12 061011C	20	700	300-100	780	340-180-150-60	*H. guilliermondii*
04 12 061011C	13	750	300-100	750	300-180-150	*H. guilliermondii*

04 12 061011D	1	425	300-100	780	340-180-150-60	*H. guilliermondii*
04 12 061011D	2	690	310-100	750	340-180-150-60	*H. guilliermondii*
04 12 061011D	3	740	320-100	780	340-180-150-60	*H. guilliermondii*
04 12 061011D	8	740	365-340-130	300-220-160-120	350-120	*S.cerevisiae*
04 12 061011D	11	670	320-100	750	350-180-150-60	*H. guilliermondii*
04 12 061011D	12	430	200-100-50	450	225-210	*Candida zemplinina*
04 12 061011D	16	760	370-340-130	300-220-160-120	350-110	*S.cerevisiae*
04 12 061011E	1	710	320-100	780	340-180-150-60	*H. guilliermondii*
04 12 061011E	3	690	300-100	780	340-180-150-60	*H. guilliermondii*
04 12 061011E	7	700	310-100	725	340-180-150-60	*H. guilliermondii*
04 12 061011E	10	760	350-330-130	300-220-160-120	350-120	*S.cerevisiae*
04 12 061011E	15	760	300-100	750	340-180-150-60	*H. guilliermondii*
04 12 061011E	18	720	300-100	750	340-180-150-60	*H. guilliermondii*
04 12 061011E	20	760	300-100	750	340-180-150-60	*H. guilliermondii*
04 12 061011E	6	750	300-100	750	320-180-150	*H. guilliermondii*
04 12 061011E	17	750	300-100	750	320-180-150	*H. guilliermondii*

Table 19. Size of amplified products of the 5.8S-ITS region and restriction fragment from non-*Saccharomyces* spp. Isolated from wines at the beginning of AF (1% EtOH, Nero di Troia), samples 04 12 061011A - B- C- D- E.

The strains isolated from samples 01 12 290911A-B (grape cultivar Uva di Troia) generated a total of 5 different banding profiles on the basis of the restriction patterns obtained using the *CfoI*, *HaeIII* and *HinfI* restriction enzymes generated. The strains analyzed were identified respectively as *H. guilliermondii* (5.8-ITS amplicon 750 bp, restriction patterns obtained with *CfoI* 300-100, *HaeIII* 750 bp and *HinfI* 320-180-150bp), *C. zemplinina* (5.8-ITS amplicon 450 bp, restriction patterns obtained with *CfoI* 120-100, *HaeIII* 280-100 bp and *HinfI* 220-210 bp), *I. terricola* (5.8-ITS amplicon 450 bp, restriction patterns obtained with *CfoI* 120-100-80-70-60, *HaeIII* 280-100 bp and *HinfI* 210-100 bp), *Zygoascus hellenicus* (5.8-ITS amplicon 700 bp, restriction patterns obtained with *CfoI* 320, *HaeIII* 700 bp and *HinfI* 350-170-120 bp) and *H. uvarum* (5.8-ITS amplicon 700 bp, restriction patterns obtained with *CfoI* 280-90, *HaeIII* 700 bp and *HinfI* 200-90).

The yeast identified from samples 01 12 290911A-B belonged all to non-.*Saccharomyces* strains. The major part of the strains analyzed are of oenological interest, such as strains of *Candida* spp. (*C. stellata* and *C. zemplinina*), *Hanseniaspora* spp. (*H. guilliermondii* and *H. uvarum*).

In **Tab. 20** is reported the size of amplified products of the 5.8S-ITS region and relative restriction patterns obtained with the endonucleases *CfoI*, *HaeIII* and *HinfI* of putative non-*Saccharomyces* strains isolated from samples 01 12 290911A-B (grape cultivar Uva di Troia) at the beginning of AF.

Sample		**ITS**	***CfoI***	***HaeIII***	***HinfI***	***Specie***
01 12 290911A	3	450	200-100-50	450	250-230	*Candida zemplinina*
01 12 290911A	5	450	120-100	280-100	220-210	*Candida zemplinina*
01 12 290911A	9	450	120-100-80-70-60	280-100	210-100	*Issatchenkia terricola*
01 12 290911A	10	450	120-100-80-70-60	290-120	230-100	*Issatchenkia terricola*
01 12 290911A	13	450	170-150	300	250-120	*Candida zemplinina*
01 12 290911A	20	450	120-100-80-70-60	290-120	210-90	*Issatchenkia terricola*
01 12 290911A	1	750	300-100	750	320-180-150	H. guilliermondii
01 12 290911A	8	750	300-100	750	320-180-150	H. guilliermondii
01 12 290911B	4	700	320	700	350-170-120	*Zygoascus hellenicus*
01 12 290911B	5	450	120-100-80-70-60	290-120	230-100	*Issatchenkia terricola*
01 12 290911B	9	470	200-100-60	450	250-230	*Candida zemplinina*
01 12 290911B	12	470	200-100-60	470	250-230	*Candida zemplinina*
01 12 290911B	13	470	200-100-60	470	250-230	*Candida zemplinina*
01 12 290911B	16	650	300	650	340-160-110	*Zygoascus hellenicus*
01 12 290911B	11	700	280-90	700	300-170-140-50	H.uvarum
01 12 290911B	20	400	100-80-70-60-50	270-115	200-90	*Issatchenkia terricola*

Table 20. Size of amplified products of the 5.8S-ITS region and restriction fragment from non-*Saccharomyces* spp. isolated from wines at the beginning of AF (1% EtOH, Nero di Troia), samples 01 12 290911A- B.

The strains isolated from samples 02 12 290911A-B (grape cultivar Uva di Troia) generated a total of 7 different banding profiles on the basis of the restriction patterns obtained using the *CfoI*, *HaeIII* and *HinfI* restriction enzymes generated.

The strains analyzed were identified respectively as *H. guilliermondii* (5.8-ITS amplicon 750 bp, restriction patterns obtained with *CfoI* 300-100, *HaeIII* 750 bp and *HinfI* 320-180-150bp), *C. zemplinina* (5.8-ITS amplicon 450 bp, restriction patterns obtained with *CfoI* 200-100-60, *HaeIII*

450 bp and *HinfI* 250-230 bp), *I. terricola* (5.8-ITS amplicon 450 bp, restriction patterns obtained with *CfoI* 120-100-80-70-60, *HaeIII* 280-100 bp and *HinfI* 210-100 bp), *Zygoascus hellenicus* (5.8-ITS amplicon 700 bp, restriction patterns obtained with *CfoI* 320, *HaeIII* 700 bp and *HinfI* 350-170-120 bp), *H. uvarum* (5.8-ITS amplicon 700 bp, restriction patterns obtained with *CfoI* 280-90, *HaeIII* 700 bp and *HinfI* 200-90) and *Yarrowia lipolitica* (5.8-ITS amplicon 600 bp, restriction patterns obtained with *CfoI* 180-50, *HaeIII* 500-360 bp and *HinfI* 200-130-120).

All the yeast identified from samples 02 12 290911A-B belonged to non-.*Saccharomyces* strains. The major part of the strains analyzed are of oenological interest, such as strains of *Candida* spp. (*C. stellata* and *C. zemplinina*), *Hanseniaspora* spp. (*H. guilliermondii* and *H. uvarum*).

In **Tab. 21** is reported the size of amplified products of the 5.8S-ITS region and relative restriction patterns obtained with the endonucleases *CfoI*, *HaeIII* and *HinfI* of putative non-*Saccharomyces* strains isolated from samples 02 12 290911A-B (grape cultivar Uva di Troia) at the beginning of AF.

Sample		**ITS**	***CfoI***	***HaeIII***	***HinfI***	
02 12 290911A	5	760	300-100	760	340-180-150-60	*H. guilliermondii*
02 12 290911A	8	460	200-100-60	460	250-230	*Candida zemplinina*
02 12 290911A	13	640	200-100-60	640	340-160-110	*Zygoascus hellenicus*
02 12 290911A	15	760	310-100	760	340-180-150-60	*H. guilliermondii*
02 12 290911A	12	400	120-100-80-70-60	280-100	210-100	Issatchenkia terricola
02 12 290911B	8	630	340	630	344-180-135	*Zygoascus hellenicus*
02 12 290911B	10	760	350-150	760	350-200-170-60	*H. uvarum*
02 12 290911B	11	760	350-150	760	350-200-170-60	*H. uvarum*
02 12 290911B	13	760	350-150	760	350-200-170-60	*H. uvarum*
02 12 290911B	16	450	120-100-80-70-60	280-100	210-100	*Issatchenkia terricola*
02 12 290911B	14	600	180-150	500-360	200-130-120	*Yarrowia lipolitica*

Table 21. Size of amplified products of the 5.8S-ITS region and restriction fragment from non-*Saccharomyces* spp. Isolated from wines at the beginning of AF (1% EtOH, Nero di Troia), samples 02 12 290911A- B.

The strains isolated from samples 05 12 120911A (grape cultivar Uva di Troia) generated a total of 5 different banding profiles on the basis of the restriction patterns obtained using the *CfoI*, *HaeIII* and *HinfI* restriction enzymes generated.

The strains analyzed were identified respectively as *H. guilliermondii* (5.8-ITS amplicon 750 bp, restriction patterns obtained with *CfoI* 300-100, *HaeIII* 750 bp and *HinfI* 320-180-150bp), *H. uvarum* (5.8-ITS amplicon 700 bp, restriction patterns obtained with *CfoI* 300-100, *HaeIII* 700 bp and *HinfI* 325-180-150-60 bp), *Zygosaccharomyces bailii* (5.8-ITS amplicon 700 bp, restriction patterns obtained with *CfoI* 380, *HaeIII* 700 bp and *HinfI* 360-200-170 bp), *H. opuntiae* (5.8-ITS amplicon 750 bp, restriction patterns obtained with *CfoI* 300-100, *HaeIII* 750 bp and *HinfI* 340-180-150) and *S.cerevisiae* (5.8-ITS amplicon 880 bp, restriction patterns obtained with *CfoI* 360-340-130, *HaeIII* 310-230-170-130 bp and *HinfI* 360-120).

In **Tab. 22** is reported the size of amplified products of the 5.8S-ITS region and relative restriction patterns obtained with the endonucleases *CfoI*, *HaeIII* and *HinfI* of putative non-*Saccharomyces* strains isolated from sample 05 12 120911A (grape cultivar Uva di Troia) at the beginning of AF.

Sample		**ITS**	***CfoI***	***HaeIII***	***HinfI***	**Specie**
05 12 120911A	1	700	300-100	700	325-180-150-60	*H.uvarum*
05 12 120911A	15	700	380	700	360-200-170	*Zygosaccharomyces bailii*
05 12 120911A	18	750	310-100	750	340-180-150	*H.opuntiae*
05 12 120911A	20	880	360-340-130	310-230-170-130	360-120	*S.cerevisiae*
05 12 120911A	5	750	300-100	750	320-180-150	H. guilliermondii
05 12 120911A	13	750	300-100	750	320-180-150	H. guilliermondii

Table 22. Size of amplified products of the 5.8S-ITS region and restriction fragment from non-*Saccharomyces* spp. Isolated from wines at the beginning of AF (1% EtOH, Nero di Troia), sample 05 12 120911A.

The strains isolated from samples 06 12 080911B (grape cultivar Uva di Troia) generated a total of 3 different banding profiles on the basis of the restriction patterns obtained using the *CfoI*, *HaeIII* and *HinfI* restriction enzymes generated.

The strains analyzed were identified respectively as *H. guilliermondii* (5.8-ITS amplicon 750 bp, restriction patterns obtained with *CfoI* 300-100, *HaeIII* 750 bp and *HinfI* 320-180-150bp), *C. boidinii* (5.8-ITS amplicon 730 bp, restriction patterns obtained with *CfoI* 300-100, *HaeIII* 730 bp

and *HinfI* 320-180-150 bp) and *S.cerevisiae* (5.8-ITS amplicon 880 bp, restriction patterns obtained with *CfoI* 340-315-130, *HaeIII* 300-220-160-120 bp and *HinfI* 340-115).

In **Tab. 23** is reported the size of amplified products of the 5.8S-ITS region and relative restriction patterns obtained with the endonucleases *CfoI*, *HaeIII* and *HinfI* of putative non-*Saccharomyces* strains isolated from sample 06 12 080911B (grape cultivar Uva di Troia) at the beginning of AF.

Sample		**ITS**	***CfoI***	***HaeIII***	***HinfI***	***Specie***
06 12 080911B	2	880	340-315-130	300-220-160-120	340-115	*S.cerevisiae*
06 12 080911B	11	730	300-100	730	320-180-150	*Candida boidinii*
06 12 080911B	5	750	310-100	750	340-180-150-60	*H. guilliermondii*
06 12 080911B	13	750	310-100	750	340-180-150-60	*H. guilliermondii*
06 12 080911B	16	750	310-100	750	340-180-150-60	*H. guilliermondii*
06 12 080911B	20	750	310-100	750	340-180-150	*H. guilliermondii*
06 12 080911B	1	750	300-90	750	320-180-150	*H. guilliermondii*
06 12 080911B	13	750	300-90	750	320-180-150	*H. guilliermondii*

Table 23. Size of amplified products of the 5.8S-ITS region and restriction fragment from non-*Saccharomyces* spp. Isolated from wines at the beginning of AF (1% EtOH, Nero di Troia), sample 06 12 080911B.

The strains isolated from samples 06 12 140911 (grape cultivar Uva di Troia) generated a total of 5 different banding profiles on the basis of the restriction patterns obtained using the *CfoI*, *HaeIII* and *HinfI* restriction enzymes generated.

The strains analyzed were identified respectively as *H. guilliermondii* (5.8-ITS amplicon 750 bp, restriction patterns obtained with *CfoI* 300-100, *HaeIII* 750 bp and *HinfI* 320-180-150bp), *C. boidinii* (5.8-ITS amplicon 720 bp, restriction patterns obtained with *CfoI* 300-100, *HaeIII* 720 bp and *HinfI* 320-180-150 bp), *S.cerevisiae* (5.8-ITS amplicon 880 bp, restriction patterns obtained with *CfoI* 335-310-130, *HaeIII* 300-220-160-120 bp and *HinfI* 380-115), *C. mogii* (5.8-ITS amplicon 380 bp, restriction patterns obtained with *CfoI* 200-170, *HaeIII* 380 bp and *HinfI* 215-

150-135) and *I. terricola* (5.8-ITS amplicon 400 bp, restriction patterns obtained with *CfoI* 120-90-80-70-50, *HaeIII* 280-110 bp and *HinfI* 230-100).

In **Tab. 24** is reported the size of amplified products of the 5.8S-ITS region and relative restriction patterns obtained with the endonucleases *CfoI*, *HaeIII* and *HinfI* of putative non-*Saccharomyces* strains isolated from sample 06 12 140911 (grape cultivar Uva di Troia) at the beginning of AF.

Sample		**ITS**	***CfoI***	***HaeIII***	***HinfI***	
06 12 140911	1	720	300-100	720	320-180-150	*Candida boidinii*
06 12 140911	2	720	300-100	720	320-180-150	*Candida boidinii*
06 12 140911	4	880	335-310-130	300-220-160-120	380-115	*S.cerevisiae*
06 12 140911	13	380	200-170	380	215-150-135	*Candida mogii*
06 12 140911	15	700	300-100	700	320-180-150	*Candida boidinii*
06 12 140911	20	700	300-100	700	320-180-150	*Candida boidinii*
06 12 140911	13	750	300-100	750	320-180-150	H. guilliermondii
06 12 140911	19	400	120-90-80-70-50	280-110	230-100	Issatchenkia terricola

Table 24. Size of amplified products of the 5.8S-ITS region and restriction fragment from non-*Saccharomyces* spp. Isolated from wines at the beginning of AF (1% EtOH, Nero di Troia), samples 06 12 140911.

The strains isolated from samples 05 27 130911-05 27 120911 B (grape cultivar Montepulciano) generated a total of 4 different banding profiles on the basis of the restriction patterns obtained using the *CfoI*, *HaeIII* and *HinfI* restriction enzymes generated.

The strains analyzed were identified respectively as *H. guilliermondii* (5.8-ITS amplicon 750 bp, restriction patterns obtained with *CfoI* 300-100, *HaeIII* 750 bp and *HinfI* 320-180-150bp), *C. zemplinina* (5.8-ITS amplicon 450 bp, restriction patterns obtained with *CfoI* 200-100, *HaeIII* 450 bp and *HinfI* 230-80 bp), *S.cerevisiae* (5.8-ITS amplicon 800 bp, restriction patterns obtained with *CfoI* 360-330-, *HaeIII* 470-320-230-170-120 bp and *HinfI* 340-100) and *I. terricola* (5.8-ITS amplicon 400 bp, restriction patterns obtained with *CfoI* 120-90-80-70-50, *HaeIII* 280-220-110 bp and *HinfI* 220-100).

In **Tab. 25** is reported the size of amplified products of the 5.8S-ITS region and relative restriction patterns obtained with the endonucleases *CfoI*, *HaeIII* and *HinfI* of putative non-*Saccharomyces* strains isolated from samples 05 27 130911- 05 27 120911 B (grape cultivar Montepulciano) at the beginning of AF.

Sample		**ITS**	***CfoI***	***HaeIII***	***HinfI***	***Specie***
05 27 130911	3	800	360-330-120	300-220-170-130	340-110	*S.cerevisiae*
05 27 130911	11	400	120-90-80-70-50	280-220-110	220-100	*Issatchenkia terricola*
01 27 290911	2	800	360-330	470-320-230-170-120	340-110	*S.cerevisiae*
01 27 290911	2	450	200-100	450	230-80	*Candida zemplinina*
01 27 290911	4	450	200-100	450	230-80	*Candida zemplinina*
05 27 120911 B	9	750	300-100	750	320-180-160	*H. guilliermondii*
05 27 120911 B	18	790	395-366-140	300-220-170-130	370-120	*S.cerevisiae*

Table 25. Size of amplified products of the 5.8S-ITS region and restriction fragment from non-*Saccharomyces* spp. Isolated from wines at the beginning of AF (1% EtOH, Montepulciano), samples 05 27 130911- 05 27 120911 B.

The strains isolated from sample 03 24 250811A (grape cultivar Sangiovese)generated a total of 2 different banding profiles on the basis of the restriction patterns obtained using the *CfoI*, *HaeIII* and *HinfI* restriction enzymes generated.

The strains analyzed were identified respectively as *C. zemplinina* (5.8-ITS amplicon 450 bp, restriction patterns obtained with *CfoI* 200-100-50, *HaeIII* 450 bp and *HinfI* 220 bp) and *S.cerevisiae* (5.8-ITS amplicon 800 bp, restriction patterns obtained with *CfoI* 380-360-170-, *HaeIII* 300-220-160-120 bp and *HinfI* 350-110).

In **Tab. 26** is reported the size of amplified products of the 5.8S-ITS region and relative restriction patterns obtained with the endonucleases *CfoI*, *HaeIII* and *HinfI* of putative non-*Saccharomyces* strains isolated from sample 03 24 250811A (grape cultivar Sangiovese) at the beginning of AF.

Sample		ITS	*CfoI*	*HaeIII*	*HinfI*	*Specie*
03 24 250811A	10	450	200-100-50	450	220	*Candida zemplinina*
03 24 250811A	17	450	200-100-50	450	220	*Candida zemplinina*
03 24 250811A	18	800	380-360-170	300-220-160-120	350-110	*S.cerevisiae*

Table 26. Size of amplified products of the 5.8S-ITS region and restriction fragment from non-*Saccharomyces* spp. Isolated from wines at the beginning of AF (1% EtOH, Sangiovese), sample 03 24 250811A.

The strains isolated from sample 03 25 250811 (grape cultivar Merlot) generated a total of 3 different banding profiles on the basis of the restriction patterns obtained using the *CfoI*, *HaeIII* and *HinfI* restriction enzymes generated.

The strains analyzed were identified respectively as *C. zemplinina* (5.8-ITS amplicon 450 bp, restriction patterns obtained with *CfoI* 230-190, *HaeIII* 450 bp and *HinfI* 250-220 bp), *H.guilliermondii* (5.8-ITS amplicon 700 bp, restriction patterns obtained with *CfoI* 300-100, *HaeIII* 750 bp and *HinfI* 320-180-150) and *S.cerevisiae* (5.8-ITS amplicon 800 bp, restriction patterns obtained with *CfoI* 350-110, *HaeIII* 300-220-160-120 bp and *HinfI* 390-150).

In **Tab. 27** is reported the size of amplified products of the 5.8S-ITS region and relative restriction patterns obtained with the endonucleases *CfoI*, *HaeIII* and *HinfI* of putative non-*Saccharomyces* strains isolated from sample 03 25 250811 (grape cultivar Merlot) at the beginning of AF.

Sample		ITS	*CfoI*	*HaeIII*	*HinfI*	*Specie*
03 25 250811	13	450	230-190	450	250-220	*Candida zemplinina*
03 25 250811	27	800	350-110	300-220-160-120	390-150	*S.cerevisiae*
03 25 230811	1	800	340-320	320-230-180-130	340-110	*S.cerevisiae*
03 25 230811	3	750	300-100	750	320-180-150	*H. guilliermondii*

Table 27. Size of amplified products of the 5.8S-ITS region and restriction fragment from non-*Saccharomyces* spp. Isolated from wines at the beginning of AF (1% EtOH, Merlot), sample 03 25 250811.

In **Fig. 8** is reported the frequency of the predominant yeast isolated from must at the beginning of AF (1% EtOH vol/vol) of several cultivar collected in the "Capitanata" area. The majority of the

strains isolated belong to *Hanseniaspora spp.* (about 34%), in particular *H. guilliermondii* (30%) and *H. uvarum* (4%).

Several authors investigated the potentiality of apiculate wine yeasts, mainly *H. uvarum* and *H. guilliermondii*, in winemaking. In fact this yeast are frequently found in grapes and are also dominators of the early stages of must fermentation (Kunkee, 1984; Gao and Fleet, 1988; Zironi *et al*., 1993; Gil *et al*., 1996; Fleet, 2003).

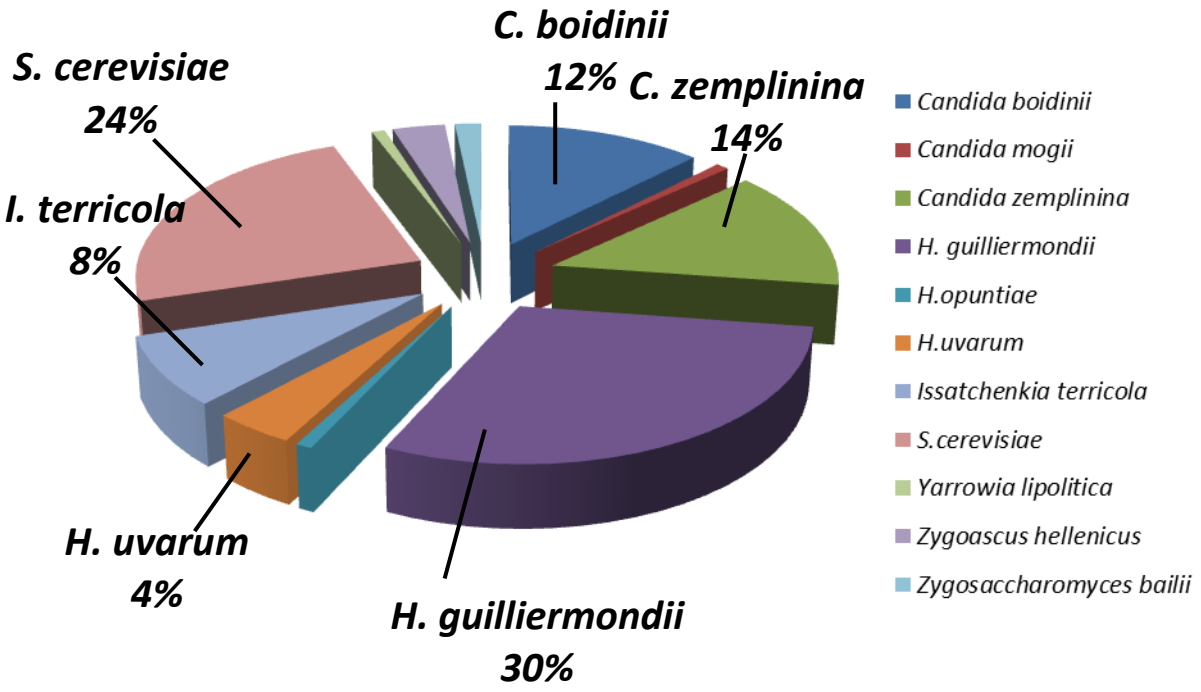


Figure 8. Identification of predominant yeast isolated from several wines at the beginning of AF (1% EtOH) and its frequency.

The major part of the studies on apiculate evaluated the production of fermentation compounds by pure, mixed or sequential cultures of apiculate yeasts with *S. cerevisiae* strains, using either grape must or basal synthetic medium (Herraiz *et al*., 1990; Mateo *et al*., 1991; Velàzquez *et al*., 1991; Zironi *et al*., 1993; Ciani and Picciotti, 1995; Gil *et al*., 1996; Romano *et al*., 1997a,b; Ciani and Maccarelli, 1998; Rojas *et al*., 2001, 2003; Zohre and Erten, 2002; Romano *et al*., 2003). These

experiments showed that there are significant differences in chemical composition of the resulting wines or fermented media.

Moreira *et al.* (2008) demonstrated that *H. uvarum* and *H. guillermondi* enhance the production of desirable compounds, such as esters, without increasing the undesirable heavy sulphur compounds, either in pure or in mixed starter cultures with *S. cerevisiae.* In particular, in mixed fermentation, *H. uvarum* increased the isoamyl acetate content in wine, whereas *H. guilliermondii* resulted in an enhancement of 2-phenylethyl acetate (Moreira *et al*. 2008; Rojas et al., 2001, 2003).

The 27% of the yeast isolated and identified belonged to *Candida spp.*. Among *Candida spp.* the species most important identified are *C. stellata* (12%) and *C. zemplinina* (2%). *C. zemplinina* is a new osmotolerant and psycrotolerant yeast, formerly identified as *C. stellata*, identified by Sipiczki (2003) that can be used in sweet wine production, thanks to its properties. Several yeast ecology studies demonstrated the frequent presence of this species in wine fermentations (Brezna *et al*., 2010; Li *et al*. 2010; Magyar & Bene, 2006; Nisiotou & Nychas, 2007; Tofalo *et al*., 2009; Tofalo *et al*., 2012; Urso *et al*., 2008; Zott *et al*., 2008), is a typical contaminant of botrytised juice fermentations but its dissemination is also spread to sound grapes (Barata *et al*., 2008).

C. zemplinina is an osmotolerant and fructophylic yeast that produces low amounts of acetic acid, together with relevant quantities of glycerol (Mills *et al*., 2002; Tofalo *et al*., 2012). Several studies focused on the potential application of *C. zemplinina* in wine fermentations (Andorrà *et al*., 2010; Magyar & Toth, 2011; Tofalo *et al*., 2011; Tofalo *et al*., 2012), mainly due to its ethanol and low temperature tolerance, osmotic resistance and fructophylic character.

In addition other studies also suggest that *C. zemplina* strains are able to produce relevant quantities of glycerol and low amounts of acetic acid (Magyar & Toth, 2011; Tofalo *et al.,* 2012), in particular when used in multistarter mixtures with *S. cerevisiae* (Rantsiou *et al*., 2012).

4.3.4 RFLP analysis and sequencing of 5.8 S rRNA gene and the two ribosomal internal transcribed region of *Saccharomyces* yeast isolated from grape juice at the end of AF

About 170 putative *Saccharomyces* strains were selected from those isolated at the end of AF, from several grape cultivar collected from various vineyard. Yeast were subjected to PCR–restriction-fragment length polymorphism (RFLP) analysis of the ITS regions of the rDNA gene.

Based on the restriction patterns generated using the *CfoI*, *HaeIII* and *HinfI* restriction enzymes, all strains analyzed were identified as *S. cerevisiae*, showing the typical restriction patterns (5.8-ITS amplicon 880 bp, restriction patterns obtained with *CfoI* 385-365, *HaeIII* 320-230-180-150 bp and *HinfI* 365-155).

In **Tab. 28** is reported the size of amplified products of the 5.8S-ITS region and relative restriction patterns obtained with the endonucleases *CfoI*, *HaeIII* and *HinfI* of putative *Saccharomyces cerevisiae* strains isolated from grape juice, obtained from several grape cultivars, at the end of AF, for each sample analyzed we reported the code and the number of strains isolated.

Sample	N° isolate	ITS	*HaeIII*	*HinfI*	*CfoI*	*Putative identification*	*Oringin*
06 12 080911B	7	880	320-230-180-150	365-155	385-365	*Saccharomyces cerevisiae*	Nero di Troia
05 12 120911A	7	880	320-230-180-150	365-155	385-365	*Saccharomyces cerevisiae*	Nero di Troia
06 12 140911	7	880	320-230-180-150	365-155	385-365	*Saccharomyces cerevisiae*	Nero di Troia
02 12 290911B	7	880	320-230-180-150	365-155	385-365	*Saccharomyces cerevisiae*	Nero di Troia
01 12 290911A	7	880	320-230-180-150	365-155	385-365	*Saccharomyces cerevisiae*	Nero di Troia
01 12 290911B	7	880	320-230-180-150	365-155	385-365	*Saccharomyces cerevisiae*	Nero di Troia
02 12 290911A	7	880	320-230-180-150	365-155	385-365	*Saccharomyces cerevisiae*	Nero di Troia
03 12 061011A	7	880	320-230-180-150	365-155	385-365	*Saccharomyces cerevisiae*	Nero di Troia
03 12 061011B	7	880	320-230-180-150	365-155	385-365	*Saccharomyces cerevisiae*	Nero di Troia
03 12 061011C	7	880	320-230-180-150	365-155	385-365	*Saccharomyces cerevisiae*	Nero di Troia
04 12 061011A	7	880	320-230-180-150	365-155	385-365	*Saccharomyces cerevisiae*	Nero di Troia
04 12 061011B	7	880	320-230-180-150	365-155	385-365	*Saccharomyces cerevisiae*	Nero di Troia
04 12 061011C	7	880	320-230-180-150	365-155	385-365	*Saccharomyces cerevisiae*	Nero di Troia
04 12 061011D	7	880	320-230-180-150	365-155	385-365	*Saccharomyces cerevisiae*	Nero di Troia
04 12 061011E	7	880	320-230-180-150	365-155	385-365	*Saccharomyces cerevisiae*	Nero di Troia
03 24 250811A	15	880	320-230-180-150	365-155	385-365	*Saccharomyces cerevisiae*	San Giovese
03 25 250811	9	880	320-230-180-150	365-155	385-365	*Saccharomyces cerevisiae*	Malbec
01 27 290911	7	880	320-230-180-150	365-155	385-365	*Saccharomyces cerevisiae*	Montepulciano
03 25 230811	19	880	320-230-180-150	365-155	385-365	*Saccharomyces cerevisiae*	Malbec
05 27 130911	7	880	320-230-180-150	365-155	385-365	*Saccharomyces cerevisiae*	Montepulciano
05 27 120911B	7	880	320-230-180-150	365-155	385-365	*Saccharomyces cerevisiae*	Montepulciano

Table.28 Size of amplified products of the 5.8S-ITS region and restriction fragment from *Saccharomyces* spp. isolated from wines at the end of AF (9%EtOH).

4.4 PCR Specie-specific for *Saccharomyces cerevisiae*

Yeast strains identified as *S. cerevisiae* by ITS PCR/RFLP analysis were confirmed by PCR specie-specific for *S. cerevisiae,* with primer SC1 (5'-AACGGTGAGAGATTTCTGTGC-3') and SC2 (5'-AGCTGGCAGTATTCCCACAG-3') (Josepa *et al*., 2000), designed on ITS-1 region and LSU gene of *S.cerevisiae.*

All the strains analyzed give a specific fragment of amplification (about 1000bp). In **Fig. 9** is reported an example of PCR specie-specific for *S. cerevisiae*.

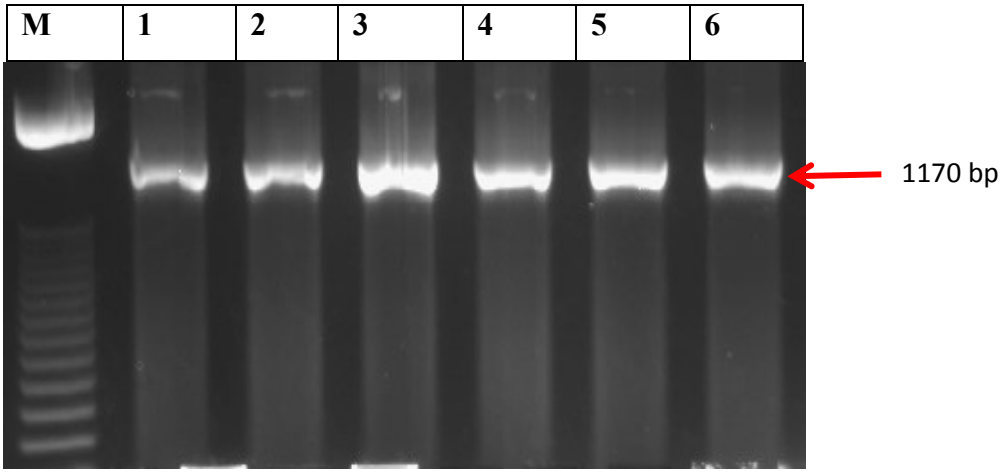


Figure 9. PCR specie-specific for *S. cerevisiae*. M marker 1Kb Promega, Lane 1-6 putative *S. cereviasiae* strains.

4.5 Genotipic characterization of Saccharomyces cerevisiae strains: analysis of δ sequences

90 strains identified as *S. cerevisiae* by RFLP analysis, ITS sequencing and PCR specie-specific were subjected to further characterization. The genetic variability was evaluated by amplification of δ region, with the primers proposed by Legras and Krast (2003), δ12 (5'TCAACAATGGAATCCCAAC3') and δ21 (5'-CATCTTAACACCGTATATGA-3').
This method is a rapid, reproducible and very sensitive to highlight intraspecific variability of yeasts (Capece et al., 2012).
In **Fig. 13** is reported the dendrogram from UPGMA clustering analysis of amplification of interdelta region patterns. Interdelta analysis produced 86 different profiles, resulting the most suitable tool to differentiate at strain leves *S. cerevisiae* strains (Capece *et al*., 2012).

According to the resulting dendrogram (Fig. 10), the strains were distributed in 13 clusters (nominated from A to O), cluster M included the higher number of strains (20 strains), while cluster A included 12 strains, clusters C and E 10 strains, clusters F and I 9 strains, clusters G and L 5 strains, cluster D 4 strains and cluster O 3 strains. Only clusters B, H and N included only a strain.

In **Tab. 29** are reported data obtained from delta analysis of 90 *S. cerevisiae* strains. The major part of the isolates collected in the same area belonged to the same delta cluster (see table 29 for details). Only seven strains shown identical profiles, respectively strains 04 061011 E 15 and 18 (delta profile 33), 04 061011 E 3-5-13 (delta profile 34) and 04 12 061011A 1-15 (delta profile 85).
The amplification of interdelta region resulted the most rapid, reproducible, sensitive and discriminative method (Legras and Karst, 2003; Schuller *et al*., 2005; Xufre *et al*., 2011). Furthermore, the analysis of interdelta region was performed directly from yeast colony, without DNA extraction, in according to Capece *et al*. (2012), so this method results the most suitable tool for a rapid evaluation of genetic variability among S. cerevisiae strains.
S. cerevisiae strains collected from several area of "heroic viticulture" shown an high polymorphism, in other this strains, all isolated from spontaneous fermentation in sample at the end of AF (9% EtOH vol/vol) represent strains resident in this habitat,i.e. truly autochthonous yeast strains, which demand increased recently. However, strains collected from the same area belonged to the same cluster.

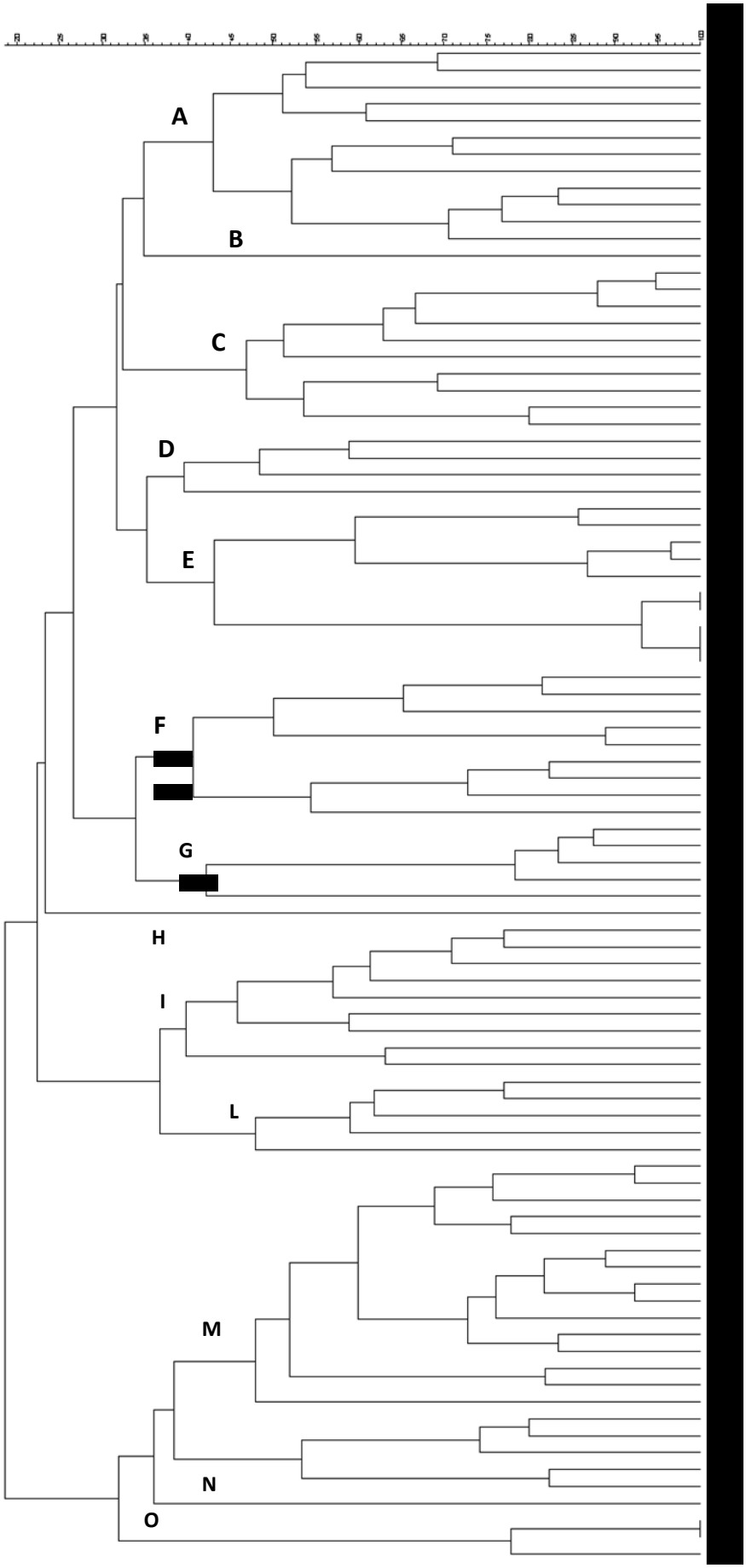


Figure 10. Cluster analysis of the profiles obtained by PCR inter-delta region from 90 *Saccharomyces cerevisiae* strains

	Strain	Cluster	Delta profile		Strain	Cluster	Delta profile
1	03 12 061011A 7	A	1	**46**	04 12 061011A 3	F	43
2	03 12 061011A 16	A	2	**47**	04 12 061011C 6	G	44
3	03 12 061011A 1	A	3	**48**	04 12 061011C 16	G	45
4	01 12 290911B 5	A	4	**49**	04 12 061011C 3	G	46
5	03 12 061011A 4	A	5	**50**	04 12 061011C 20	G	47
6	03 12 061011B 2	A	6	**51**	04 12 061011D 6	G	48
7	03 12 061011 C 1	A	7	**52**	03 12 061011A 13	H	49
8	03 12 061011B 10	A	8	**53**	03 12 061011C 7	I	50
9	03 12 061011A 13	A	9	**54**	02 12 290911A 1	I	51
10	03 12 061011B 11	A	10	**55**	06 12 140911 10	I	52
11	03 12 061011B 1	A	11	**56**	05 27 120911B 2	I	53
12	03 12 061011 C 16	A	12	**57**	03 24 250811A 19	I	54
13	03 12 061011 C 8	B	13	**58**	05 27 120911B 14	I	55
14	01 12 290911A 1	C	14	**59**	03 24 250811A 15	I	56
15	01 12 290911A 14	C	15	**60**	03 12 061011C 1	I	57
16	01 12 290911A 18	C	16	**61**	03 12 061011A 7	I	58
17	01 12 290911A 15	C	17	**62**	02 12 290911A 6	L	59
18	01 12 290911A 12	C	18	**63**	01 27 290911A 4	L	60
19	01 12 290911B 12	C	19	**64**	05 27 130911B 16	L	61
20	01 12 290911B 16	C	20	**65**	03 25 250811 22	L	62
21	03 12 061011B 7	C	21	**66**	01 12 290911B 1	L	63
22	01 12 290911B 1	C	22	**67**	04 12 061011B 4	M	64
23	01 12 290911B 4	C	23	**68**	04 12 061011B 12	M	65
24	04 12 061011D 18	D	24	**69**	04 12 061011D 6	M	66
25	03 12 061011 C 10	D	25	**70**	04 12 061011D 18	M	67
26	04 12 061011C 16	D	26	**71**	04 061011 E 13	M	68
27	03 12 061011 C 6	D	27	**72**	04 12 061011A 16	M	69
28	04 12 061011B 17	E	28	**73**	04 12 061011B 16	M	70
29	04 12 061011B 19	E	29	**74**	04 12 061011C 3	M	71
30	04 12 061011B 4	E	30	**75**	04 12 061011C 6	M	72
31	04 12 061011B 16	E	31	**76**	04 12 061011D 7	M	73
32	04 12 061011B 12	E	32	**77**	04 12 061011A 3	M	74
33	04 12 061011 E 15	E	33	**78**	04 12 061011B 17	M	75
34	04 12 061011 E 18	E	33	**79**	04 12 061011B 19	M	76
35	04 12 061011 E 3	E	34	**80**	04 12 061011C 5	M	77
36	04 12 061011 E 5	E	34	**81**	04 12 061011D 11	M	78
37	04 12 061011 E 13	E	34	**82**	04 12 061011C 20	M	79
38	04 12 061011A 15	F	35	**83**	04 061011 E 5	M	80
39	04 12 061011D 7	F	36	**84**	04 061011 E 18	M	81
40	04 12 061011C 5	F	37	**85**	04 12 061011D 16	M	82
41	04 12 061011D 11	F	38	**86**	04 061011 E 3	M	83
42	04 12 061011D 16	F	39	**87**	04 061011 E 15	N	84
43	04 12 061011A 2	F	40	**88**	04 12 061011A 1	O	85
44	04 12 061011A 16	F	41	**89**	04 12 061011A 15	O	85
45	04 12 061011A 1	F	42	**90**	04 12 061011A 2	O	86

Table 29. Molecular profiles obtained from interdelta analysis of 90 *S. cerevisiae* strains.

4.6 Technological characterization of yeast strains

With the aim to analyse the technological performances of the identified isolates, thirteen strains, representative of each of the clusters obtained by δ-analysis were chosen: 04 12 061011A 1, 04 061011 E 15, 04 12 061011B 4, 01 27 290911A 4, 03 12 061011C 7, 03 12 061011A 13, 04 12 061011D 6, 04 12 061011C 5, 04 12 061011B 17, 04 12 061011D 18, 01 12 290911A 14, , 03 12 061011 C 803 12 061011A 7. The first step of technological characterization concerned: killer activity/sensitivity, H_2S production, fermentation kinetics in model synthetic medium and in must, cytofluorometric analysis, and esters production (**Figure 11**).

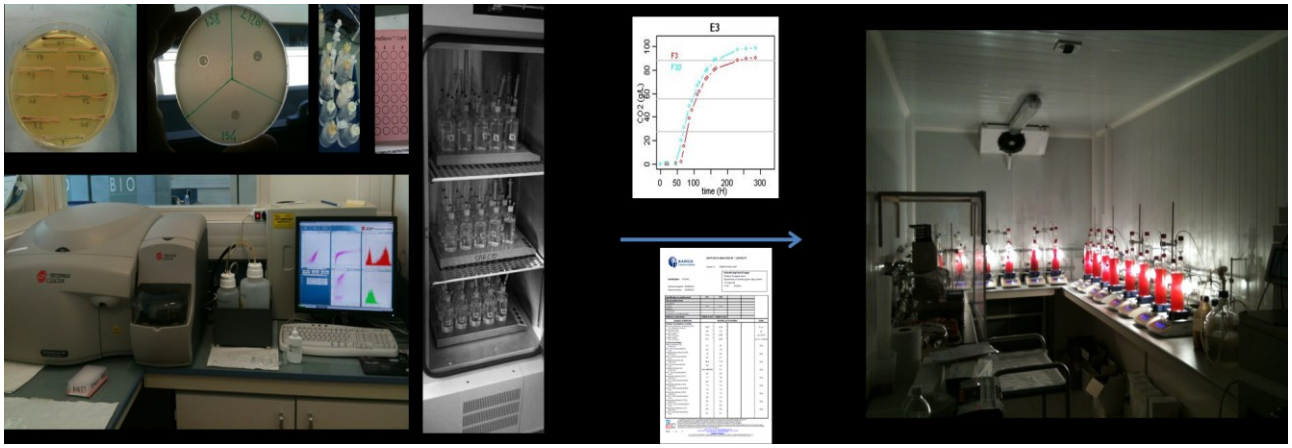
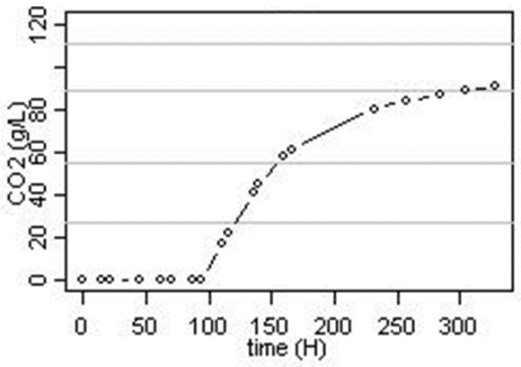
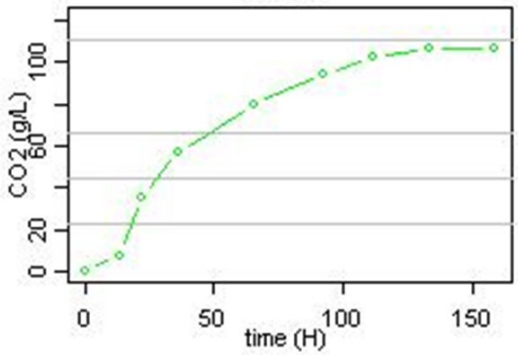
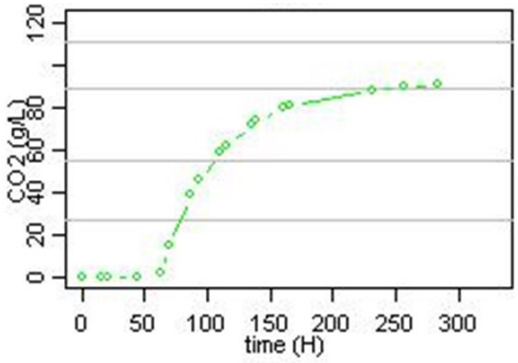
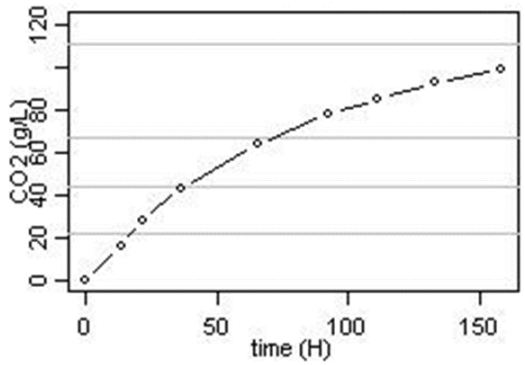
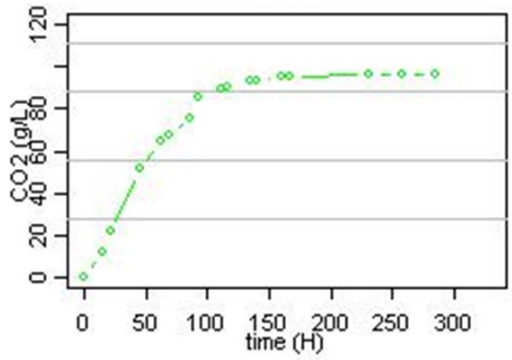
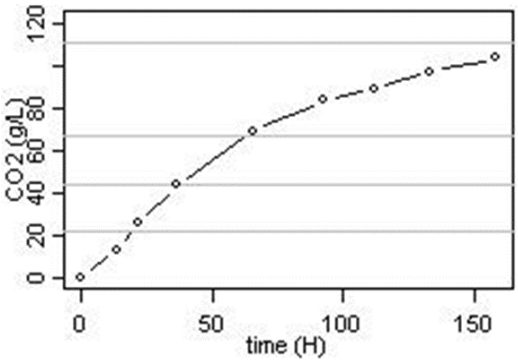
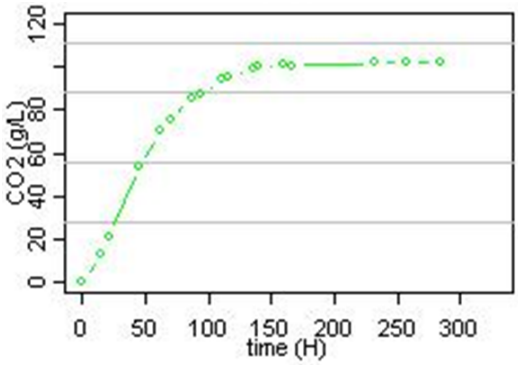
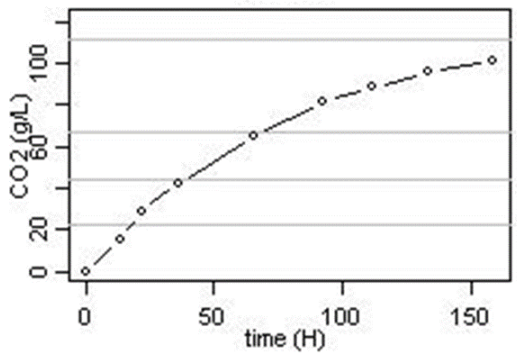
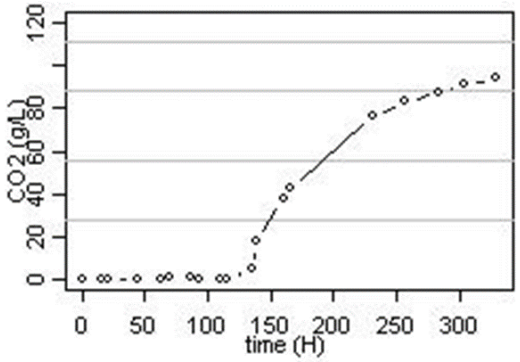


Figure 11. Schematic representation of technological characterization of oenological yeasts from Nero di Troia wines (killer activity/sensitivity, H_2S production, fermentation kinetics in model synthetic medium and in must, cytofluorometric analysis, alcohol content, reducing sugar content, volatile acidity, glycerol content).

The analysis of fermentation kinetics (**Figure 12**), conveniently integrated with the results of killer activity/sensitivity, H_2S production, and population dynamics (data not shown), led us to select the most promising *S. cerevisiae* strains: I6 and E4.

	model synthetic medium	must
G6		
E3		
G2		
G3		

F2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40
60
80
120
0
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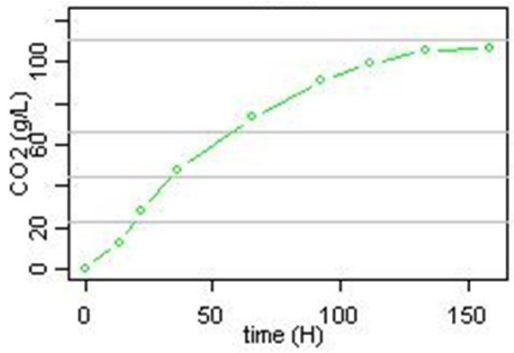
100
150
200
250
300
time (H)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100
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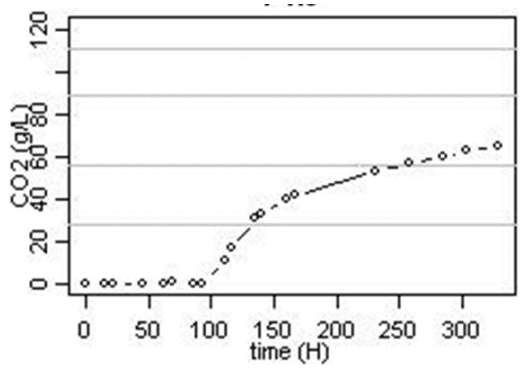
time (H)

H5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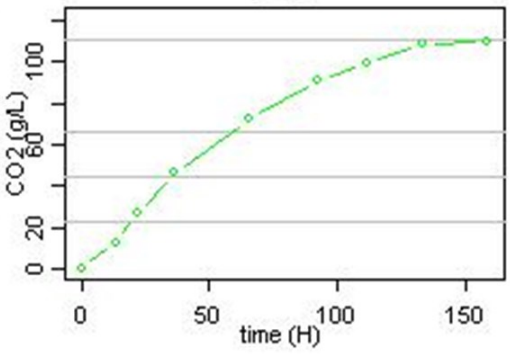
time (H)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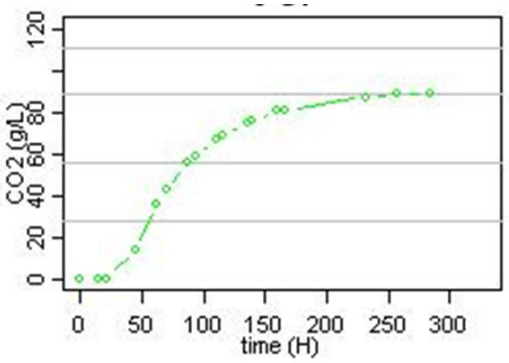
0
50
100
150
time (H)

G1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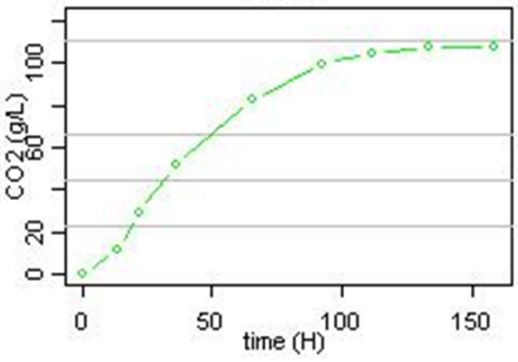
time (H)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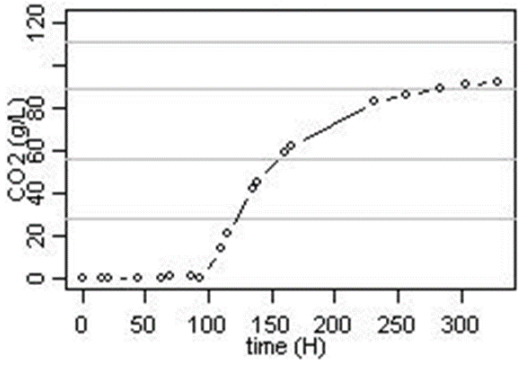
150
time (H)

F8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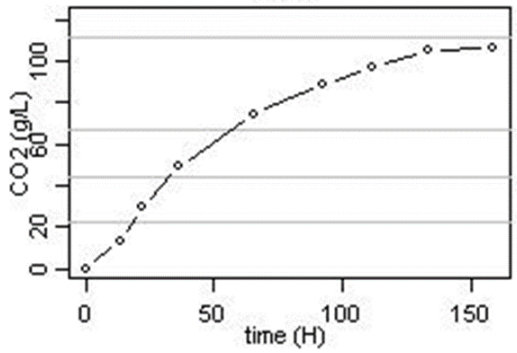
time (H)


CO2 (g/L)
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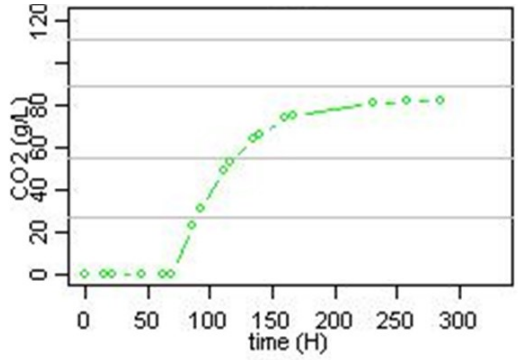
0
50
100
150
time (H)

I2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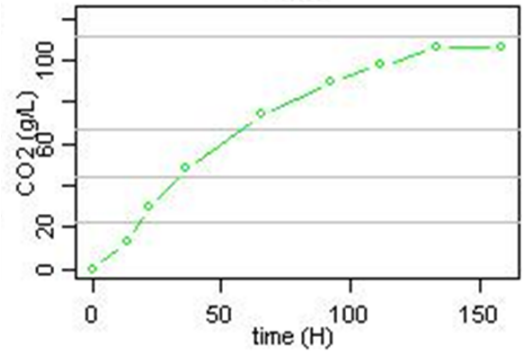
time (H)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50
100
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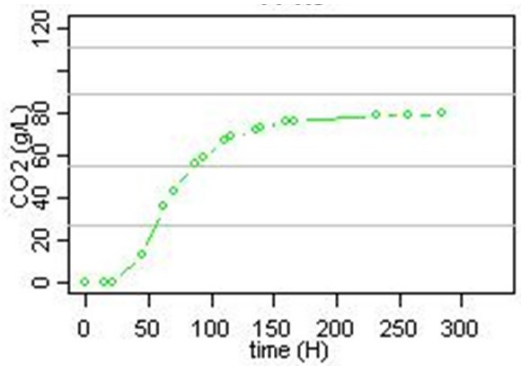
time (H)

H3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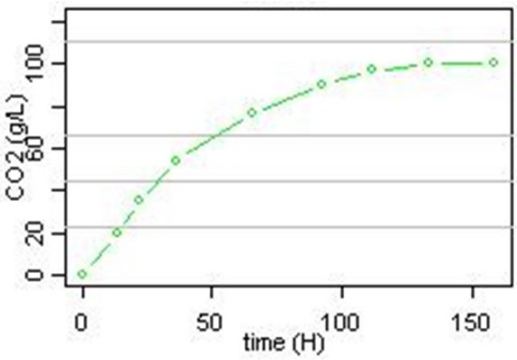
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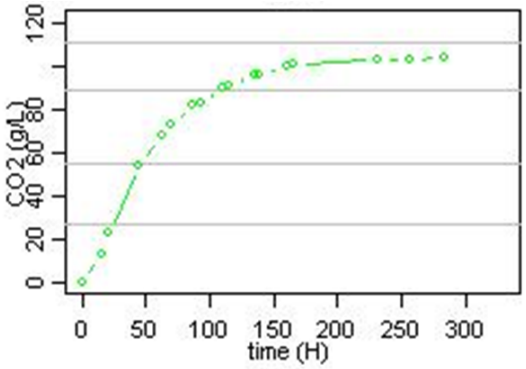
time (H)

I6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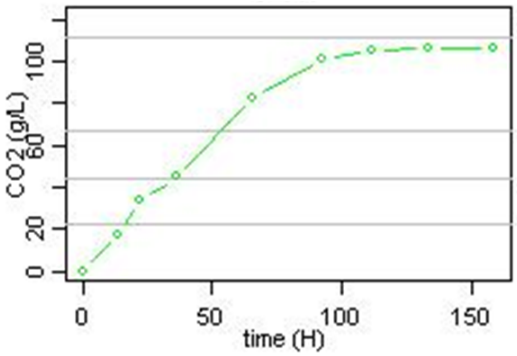
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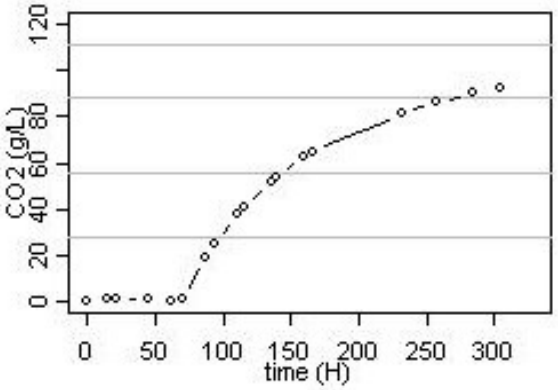
time (H)

F7


CO2 (g/L)
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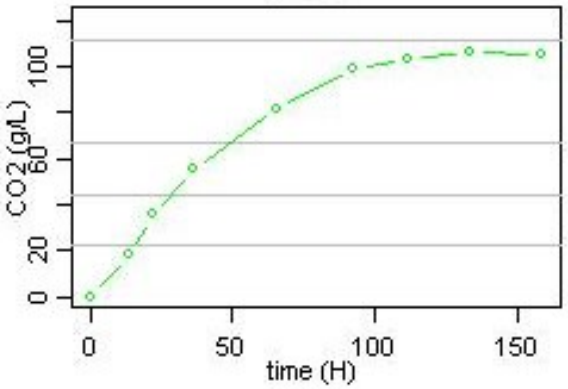
time (H)


CO2 (g/L)
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time (H)

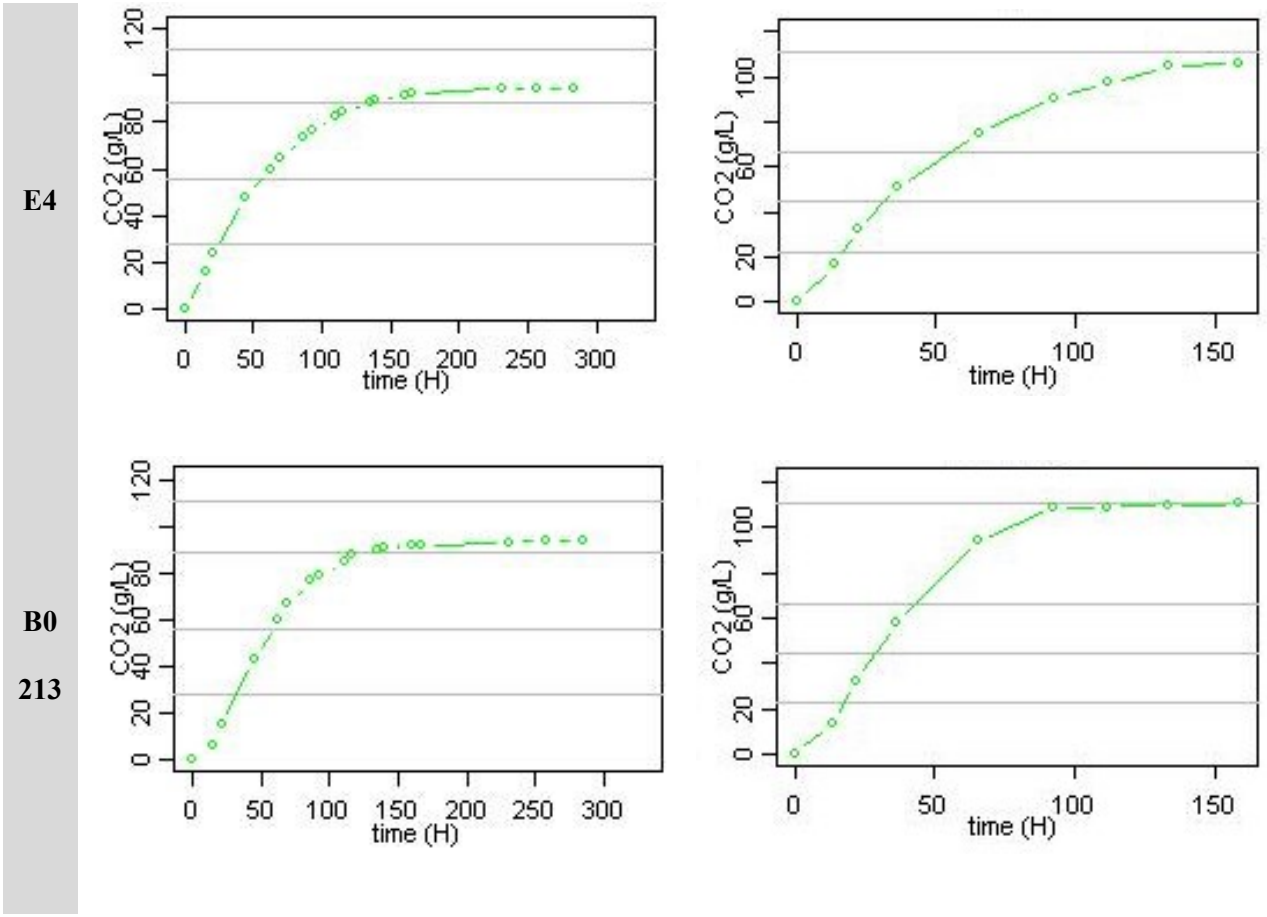
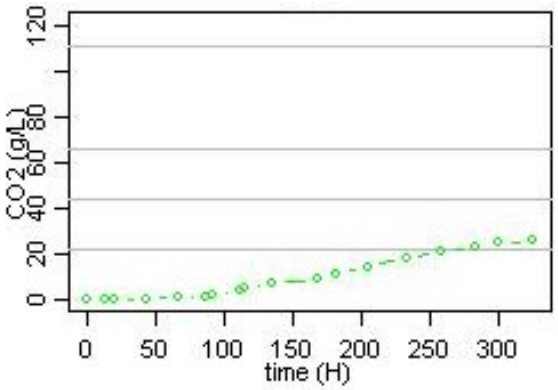
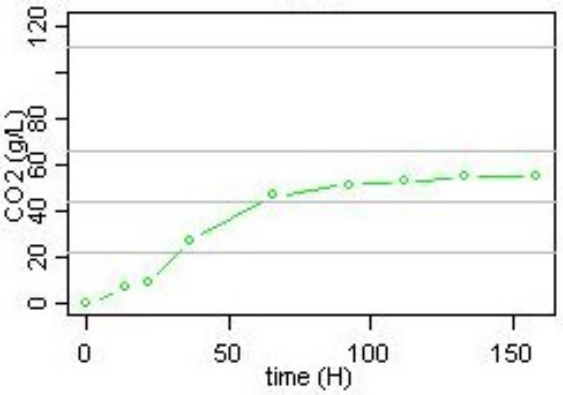
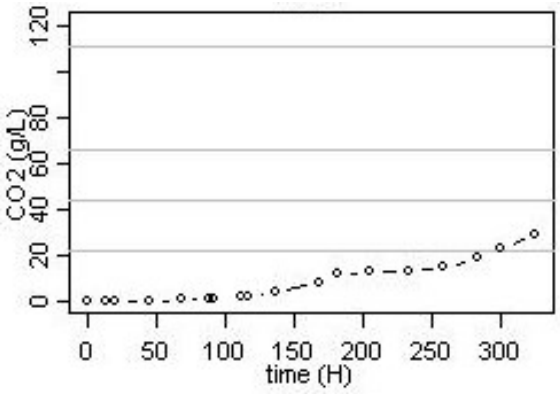
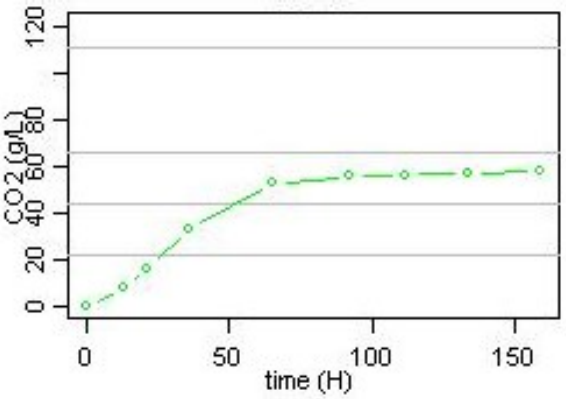
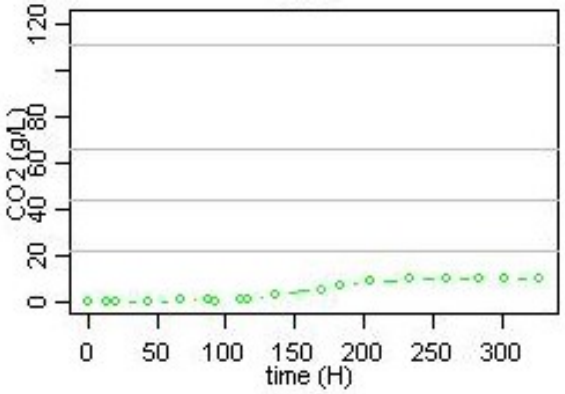
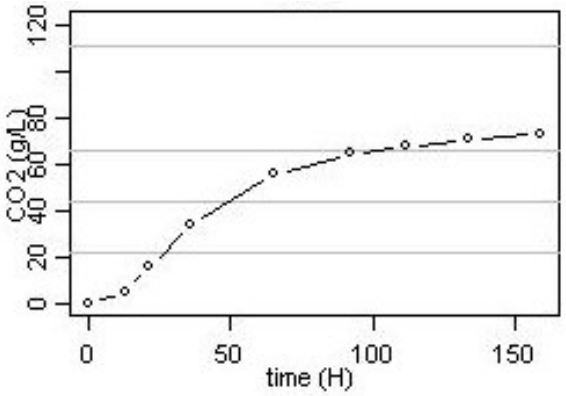
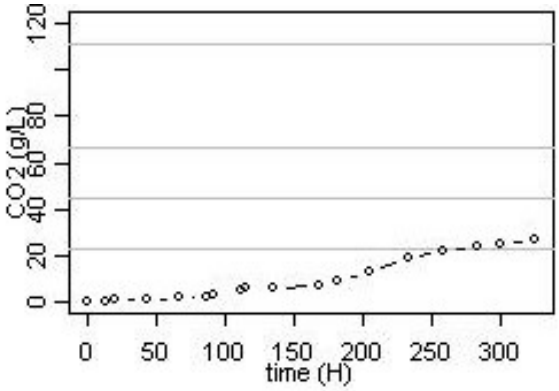


Figure 12. Fermentation kinetics in model synthetic medium and in must of 13 strains selected from Nero di Troia wines (04 061011 E 15 (G6), 04 12 061011B 4 (E3), 01 27 290911A 4 (G2), 03 12 061011C 7 (G3), 03 12 061011A 13 (F2), 04 12 061011D 6 (H5), 04 12 061011C 5 (G1), 04 12 061011B 17 (F8), 04 12 061011D 18 (I2), 01 12 290911A 14 (H3) , 03 12 061011 C 8 (I6), 03 12 061011A 7 (F7) and 04 12 061011A 1 (E4)) and a commercial strain ACTVIVEFLORE® B0213 (Laffort).

Respecting the percentages reported in **Figure 8**, we selected the two dominant non-*Saccharomyces* species *Candida zemplinina* and *Hanseniaspora guillermondii*. Ten strains, 7 *C. zemplinina* spp (04 12 061011A 5(1C), 01 12 290911A 3(2C), 01 12 290911B 12(6C), 02 12 290911A 8(7C), 01 27 290911 2(10C), 03 24 250811A 10(13C), 03 25 250811 13(3M) and 3 *H. guilliermondii* spp. (04 12 061011A 14(4H), 03 25 230811 3(5H) and 05 27 120911 9(D3)) were subjected to technological characterization (killer activity/sensitivity, H_2S production, fermentation kinetics in model synthetic medium and in must, cytofluorometric analysis, alcohol content, reducing sugar content, volatile acidity, glycerol content).

	model synthetic medium	must
1C		
2C		
6C		

7C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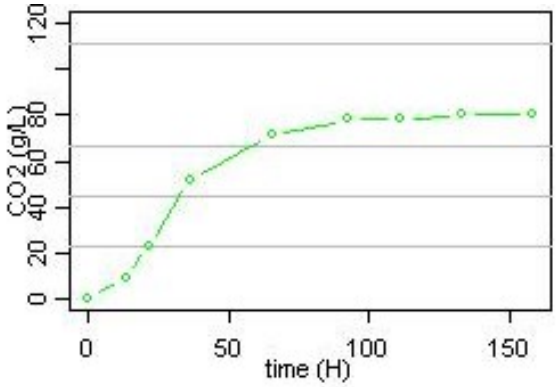
60
80
120
0
50
100
150
200
250
300
time (H)


CO2 (g/L)
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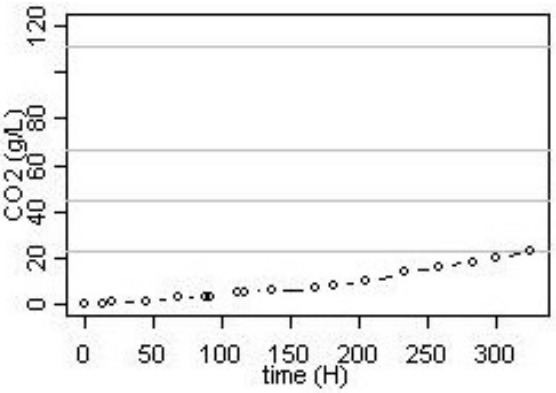
time (H)

10C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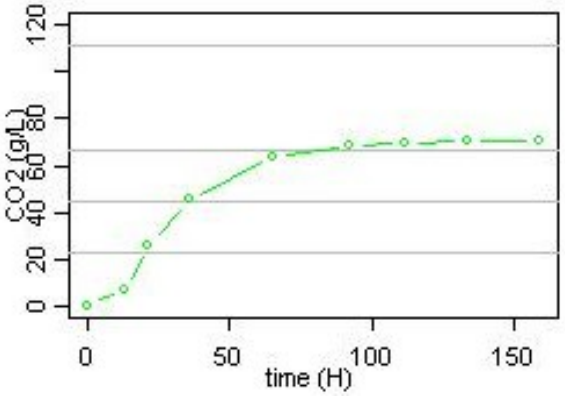
time (H)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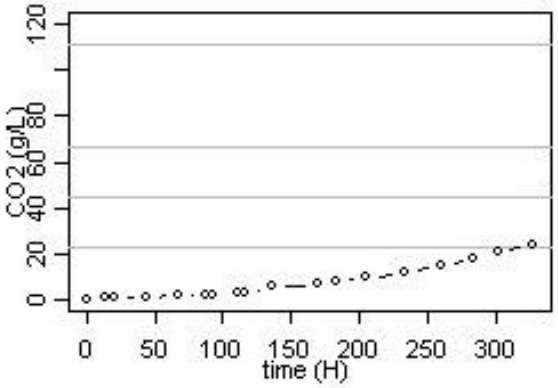
50
100
150
time (H)

13C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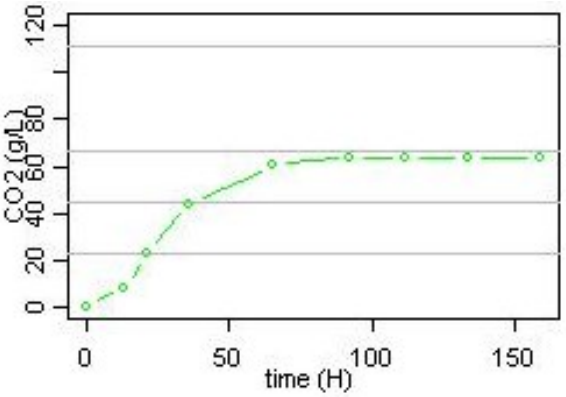
time (H)


CO2 (g/L)
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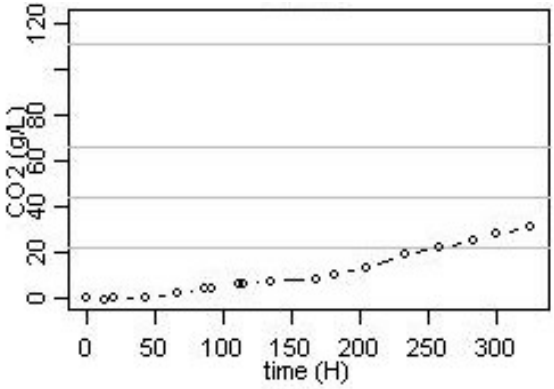
time (H)

3M


CO2 (g/L)
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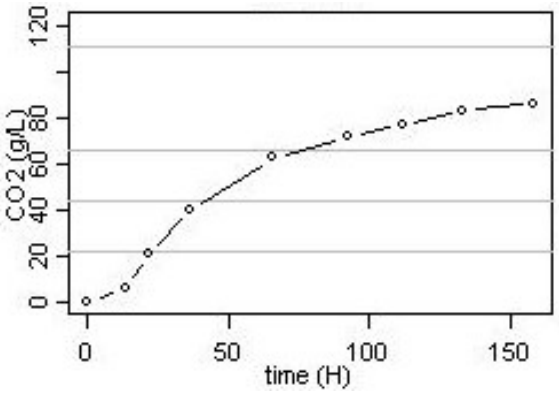
time (H)


CO2 (g/L)
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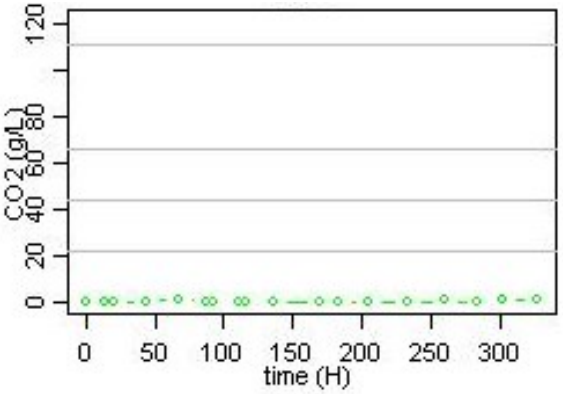
60
80
120
0
50
100
150
time (H)

4H


CO2 (g/L)
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200
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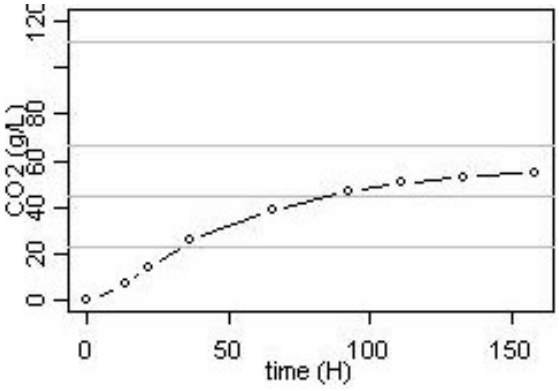
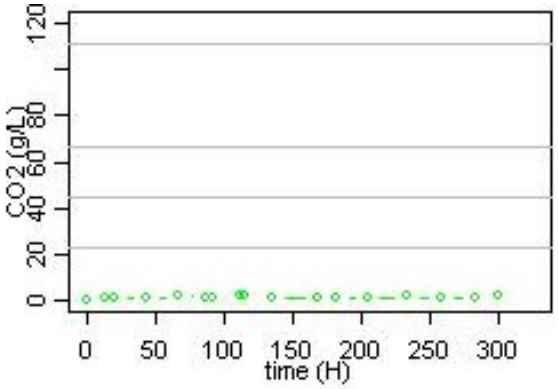
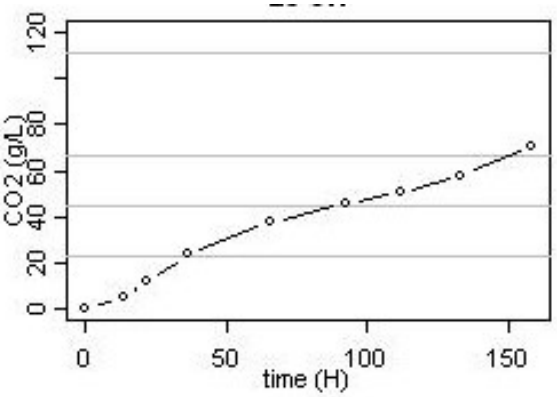
time (H)

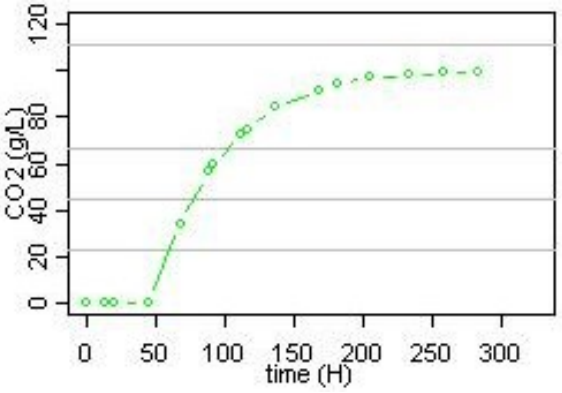

CO2 (g/L)
0
20
40
60
80
120
0
50
100
150
time (H)

5H


CO2 (g/L)
0
20
40
60
80
120
0
50
100
150
200
250
300
time (H)


CO2 (g/L)
0
20
40
60
80
120
0
50
100
150
time (H)

D3


CO2 (g/L)
0
20
40
60
80
120
0
50
100
150
200
250
300
time (H)

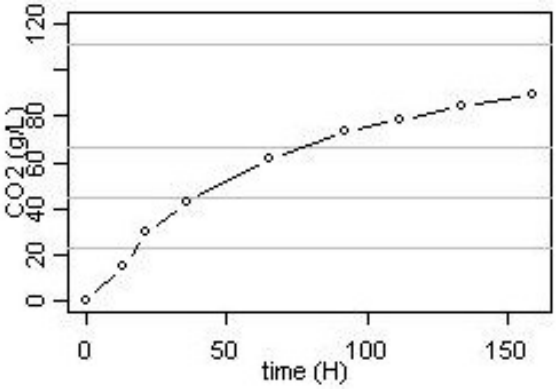

CO2 (g/L)
0
20
40
60
80
120
0
50
100
150
time (H)

Figure 13. Fermentation kinetics in synthetic medium and in must of non-*Saccharomyces* strains 04 12 061011A 5(1C), 01 12 290911A 3(2C), 01 12 290911B 12(6C), 02 12 290911A 8(7C), 01 27 290911 2(10C), 03 24 250811A 10(13C), 03 25 250811 13(3M), 04 12 061011A 14(4H), 03 25 230811 3(5H) and 05 27 120911 9(D3).

On the basis of fermentation profiles (**Fig.s 13, 14B, 14C**), complemented with the results of killer activity/sensitivity, H_2S production, and population dynamics (data not shown), we selected the most performant non-*Saccharomyces* strains: 7C (*C. zemplinina*) and D3 (*H. guillermondii*).

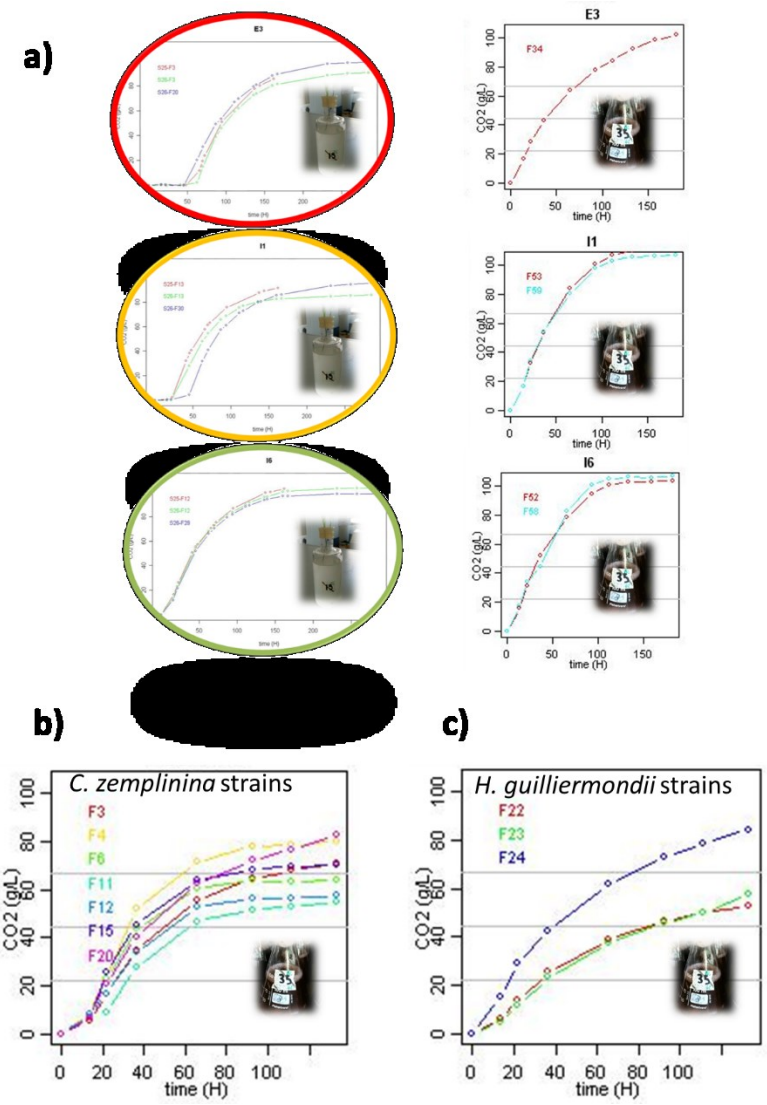
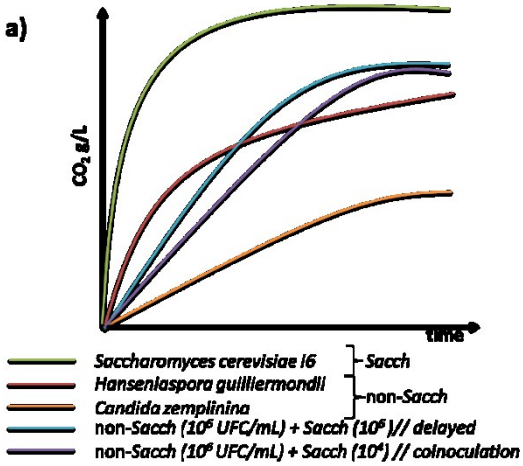


Figure 14. a) Comparison of selected *S. cerevisiae* fermentation profiles in synthetic medium and in must; b) comparison *C. zemplinina* fermentation profiles in must (F3=7C, F4=2C, F6=13C, F11=3M, F12=10C, F16=6C, F20=1C); c) comparison *H. guillermondii* fermentation profiles in must (F22=D3, F23=4H, F24=5H).

Comparing the fermentation profiles in synthetic medium and in must, we highlighted differences between *S. cerevisiae* and non-*Saccharomyces* behaviors. In the case of *S. cerevisiae* strains, the synthetic medium was useful to better highpoint the differences in the fermentation performances (we distinguished in short latency strains [circled in green], average latency strains [circled in yellow], and long latency strains [circled in red]), while these differences in must were not detected (Figure 13 and **Figure 14a**). On the contrary, in the case of non-*Saccharomyces*, the synthetic medium was ineffective (a phenomenon probably due to the fact that the formulation of the synthetic medium is designed for the nutritional requirements of *S. cerevisiae*), while the fermentation profiles in grape must showed differences useful to distinguish strains (Figure 13, Figure 14b, Figure 14c).



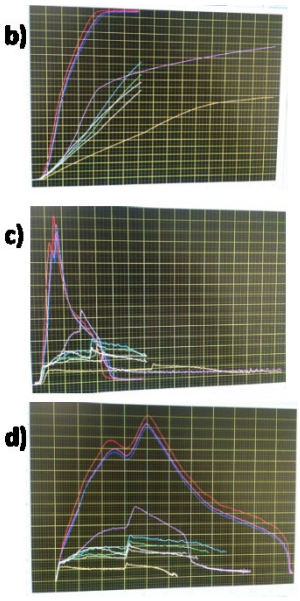


Figure 15. Glass 1.2-L fermenters used for final part of technological characterization. a) Schematic representation of carbon dioxide release as a function of time during fermentations conducted using single strains and multi-strains (delayed and coinoculation); a) carbon dioxide release as a function of time (each square corresponds to 20 hours) during fermentations conducted using single strains and multi-strains (delayed and coinoculation); b) velocity of fermentation as a function of time during fermentations conducted using single strains and multi-strains (delayed and coinoculation); c) velocity of fermentation as a function of carbon dioxide release during fermentations conducted using single strains and multi-strains (delayed and coinoculation).

Using glass 1.2-L fermentors (**Figure 15**), we assessed the performance of possible multi-strains formulations using the selected *S. cerevisiae*/non-*Saccharomyces* strains. In particular, we used two classical strategies of inoculum planned to promote the non-*Saccharomyces* 'expression': a 'delayed' strategy with same cell concentration delayed in the time (10^6 UFC/mL non-*Saccharomyces* strains; after one day, 10^6 UFC/mL *S. cerevisiae* strain), and a 'coinoculation' strategy with different cell concentration inoculated at the same time (10^6 UFC/mL non-*Saccharomyces* strains, 10^4 UFC/mL *S. cerevisiae* strain). In all the cases, a strong competition took place with the non-*Saccharomyces* strains, compromising an efficient alcoholic fermentation. An effective development of the *S. cerevisiae* strains was only reached when the concentration of the non-*Saccharomyces* strains in the co-inoculation approaches was strongly reduced (data not shown). Our observations suggest the possible presence of "robust" competitors between non-*Saccharomyces* strains of oenological importance that may negatively affect wine fermentation.

4.7 Isolation of Lactic Acid Bacteria from spontaneous MLF wines

LAB from different wine undergoing spontaneous MLF were isolated by plating wine onto MRS (pH 5.5) And FT80 plates, about 300 strains were randomly isolated.

Usually, during the initial phases of winemaking (the must phase and onset of alcoholic fermentation) LAB populations ranges from 10^3 to 10^4 CFU/mL. LAB population consist mainly in homofermentative species. The most abundant species are *Lactobacillus plantarum*, *Lactobacillus*

casei, *Lactobacillus hilgardii*, *Leuconostoc mesenteroides* and *Pediococcus damnosus*, while less common are *O. oeni* and *Lactobacillus brevis*.

During AF the bacterial population decreases to between approximately 10^2 and 10^3 CFU/ml, once AF is complete, there is a lag phase (about 10-15 d) during which the population of LAB remains unchanged as their growth is inhibited by the presence of live yeasts and inhibitory substances secreted by these, then during malolactic fermentation, the concentration of lactic acid bacteria reaches approximately 10^6-10^7CFU/ml.

In **Tab.30** are reported the LAB population isolated from several wine undergoing spontaneous MLF, LAB population of wines analyzed ranges from 10^5 to 10^7 CFU/ml. Our results confirmed result previously reported in literature for wine during MLF.

100 putative *O. oeni* strains (gram-positive, catalase-negative and coccoid-shaped) were stored for further analysis. Is important underline that the composition of the bacterial population changes during winemaking and strains that are better equipped to resist the wine hostile environment are gradually selected.

The first species to disappear are homofermentative LAB, followed by their heterofermentative counterparts and *Pediococcus* species. The dominant species at the end of alcoholic fermentation is *O. oeni*.

Code	CFU/ml	N° isolate	Sample	Cultivar
V 03 12 111111A	4,27E+06	15	MLF wine	Uva di Troia
V 03 12 111111B	1,10E+07	15	MLF wine	Uva di Troia
V 03 12 111111C	1,56E+06	15	MLF wine	Uva di Troia
V 03 12 111111D	1,27E+06	15	MLF wine	Uva di Troia
V 01 12 211111	6,75E+05	15	MLF wine	Uva di Troia
V 01 30 211111	2,42E+06	15	MLF wine	San Severo Rosso DOC
V 01 31 211111	5,25E+06	15	MLF wine	Cabernet
V 01 27 211111	4,65E+05	15	MLF wine	Montepulciano
V 01 04 021211A	5,32E+06	15	MLF wine	Bombino Bianco
V 01 04 021211B	3,77E+06	15	MLF wine	Bombino Bianco
V 01 12 021211A	3,18E+04	15	MLF wine	Uva di Troia
V 01 12 021211B	1,27E+06	15	MLF wine	Uva di Troia
V 05 32 141211	2,19E+06	15	MLF wine	Cacc'e mmitte
V 05 12 141211A	1,99E+06	15	MLF wine	Uva di Troia

V 05 33 141211	1,62E+07	15	MLF wine	Syrah
V 05 33 270112	2,76E+06	15	MLF wine	Syrah
V 05 12 270112 B	3,12E+05	15	MLF wine	Uva di Troia
V 05 32 270112	5,30E+06	15	MLF wine	Cacc'e mmitte
V 01 26 120312	9,92E+05	15	MLF wine	Trebbiano
V 01 25 120312A	6,92E+05	15	MLF wine	Merlot
V 01 25 120312B	6,36E+05	15	MLF wine	Merlot
V 01 25 130412A	2,00E+04	15	MLF wine	Merlot
V 01 25 130412B	2,39E+05	15	MLF wine	Merlot
V 01 25 130412C	2,66E+05	15	MLF wine	Merlot

Table 30. LAB population isolated from grape wine undergoing spontaneous MLF

4.8 Molecular identification and characterization of wine Lactic Acid Bacteria

4.8.1 *Oenococcus oeni* specie-specific PCR

The identification of putative *O. oeni* strains was performed by two species-specific PCR, the first PCR of the malolactic enzyme (MLE), the 2nd PCR specie-specific amplified the internal transcribed region of *O. oeni* strains. The size of specific PCR products are respectively 1000 and 125 bp.

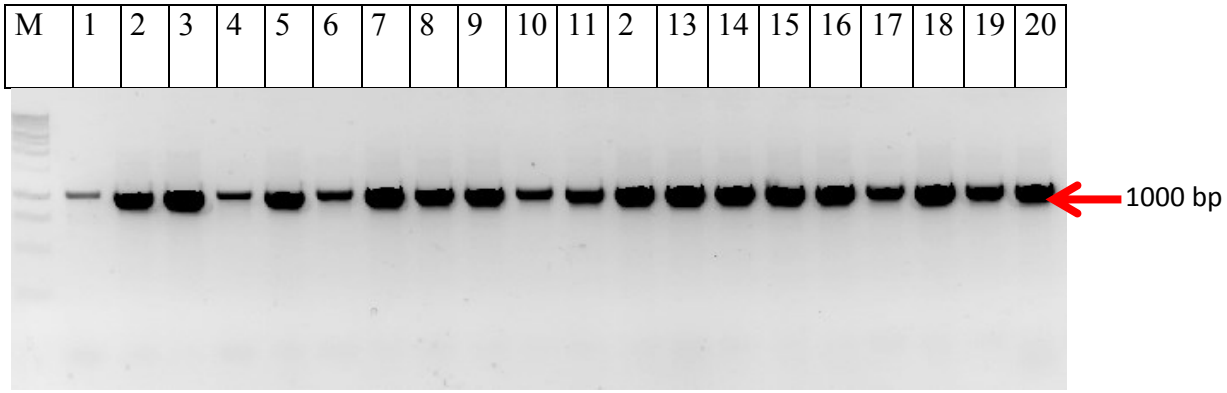


Figure 16. Species-specific amplification of DNA extracted from *O. oeni* strains and amplified with primers On1-On2 (Malolactic enzyme MLE). M, marker 1kb Promega. Line 1-20, putative strains of *O. oeni*.

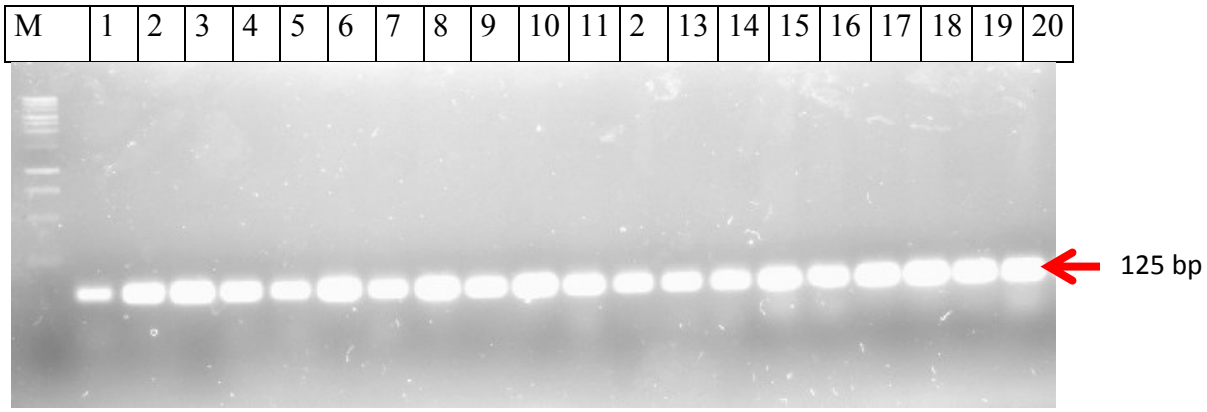


Figure 17. Species-specific amplification of DNA extracted from *O. oeni* strains and amplified with primers Oo_smISRf and Oo_smISRr (ITS region). M, marker 1kb Promega. Line 1-20, putative strains of *O. oeni*.

In **Fig.s 16** and **17** is reported an example of the specific fragment of amplification obtained for *O. oeni* strains, respectively the amplification of malolactic enzyme and the internal transcribed region of *O. oeni*, 50 strains, that shown the typical fragment of amplification, were identified as *O. oeni*.

4.8.2 Variable number of tandem repeat (VNTR) analysis

Variable number of tandem repeat (VNTR) analysis is a PCR-based method that can be used to discriminate between different strains of a bacterial species and can therefore infer genetic relationships between them.

This approach, based on the presence of a variable number of tandem repeats (TR) at a specific locus in the genome of a microorganism, was applied for the first time to the *O. oeni* species by Claisse & Lonvaud-Funel (2012).

The VNTR method is highly discriminating and easy to interpret. It facilitates the rapid and reliable typing of *O. oeni* strains, using only five tandem repeat regions (designated TR1 through TR5). All five of the TR regions are located within ORFs encoding surface anchored cell wall proteins.

Claisse & Lonvaud-Funel (2012) showed that VNTR has a more discriminatory power, than both PFGE and MLST and allowed to distinguish a total of 201 different VNTR types from 236 strains analyzed.

In **Table 31** the TR loci characteristics of the 50 strains analyzed are reported. For the 50 *O. oeni* strains analyzed TR-containing amplicons were produced for all of the strains at all of the loci, with the exceptions of TR2 for two strains and TR3 for three strain, in literature has been reported the amplification of TR loci for all of the strains tested, with the exceptions of TR2 for 11 strains and TR4 for one strain. In our strains for five TR loci, the number of repeats varied from 15 to 42 for TR1, 3 to 12 for TR2, 3 to 5 for TR3, and 1 to 3 for TR4 and 1 to 4 TR5, while for the 236 strains analyzed by Claisse & Lonvaud-Funel (2012) the number of repeats for the five TR loci varied from 6 to 53 for TR1, 2 to 14 for TR2, 1 to 6 for TR3, and 2 to 4 for TR4 and TR5.

Locus	Number of different allele	Allele distribution	Dominant allele and (strain frequency %)	Simpson's index of diversity
TR1	42	15-42	41-(44.68)	0.969
TR2	12	3-12	11-(76.59)	0.830
TR3	5	3-5	4- (85.10)	0.578
TR4	3	1-3	2- (59.57)	0.554
TR5	4	1-4	2-(57.44)	0.638

Table 31. Characteristics of TR loci used for VNTR analysis of the *O. oeni* strains isolated.

The deduced allele numbers were 42, 12, 5, 3 and 4 alleles for TR1, TR2, TR3, TR4 and TR5, respectively. Similar results were reported also in literature, in fact Claisse & Lonvaud-Funel (2012) affirmed that the allele numbers of the strains analyzed were 43, 14, 6, 4 and 3 alleles for TR1, TR2, TR3, TR4 and TR5, respectively. In our samples unique alleles were observed only for all the loci except TR3 and TR4 and some alleles were frequently found: for example, allele 11 from TR2, allele 4 from TR3, allele 2 from TR4 and TR5 were found in more than half of the

strains tested. Farther in literature were observed unique alleles for all of the loci except for TR5 and some of the alleles were more frequently found: for example, allele 4 from TR3 and allele 3 from TR4 were found in more than half of the strains tested.

When analyzed separately, TR1 showed the highest discriminatory power, followed by TR2, TR5, TR3 and TR4, these results confirmed those reported by Claisse & Lonvaud-Funel (2012), that applied for the first time this methods to typing *O. oeni* species.

In **Table 32**, the number of repeats for loci TR1, TR2, TR3, TR4 and TR5 obtained for the 50 strains analyzed are reported. Strains V0532141211 1, V0532141211 16 and OT25 were eliminated from the VNTR analysis because failed to give amplification for loci TR2 and TR3.

Strains	**TR 1**	**TR2**	**TR3**	**TR4**	**TR5**	**Strains**	**TR 1**	**TR2**	**TR3**	**TR4**	**TR5**
UniFG3	15	10	3	2	3	**UniFG 28**		10	4	2	3
UniFG 4	15	8	4	2	1	**UniFG 29**	41	10	4	2	3
UniFG 5	16	11	4	3	3	**UniFG 30**	41	11	4	2	2
UniFG 6	16	11	4	3	2	**UniFG 31**	41	11	4	2	2
UniFG 7	16	11	4	3	3	**UniFG 32**	41	11	4	2	2
UniFG 8	17	11	4	3	3	**UniFG 33**	41	11	4	2	2
UniFG 9	17	11	4	3	3	**UniFG 34**	41	7	3	2	2
UniFG 11	19	12	5	3	2	**UniFG 35**	41	11	4	2	3
UniFG 12	20	11	5	1	2	**UniFG 36**	41	11	4	3	2
UniFG 13	21	8	5	2	2	**UniFG 37**	41	11	4	3	2
UniFG 14	21	8	4	1	4	**UniFG 38**	41	11	4	1	2
UniFG 15	21	11	5	2	1	**UniFG 39**	41	11	4	1	1
UniFG 16	22	8	5	2	2	**UniFG 40**	41	11	4	1	1
UniFG 17	23	11	4	2	3	**UniFG 41**	41	11	4	1	2
UniFG 18	28	11	4	3	3	**UniFG 42**	41	11	4	1	2
UniFG 19	28	11	4	2	2	**UniFG 43**	41	11	4	2	2

UniFG 20	28	11	4	2	2	**UniFG 44**	41	11	4	2	2
UniFG 21	28	11	4	2	2	**UniFG 45**	41	11	4	2	2
UniFG 22	30	3	4	1	2	**UniFG 46**	41	11	4	2	2
UniFG 23	37	11	4	3	3	**UniFG 47**	41	11	4	2	2
UniFG 24	37	11	4	2	2	**UniFG 48**	42	11	4	2	2
UniFG 25	37	11	4	3	3	**UniFG 49**	42	11	4	2	3
UniFG 26	40	12	4	2	2	**UniFG 50**	42	11	4	2	3
UniFG 27	41	11	4	2	3						

Table 32. Number of repeats for loci TR1, TR2, TR3, TR4 and TR5 for the *O. oeni* strains analyzed

When each locus was used in descending order with respect to its discriminatory power, it led respectively to 3, 3, 4, 6 and 17 VNTR profiles. Finally, when all five TR regions were combined together, the 50 strains were assigned to 30 VNTR profiles (see Table 32).

4.8.3 Multi locus sequence typing (MLST) analysis

The 20 isolates were also submitted to MLST typing technique. Seven housekeeping genes *rpoB*, *purK*, *g6pd*, *pgm*, *dnaE*, *gyrB* and *recP*, were targeted in this MLST analysis (see **Tab. 11**).

The MLST scheme used in this study was those reported by Bridier *et al*. (2010), that suggested that better results can be obtained by increasing the number of housekeeping genes. However, they shown that there is a point where it is not worth studying more loci because results don't become more discriminating. Therefore, genes and primers that provide more information about *O. oeni* typing were chosen.

A consensus dendrogram was generated using the obtained sequences, isolates with 100% similarity level were assigned the same ST and eight STs were identified. The STs were separated in two main branches at 98% similarity level (Fig. 18). Branch A included most of the typed strains, eighteen isolates represented by six STs, and branch B was formed by two strains represented by two STs.

In **Fig. 18** are also reported the polymorphic sites of each genes analyzed. Mutation point are 311 and 553 for gene *dnaE,* 445 and 462 for *g6pD*, 224, 440 and 541 for *gyrB*, 184 and 209 for *purK* and 72, 150, 233, 268 and 381 for *rpoB*. No mutation point are present in all samples analyzed for gene recP.

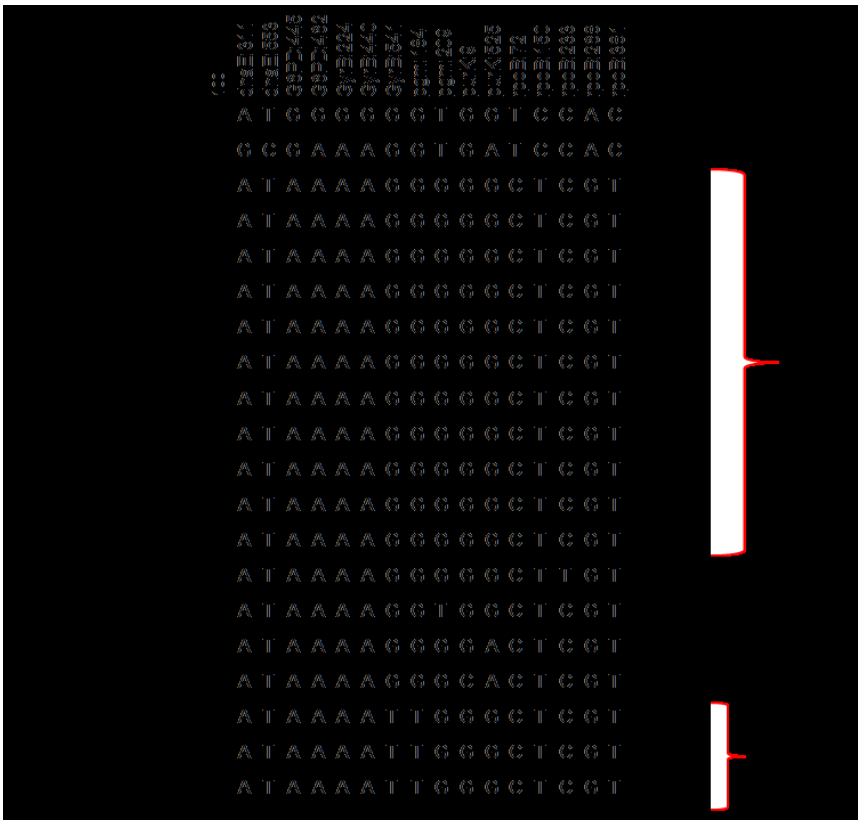


Figure 18. Consensus dendrogram obtained by combining sequences of the seven housekeeping genes (*rpoB*, *purK*, *pgm*, *g6pd*, *dnaE*, *gyrB* and *recP*) amplified from the 20 *O. oeni* strains genomes.

In **Table 33** are reported data obtained for each gene used. The seven housekeeping genes allowed to determine from one to three alleles. *RecP* was the gene that showed less alleles, while the other genes analyzed showed more alleles, three and two different alleles respectively for the genes *g6pD*, *gyrB*, *pgm*, *purK*, *rpoB* and *dnaE*.

Among the eight ST identified the most widespread was ST 4 that included 11 isolates with identical allelic profiles obtained from different wineries. STs 1, 3, 5, 6, 7, 8 are unique allelic

profiles that included one isolate for each ST. ST 2 included three strains that shown the same allelic profiles, isolated from the same winery.

The comparison of the sequences from this study with the ones already deposited to date in the GenBank database revealed two new alleles that had never been described before and that were represented by Roman numerals, these alleles are allele I of *purK* and allele I of *rpoB* (**Table 32**), while the alleles already deposited in GenBank were named with the numbers that other authors had previously established.

Strain	**Alleles**							**Genotypes MLST**
	dnaE	*g6pD*	*gyrB*	*pgm*	*purK*	*recP*	*rpoB*	
UniFG2	2	1	10	4	2	11	1	ST 1
UniFG 4	12	3	2	2	12	11	5	ST 2
UniFG 6	12	3	2	2	12	11	5	ST 2
UniFG 8	12	3	2	2	12	11	5	ST 2
UniFG 9	12	3	10	5	2	11	5	ST 3
UniFG 12	12	3	10	5	12	11	5	ST 4
UniFg 14	12	3	10	5	12	11	**I**	ST 5
UniFG 15	12	3	10	5	12	11	5	ST 4
UniFG 16	12	3	10	5	12	11	5	ST 4
UniFG 17	12	3	10	5	12	11	5	ST 4
UniFG 21	12	3	10	5	12	11	5	ST 4
UniFG23	12	3	10	4	12	11	5	ST 6
UniFG 24	12	4	5	4	12	11	1	ST 7
UniFG 25	12	3	10	5	12	11	5	ST 4
UniFG 28	12	3	10	5	12	11	5	ST 4
UniFG 31	12	3	10	5	12	11	5	ST 4
UniFG 36	12	3	10	5	12	11	5	ST 4
UniFG 38	12	3	10	5	12	11	5	ST 4
UniFG 46	12	3	10	5	12	11	5	ST 4
UniFG 48	12	3	10	5	**I**	11	5	ST 8
Total number[a]	2	3	3	3	3	1	3	8

Table 33. Typing data of the 20 *O. oeni* isolates analyzed in this study. Alleles in numerical character were described by Bilhère *et al*. (2009) and alleles in bold Roman numbers were not previously described.
[a] Total number of differentiated alleles of genotypes.

Every targeted gene showed its polymorphic condition so that the strategy for typing with the MLST scheme used in this study gave a suitable result.

Cluster A included most of the typed strains, it shown six different STs, two STs, named ST 2 and ST 4 shown the same allelic profiles described as ST 70 and ST 52 by Bridier *et al*. (2010), while STs 3, 5, 6 and 8 shown new allelic profiles. ST 5 and ST 8 shared new mutations, respectively in the polymorphic *rpoB* and *purK* genes. Some studies have described similar results for strains that underwent similar conditions as a result of adaptation to a new or different niche (Bilhère et al., 2009; Bridier et al., 2010). Whereas cluster B included only two strains, ST 1 and ST 7, that shown both new allelic profiles (see **Fig.18**).

4.9 Microvinification assays

50 *O. oeni* strains were studied, focusing attention on their malolactic performances and their interaction with autochthonous *Saccharomices cerevisiae* strains. Preliminary microvinification assays were performed in grape must of "Nero di Troia", fermentation was carried out on magnetic stirrers at 25 °C for 45 days.

To induce simultaneous AF/MLF, bacteria were co-inoculated with yeast, while to induce sequential AF/MLF, bacteria were inoculated at the end of alcoholic fermentation. Bacteria were inoculated, in both, sequential or co-inoculation approaches, to a final concentration of $2x10^6$ CFU/ml.

MLF was monitored by measuring the consumption of malic acid and the production of lactic acid, through the use of enzymatic kit for L-lactic and L-malic acid (BioGamma). Samples were analyzed at 0, 6, 10, 16, 22, 29 and 41 days after the beginning of AF.

This approach allowed to investigate the malolactic performances of the *O. oeni* strains co-inoculated.

Different results related to the efficiency of MLF were observed when different association of yeast and bacteria were analyzed. For instance, some *O. oeni* strains performed an improved MFL when

associated with *S. cerevisiae* I6, while an improved MFL was observed when others *O. oeni* strains were associated with *S. cerevisiae* E4.

O. oeni strains were classified for their malolactic performances. Generally, simultaneous inoculation allows to reach a quick, complete and efficient MLF, while for sequential inoculation the behavior in terms of efficiency of MLF was quite different for each strain. Indeed, for some strains, the complete degradation of malic acid failed, while other strains complete MLF in about 35-40 days.

In **Table 34**. is reported the characteristic of wine obtained with *S. cerevisiae* E4 and 50 different *O. oeni* strains, time required to complete MLF (L-malic acid concentration below 0.5 g/L), malic and lactic acid concentration (g/L) after MLF in Nero di Troia must.

Malolactic performances of *O. oeni* strains investigated were quite different depending on both *S. cerevisiae* strain and time of inoculum (simultaneous or sequential).

The major part of the *O. oeni* strains co-inoculated with *S. cerevisiae* E4 weren't able to completely degrade malic acid (see Table 34.), in fact malic acid residual concentration is high (about 1.5 g/L), a value much higher than those generally accepted for a complete degradation of malic acid (0.5 g/L).

O. oeni strains that has been able to complete MLF when associated with *S. cerevisiae* E4 need

O. oeni strain	Inoculation Time	Malic acid (g/L)	Lactic acid (g/L)	MLF time after LAB inoculation (days)	*O. oeni* strain	Inoculation Time	Malic acid (g/L)	Lactic acid (g/L)	MLF time after LAB inoculation (days)
UniFG 1	SEQ	1.51	0.76	NR	**UniFG 26**	SEQ	1.51	0.70	NR
	SIM	1.24	0.89	NR		SIM	0.20	0.87	30
UniFG 2	SEQ	1.55	1.63	NR	**UniFG 27**	SEQ	1.50	0.70	NR
	SIM	1.30	1.00	NR		SIM	1.46	0.76	NR
UniFG 3	SEQ	1.45	0.87	NR	**UniFG 28**	SEQ	2.05	0.81	NR
	SIM	1.52	0.90	NR		SIM	0.44	2.33	40
UniFG 4	SEQ	1.51	0.79	NR	**UniFG 29**	SEQ	1.63	1.70	NR
	SIM	0.52	1.91	40		SIM	1.11	1.50	NR
UniFG 5	SEQ	1.48	0.51	NR	**UniFG 30**	SEQ	1.56	1.63	NR
	SIM	1.45	0.86	NR		SIM	0.98	1.29	NR
UniFG 6	SEQ	1.72	0.78	NR	**UniFG 31**	SEQ	1.54	0.83	NR
	SIM	1.32	0.95	NR		SIM	0.52	2.79	30
UniFG 7	SEQ	1.54	0.61	NR	**UniFG 32**	SEQ	1.54	0.83	NR
	SIM	0.69	1.56	40		SIM	0.64	2.26	40
UniFG 8	SEQ	1.54	1.02	NR	**UniFG 33**	SEQ	1.60	0.78	NR
	SIM	1.19	1.07	NR		SIM	1.54	0.82	NR
UniFG 9	SEQ	1.54	1.02	NR	**UniFG 34**	SEQ	1.04	1.09	NR
	SIM	0.13	2.70	20		SIM	0.25	0.78	NR
UniFG 10	SEQ	1.66	0.96	NR	**UniFG 35**	SEQ	0.92	0.84	NR
	SIM	1.53	0.75	NR		SIM	0.18	2.06	30
UniFG 11	SEQ	1.59	0.80	NR	**UniFG 36**	SEQ	0.10	0.75	14 (TOT 30)
	SIM	1.56	0.76	NR		SIM	0.26	1.76	30
UniFG 12	SEQ	1.60	0.80	NR	**UniFG 37**	SEQ	0.19	1.20	24 (TOT 40)
	SIM	0.80	1.32	40		SIM	0.26	1.94	25
UniFG 13	SEQ	1.65	1.02	NR	**UniFG 38**	SEQ	1.66	0.96	NR
	SIM	1.05	0.97	NR		SIM	1.31	0.91	NR

UniFG 14	SEQ	1.12	1.17	NR	**UniFG 39**	SEQ	1.66	0.87	NR
	SIM	0.13	2.50	20		SIM	1.34	0.97	NR
UniFG 15	SEQ	1.70	0.54	NR	**UniFG 40**	SEQ	1.55	0.80	NR
	SIM	1.17	1.43	NR		SIM	0.28	2.72	30
UniFG 16	SEQ	1.25	1.31	NR	**UniFG 41**	SEQ	1.54	0.83	NR
	SIM	1.29	1.20	NR		SIM	1.41	0.99	NR
UniFG 17	SEQ	1.63	0.86	NR	**UniFG 42**	SEQ	0.78	0.82	NR
	SIM	1.22	0.79	NR		SIM	1.51	0.61	NR
UniFG 18	SEQ	1.21	0.80	NR	**UniFG 43**	SEQ	1.52	1.69	NR
	SIM	1.48	0.77	NR		SIM	0.22	2.54	30
UniFG 19	SEQ	1.55	0.62	NR	**UniFG 44**	SEQ	1.45	2.03	NR
	SIM	1.41	0.83	NR		SIM	0.15	2.56	40
UniFG 20	SEQ	0.25	1.69	14 (TOT 30)	**UniFG 45**	SEQ	0.31	0.80	24 (TOT 40)
	SIM	0.13	2.48	30		SIM	0.16	2.49	20
UniFG 21	SEQ	1.54	0.83	NR	**UniFG 46**	SEQ	0.16	1.21	NR
	SIM	0.21	2.19	40		SIM	1.01	1.54	NR
UniFG 22	SEQ	0.15	2.06	14 (TOT 30)	**UniFG 47**	SEQ	0.18	1.02	24 (TOT 40)
	SIM	0.11	2.34	10		SIM	0.15	2.92	30
UniFG 23	SEQ	1.51	0.76	NR	**UniFG 48**	SEQ	1.28	1.34	NR
	SIM	1.11	0.97	NR		SIM	1.53	0.91	NR
UniFG 24	SEQ	1.52	0.75	NR	**UniFG 49**	SEQ	1.49	0.93	NR
	SIM	1.31	1.53	NR		SIM	1.52	0.76	NR
UniFG 25	SEQ	1.51	0.75	NR	**UniFG 50**	SEQ	1.45	0.85	NR
	SIM	0.24	2.26	40		SIM	1.41	1.03	NR

Table 34. Time required to complete MLF (L-malic acid concentration below 0,5 g/L) and average of chemical analysis data recorded after MLF in Nero di Troia must fermented with ***S. cerevisiae*** **E4** and with 50 *O. oeni* strains (**UniFG1-50**), comparing two inoculation times (SIM: simultaneous, SEQ: sequential). NR: Not reached.

In **Fig. 19** degradation of L-malic acid and production of L-lactic acid for an exemplificative associations yeast (*S. cerevisiae* E4)-bacteria, where has not been a complete degradation of malic acid, is reported. *O. oeni* strain is not able to completely degrade malic acid, both when used in simultaneous or sequential inoculation. In fact residual concentration of malic acid is about 1.5 g/L, in addition also the amount of lactic acid produced is low, about 0.8 g/L.

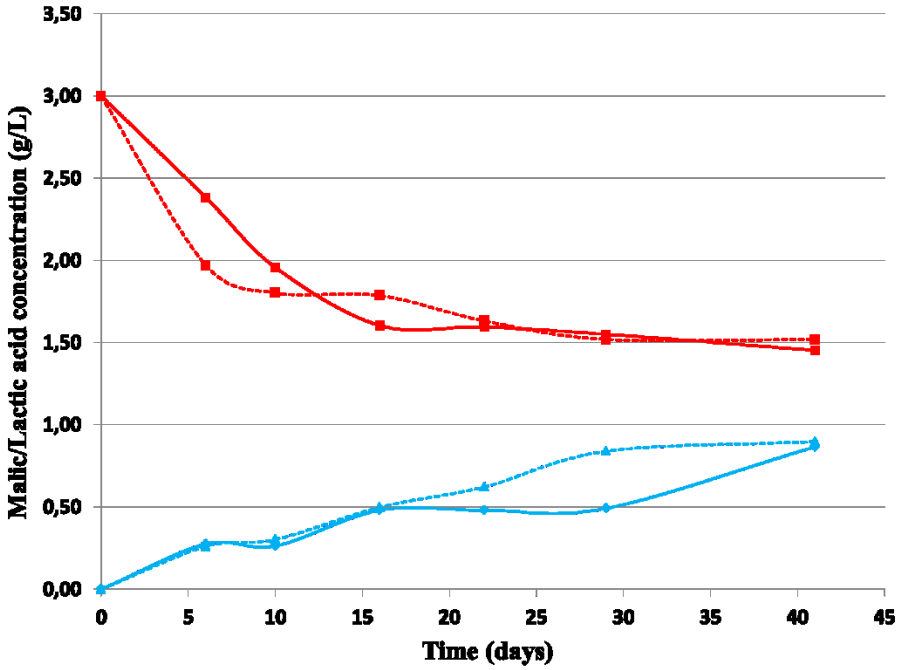


Figure 19. Monitoring of malolactic fermentation with co-inoculation and sequential inoculation of yeast *S. cerevisiae* E4 and *O. oeni* UniFG 3. In red, is reported the concentration of L-malic acid, while in blue, is reported the concentration of L-lactic acid. Continuous line represent the sequential inoculation, while dashed line represent the co-inoculation approach.

In **Fig. 20** degradation of L-malic acid and production of L-lactic acid for exemplificative associations yeast (*S. cerevisiae* E4)-bacteria, where has been a complete degradation of malic acid, in about 40 days, is reported.

All *O. oeni* strains completely degrade malic acid, about in 40 days, when used in simultaneous inoculation. In fact residual concentration of malic acid is low (about <0.5 g/L), in addition also the amount of lactic acid produced is high, about 1.5-2 g/L. While the same *O. oeni* strains when used in sequential inoculation are not able to completely degrade malic acid.

O. oeni UniFG 46 (see **Fig.20H**)) constitutes the only exception, in fact these strain completely degrade malic acid when used in sequential inoculation, about in 40 days. The residual malic acid concentration for this association is 0.15 g/L and the amount of lactic acid produced is 1.2 g/L.

While when used in simultaneous inoculation strain UniFG 46 is not able to complete degrade malic acid.

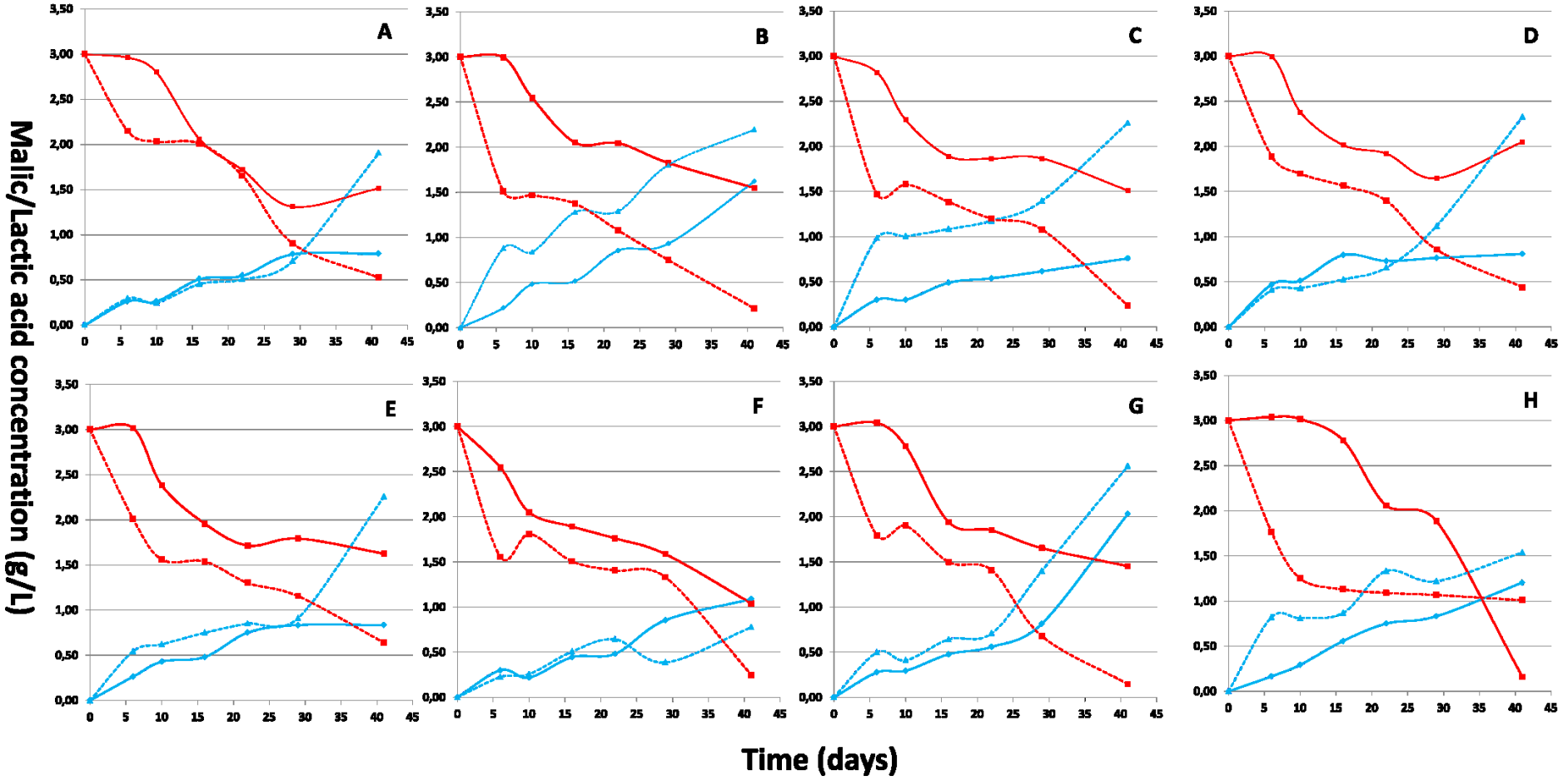


Figure 20. Monitoring of malolactic fermentation with co-inoculation and sequential inoculation of yeast *S. cerevisiae* E4 and *O. oeni* UniFG 4 (A), *O. oeni* UniFG 21 (B), *O. oeni* UniFG 25 (C), *O. oeni* UniFG 28 (D), *O. oeni* UniFG 32 (E), *O. oeni* UniFG 34 (F), *O. oeni* UniFG 44 (G) and *O. oeni* UniFG 46 (H). In red, is reported the concentration of L-malic acid, while in blue, is reported the concentration of L-lactic acid. Continuous line represent the sequential inoculation, while dashed line represent the co-inoculation approach.

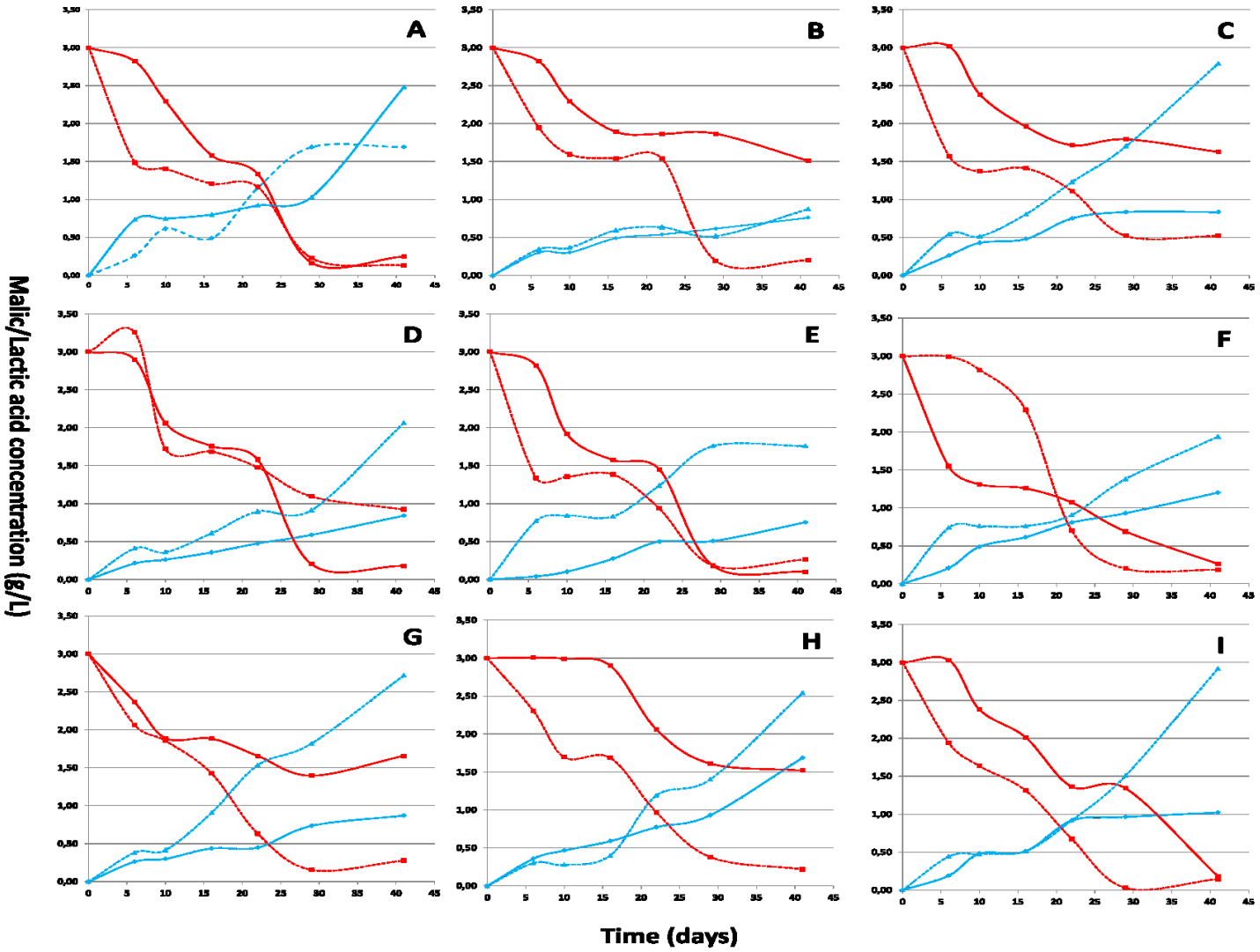


Figure 21. Monitoring of malolactic fermentation with co-inoculation and sequential inoculation of yeast *S. cerevisiae* E4 and *O. oeni* UniFG 20 (A), *O. oeni* UniFG 26 (B), *O. oeni* UniFG 31 (C), *O. oeni* UniFG 35 (D), *O. oeni* UniFG 36 (E), *O. oeni* UniFG 37 (F), *O. oeni* UniFG 40 (G), *O. oeni* UniFG 43 (H) and *O. oeni* UniFG 47. In red, is reported the concentration of L-malic acid, while in blue, is reported the concentration of L-lactic acid. Continuous line represent the sequential inoculation, while dashed line represent the co-inoculation approach.

In **Fig. 21** degradation of L-malic acid and production of L-lactic acid for exemplificative associations yeast (*S. cerevisiae* E4)-bacteria, where has been a complete degradation of malic acid, in about 30 days, is reported.

O. oeni **UniFG 20** (see **Fig.21A**) completely degrade malic acid, about in 30 days, both when used in simultaneous or sequential inoculation. Residual concentration of malic acid is low, 0.12 and 0.24 g/L, respectively in simultaneous and sequential inoculation, while the amount of lactic acid produced is high, about 2.5 g/L in co-inocultation and 1,7 in sequential inoculation.

O. oeni **UniFG 26** (see **Fig.21B**) completely degrade malic acid, about in 30 days, only when used in simultaneous inoculation, in fact these strain consume all malic acid present in grape juice, the residual concentration of malic acid is low (about 0.19 g/L), while the amount of lactic acid produced is quite high, about 0.9 g/L. Moreover these strain isn't able to completely degrade malic acid in sequential inoculation, residual concentration of malic acid is high, about 1.51 g/L.

O. oeni **UniFG 31** (**Fig.21C**) show a behavior similar to those reported for the strain UniFG 26, in fact it completely degrade malic acid in 30 days when used in co-inoculation, while in simultaneous inoculation isn't able to completely degrade malic acid.

O. oeni **UniFG 35** (see **Fig.21D**) constitutes the only exception, in fact these strain completely degrade malic acid only when used in sequential inoculation, about in 30 days. The residual malic acid concentration for these association is 0.17 g/L and the amount of lactic acid produced is 2 g/L.

O. oeni **UniFG 36** (see **Fig.21E**) completely degrade malic acid, about in 30 days, both in simultaneous or sequential inoculation, in fact these strain consume all malic acid present in grape juice and residual concentration of malic acid is low in both assay (about 0.1-0.2 g/L), while the amount of lactic acid produced is quite high, about 1.75 and 0.75 g/L, respectively in simultaneous and sequential inoculation.

In **Fig.21F** is reported the association *S. cerevisiae* E4-*O. oeni* **UniFG 37**, that allows a complete degradation of malic acid in 30 days when co-inoculated, reaching a low residual concentration of malic acid (about 0.18 g/L) and an high production of lactic acid (about 1.9 g/L). The same strain

used in sequential inoculation completely degrade malic acid in 40 days, but the amount of lactic acid is lower than those produced in simultaneous inoculation (about 1.2 g/L), while residual concentration of malic acid is comparable with those of co-inoculation.

O. oeni **UniFG 40** and **43** (see **Fig.s 21G-H**) show a similar behavior, both completely degrade malic acid in simultaneous inoculation, the two assays shown a low residual concentration of malic acid (about 0.2-0.3 g/L) and an high amount of lactic acid (about 2.5 g/L). While the same strains when used in sequential inoculation are not able to complete degrade malic acid. O. oeni UniFG 47 completely degrade malic acid both in simultaneous or sequential inoculation, respectively in 30 and 40 days. Residual concentration of malic acid is low in both assay (about 0.15 g/L), however the amount of lactic acid produced is quite different in simultaneous and sequential inoculation, respectively 2.9 and 1 g/L.

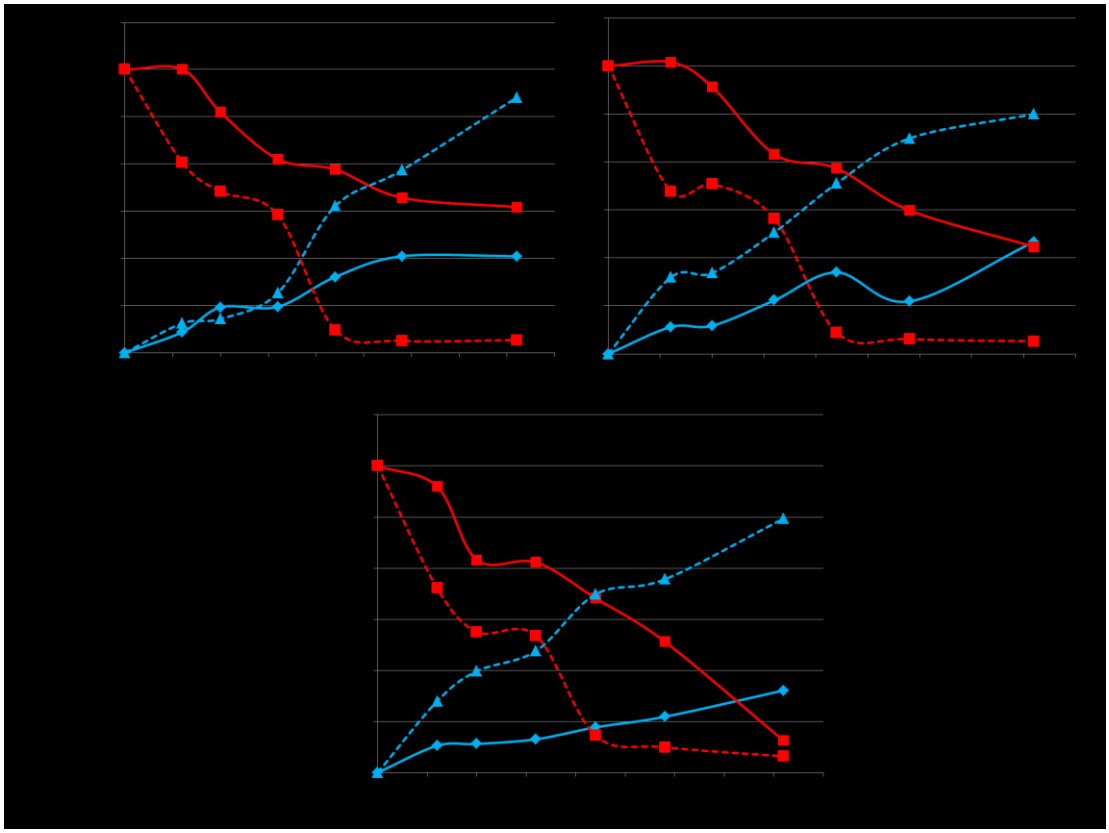


Figure 22. Monitoring of malolactic fermentation with co-inoculation and sequential inoculation of yeast *S. cerevisiae* E4 and *O. oeni* UniFG 9 (A), *O. oeni* UniFG 14 (B) and *O. oeni* UniFG 45 (C). In red, is reported the concentration of L-malic acid, while in blue, is reported the concentration of L-lactic acid. Continuous line represent the sequential inoculation, while dashed line represent the co-inoculation approach.

In **Fig. 22** degradation of L-malic acid and production of L-lactic acid for exemplificative associations yeast (*S. cerevisiae* E4)-bacteria, where has been a complete degradation of malic acid, in about 20-25 days, is reported.

Strains **UniFG 9** and **14** (see **Fig.s 22A-B**) completely degrade malic acid in about 20-25 days in simultaneous inoculation, although the same strains in sequential inoculation aren't able to completely degrade malic acid. In co-inoculation residual concentration of malic acid is low in both assays (about 0.10 g/L), also the amount of lactic acid is high (about 2.7-2.5 g/L). Instead in sequential inoculation residual amount of malic acid is high, about 1.5- 1 g/L, and the production of lactic acid is low (about 1 g/L).

Strain **UniFG 45 (Fig. 22C)** completely degrade malic acid both in simultaneous or sequential inoculation, respectively in 20-25 and 40 days. In co-inoculation residual concentration of malic acid is low both in simultaneous or sequential inoculation (about 0.15 g/L), while the amount of lactic acid produced is quite different in simultaneous and sequential inoculation, respectively 2.5 and 0.8 g/L.

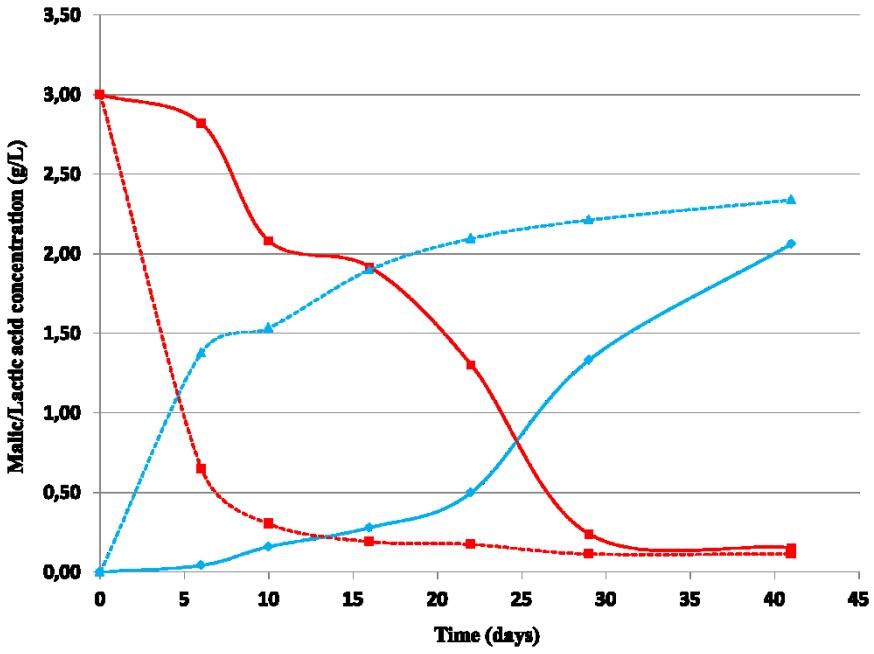


Figure 23. Monitoring of malolactic fermentation with co-inoculation and sequential inoculation of yeast *S. cerevisiae* E4 and *O. oeni* UniFG 22. In red, is reported the concentration of L-malic acid, while in blue, is reported the concentration of L-lactic acid. Continuous line represent the sequential inoculation, while dashed line represent the co-inoculation approach.

In **Fig. 23** degradation of L-malic acid and production of L-lactic acid for associations *S. cerevisiae* E4-**UniFG 22**, where has been a complete degradation of malic acid, in about 10 days, is reported.

These strain has been the best one for its malolactic performances in association with *S. cerevisiae* E4, in fact *O. oeni* UniFG 22 in co-inoculation allows a quick and complete degradation of malic acid about in 10 days, residual concentration of malic acid is low, about 0.10 g/L, also the production of lactic acid is high (about 2.3 g/L). This strain completely degrade malic acid also when used in sequential inoculation, but about in 30 days, with low residual concentration of malic acid (about 0.10 g/L) and high amount of lactic acid produced (about 2 g/L).

In **Table 35**. is reported the characteristic of wine obtained with *S. cerevisiae* I6 and 50 different *O. oeni* strains, time required to complete MLF (L-malic acid concentration below 0.5 g/L), malic and lactic acid concentration (g/L) after MLF in Nero di Troia must.

Malolactic performances of *O. oeni* strains investigated were quite different with different time of inoculum used (simultaneous or sequential).

The major part of the *O. oeni* strains co-inoculated with *S. cerevisiae* E4 completely degrade malic acid in 40 days(see **Table 35**.), in fact malic acid residual concentration is low, about <0.5 g/L, a value generally accepted in literature for an efficient and complete MLF.

Other *O. oeni* strains that has been able to complete MLF when associated with *S. cerevisiae* I6 need from 15 to 30 days to completely degrade malic acid in co-inoculation(for details see Fig.s 24, 25, 26, 27, 28, 28 and 30).

In sequential inoculation *O. oeni* behavior is quite different, the major part of the strains analyzed aren't able to completely degrade malic acid, however some strains complete MLF also in sequential inoculation, about in 40 or 30 days.

O. oeni strain	Inoculation Time	Malic acid (g/L)	Lactic acid (g/L)	MLF time after LAB inoculation (days)	*O. oeni* strain	Inoculation Time	Malic acid (g/L)	Lactic acid (g/L)	MLF time after LAB inoculation (days)
UniFG 1	SEQ	1.01	0.81	NR	**UniFG 26**	SEQ	1.30	0.72	NR
	SIM	0.26	1.93	40		SIM	0.17	2.07	30
UniFG 2	SEQ	0.86	1.18	NR	**UniFG 27**	SEQ	1.32	0.70	NR
	SIM	0.16	1.86	40		SIM	0.13	2.29	30
UniFG 3	SEQ	1.20	0.88	NR	**UniFG 28**	SEQ	0.13	2.14	24 (TOT 40)
	SIM	0.19	2.08	40		SIM	0.08	2.13	30
UniFG 4	SEQ	0.75	1.03	NR	**UniFG 29**	SEQ	0.18	2.05	24 (TOT 40)
	SIM	0.18	1.99	40		SIM	0.17	2.47	30
UniFG 5	SEQ	0.18	2.02	24 (TOT 40)	**UniFG 30**	SEQ	0.16	2.00	24 (TOT 40)
	SIM	0.11	1.75	20		SIM	0.38	1.97	40
UniFG 6	SEQ	0.75	1.16	NR	**UniFG 31**	SEQ	0.16	2.00	24 (TOT 40)
	SIM	0.01	2.13	20		SIM	0.14	2.32	40
UniFG 7	SEQ	0.79	0.73	NR	**UniFG 32**	SEQ	0.16	2.00	24 (TOT 40)
	SIM	0.16	2.57	30		SIM	0.11	2.10	40
UniFG 8	SEQ	0.79	0.73	NR	**UniFG 33**	SEQ	1.40	0.71	NR
	SIM	0.18	2.28	30		SIM	0.36	1.67	40
UniFG 9	SEQ	0.80	0.75	NR	**UniFG 34**	SEQ	0.79	0.87	NR
	SIM	0.16	2.13	20		SIM	0.80	1.19	NR
UniFG 10	SEQ	0.99	0.79	NR	**UniFG 35**	SEQ	0.54	1.67	24 (TOT 40)
	SIM	0.25	1.73	40		SIM	0.20	2.06	40
UniFG 11	SEQ	1.04	0.73	NR	**UniFG 36**	SEQ	0.53	1.61	24 (TOT 40)
	SIM	0.18	2.04	40		SIM	0.00	2.23	40
UniFG 12	SEQ	1.01	0.75	NR	**UniFG 37**	SEQ	0.21	2.56	24 (TOT 40)
	SIM	0.16	2.68	20		SIM	0.16	2.59	40
UniFG 13	SEQ	1.03	0.61	NR	**UniFG 38**	SEQ	0.14	2.01	24 (TOT 40)
	SIM	0.17	1.90	40		SIM	0.29	2.01	40

UniFG 14	SEQ	0.82	1.17	NR	**UniFG 39**	SEQ	0.25	2.01	24 (TOT 40)
	SIM	0.12	2.38	20		SIM	0.09	1.92	30
UniFG 15	SEQ	0.82	1.17	NR	**UniFG 40**	SEQ	0.21	2.56	24 (TOT 40)
	SIM	0.17	2.23	40		SIM	0.13	2.02	40
UniFG 16	SEQ	0.24	2.01	24 (TOT 40)	**UniFG 41**	SEQ	0.21	2.56	24 (TOT 40)
	SIM	0.23	2.41	40		SIM	0.15	2.07	40
UniFG 17	SEQ	0.82	1.31	NR	**UniFG 42**	SEQ	0.32	1.83	24 (TOT 40)
	SIM	0.13	2.02	40		SIM	0.25	2.37	40
UniFG 18	SEQ	0.33	1.84	24 (TOT 40)	**UniFG 43**	SEQ	0.39	1.73	24 (TOT 40)
	SIM	0.48	1.62	40		SIM	0.17	1.48	30
UniFG 19	SEQ	0.79	0.97	NR	**UniFG 44**	SEQ	0.20	2.24	24 (TOT 40)
	SIM	0.46	1.60	40		SIM	0.34	1.83	40
UniFG 20	SEQ	0.15	1.99	24 (TOT 40)	**UniFG 45**	SEQ	0.87	1.18	24 (TOT 40)
	SIM	0.14	2.65	30		SIM	0.13	2.39	40
UniFG 21	SEQ	1.00	0.62	NR	**UniFG 46**	SEQ	0.21	2.02	24 (TOT 40)
	SIM	0.16	2.22	40		SIM	0.17	2.25	40
UniFG 22	SEQ	0.19	1.93	15 (TOT 30)	**UniFG 47**	SEQ	1.14	0.69	NR
	SIM	0.14	2.22	15		SIM	0.21	2.11	30
UniFG 23	SEQ	1.32	0.71	NR	**UniFG 48**	SEQ	1.06	0.75	NR
	SIM	0.24	2.22	30		SIM	0.03	2.17	30
UniFG 24	SEQ	1.40	0.68	NR	**UniFG 49**	SEQ	1.05	0.67	NR
	SIM	0.19	2.37	30		SIM	0.16	2.26	30
UniFG 25	SEQ	1.42	0.72	NR	**UniFG 50**	SEQ	0.73	0.96	NR
	SIM	0.20	2.57	30		SIM	0.16	1.89	40

Table 35. Time required to complete MLF (L-malic acid concentration below 0.5 g/L) and average of chemical analysis data recorded after MLF in Nero di Troia must fermented with ***S. cerevisiae*** **I6** and with 50 *O. oeni* strains (**UniFG1-50**), comparing two inoculation times (SIM: simultaneous, SEQ: sequential). NR: Not reached.

In **Fig. 24** degradation of malic acid and production of lactic acid is reported for an esemplificative association between yeast –bacteria, that aren't able to complete degrade malic acid, both in simultaneous or sequential inoculation.

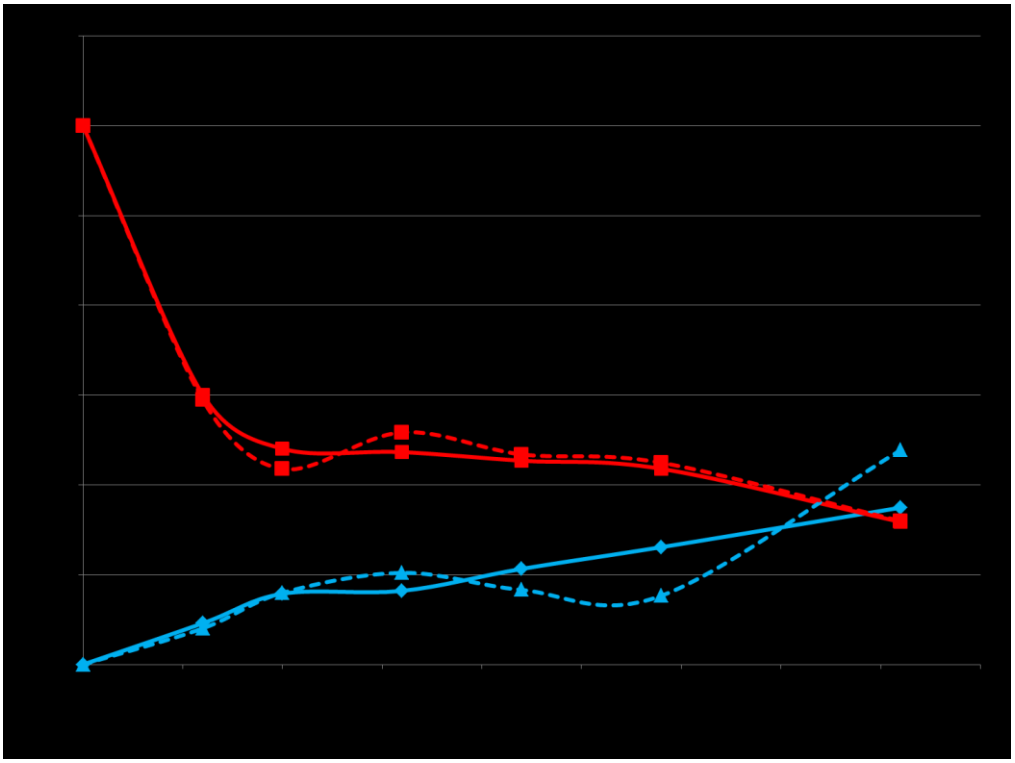


Figure 24. Monitoring of malolactic fermentation with co-inoculation and sequential inoculation of yeast *S. cerevisiae* I6 and *O. oeni* UniFG 34. In red, is reported the concentration of L-malic acid, while in blue, is reported the concentration of L-lactic acid. Continuous line represent the sequential inoculation, while dashed line represent the co-inoculation approach.

Strains **UniFG 19** and **34** shown a behavior similar to those reported in Fig.24, they are not able to induce a successful MLF in both assays investigated, i.e. simultaneous or sequential inoculation.

In **Fig. 25** degradation of malic acid and production of lactic acid is reported for an esemplificative association between yeast–bacteria, that need 40 days to completely degrade malic acid in co-inoculation. These assay shown low residual concentration of malic acid (about 0.2g/L) and high amount of lactic acid (about 2 g/L). In other terms, the same strains are not able to complete MLF when used in sequential inoculation.

Strains *O. oeni* **UniFG 1**, **2**, **3**, **4**, **10**, **11**, **13**, **15**, **17**, **21** and **50** shown the same behavior reported in Fig.24, with a successful MLF only in co-inoculation, about in 40 days.

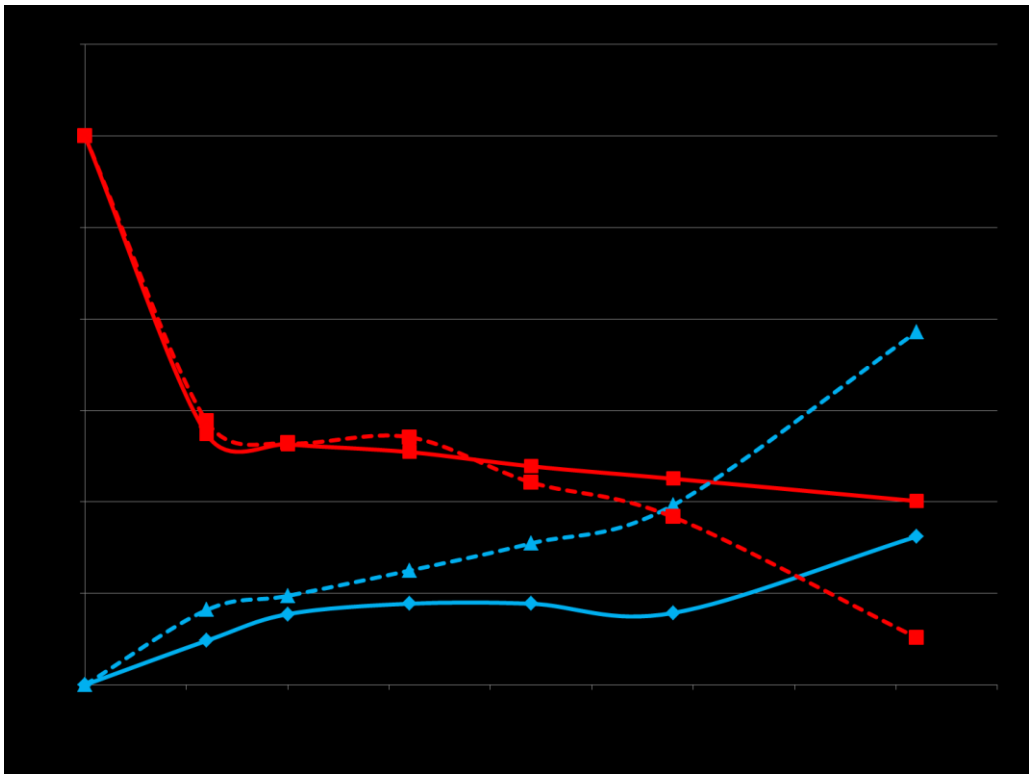


Figure 25. Monitoring of malolactic fermentation with co-inoculation and sequential inoculation of yeast *S. cerevisiae* I6 and *O. oeni* UniFG 1. In red, is reported the concentration of L-malic acid, while in blue, is reported the concentration of L-lactic acid. Continuous line represent the sequential inoculation, while dashed line represent the co-inoculation approach.

In **Fig. 26,** degradation of malic acid and production of lactic acid is reported for an esemplificative association between yeast –bacteria, that need 40 days to completely degrade malic acid both in co- and sequential inoculation. These assay shown low residual concentration of malic acid (about 0.2g/L) and high amount of lactic acid (about 2 g/L).

Strains *O. oeni* **UniFG 16**, **18**, **30**, **31**, **32**, **33**, **35**, **36**, **37**, **38, 40, 41, 42, 44, 45** and **46** shown the same behavior reported in Fig.25, with a successful MLF about in 40 days, both when used in co or sequential inoculation.

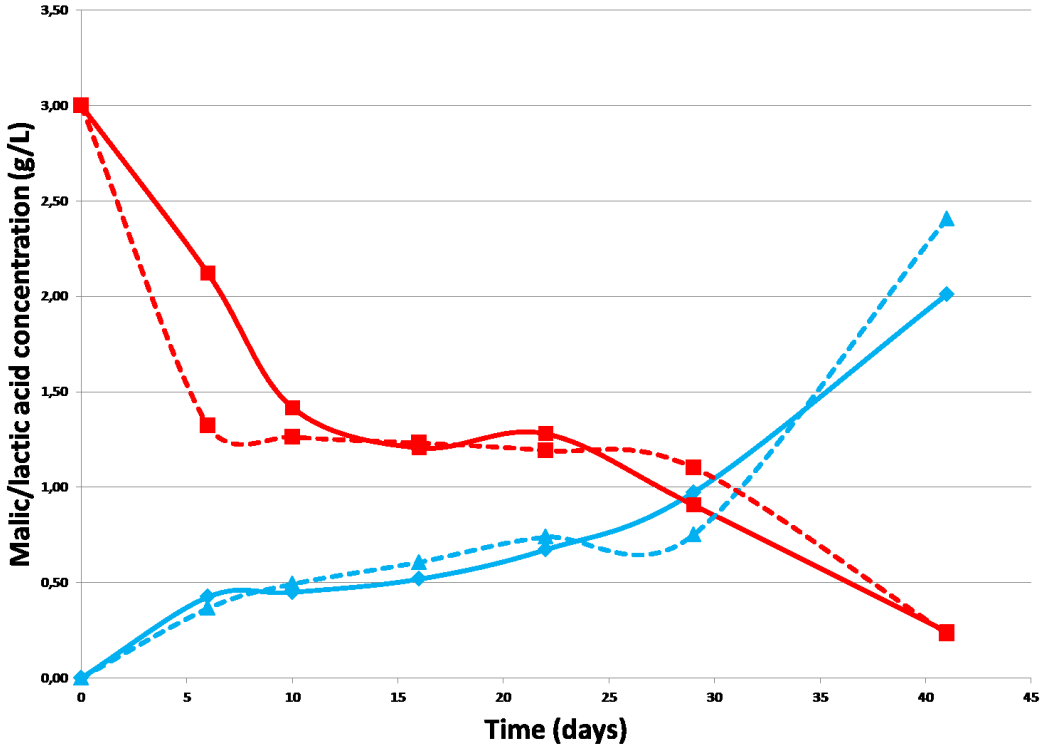


Figure 26. Monitoring of malolactic fermentation with co-inoculation and sequential inoculation of yeast *S. cerevisiae* I6 and *O. oeni* UniFG 16. In red, is reported the concentration of L-malic acid, while in blue, is reported the concentration of L-lactic acid. Continuous line represent the sequential inoculation, while dashed line represent the co-inoculation approach

In **Fig. 27,** degradation of malic acid and production of lactic acid is reported for esemplificative associations between yeast –bacteria, that need 30 days to completely degrade malic acid in co-inoculation. The same strains are not able to complete MLF when used in sequential inoculation.

In co-inoculation *O. oeni* strains (strains UniFG 7, 8, 23, 24, 25, 26, 17 and 49) completely degrade malic acid, its residual concentration is low, about 0.2 g/L, in addition also the lactic acid production is high (about 2-2.5 g/L). The same strains when used in sequential inoculation aren't able to induce a complete and successful MLF, in fact residual concentration of malic acid is higher than those obtained in co-inoculation (about 1 g/L) and the amount of lactic acid produced is lower (about 0.5-0.8 g/L).

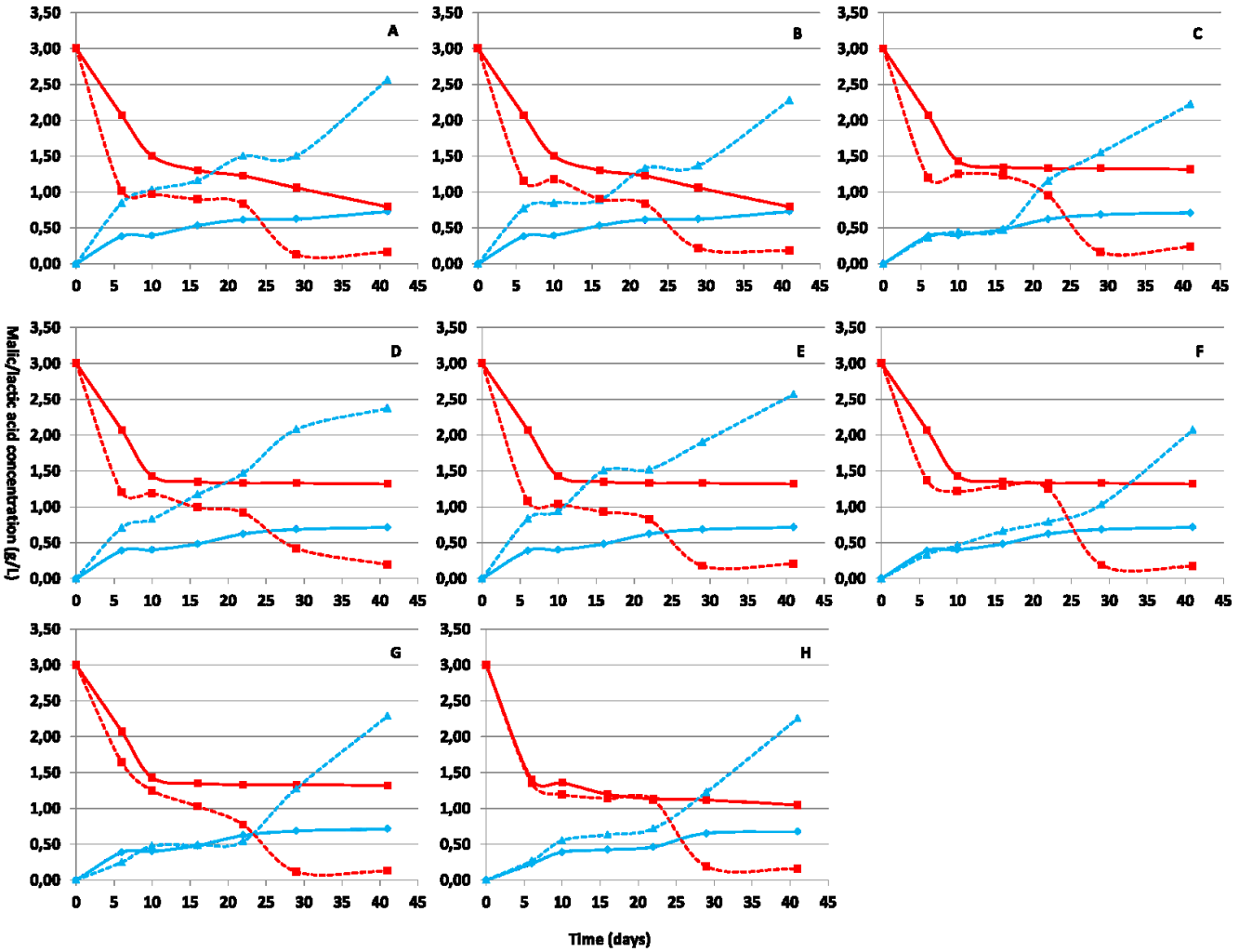


Figure 27. Monitoring of malolactic fermentation with co-inoculation and sequential inoculation of yeast *S. cerevisiae* I6 and *O. oeni* UniFG 7(A), *O. oeni* UniFG 8 (B), *O. oeni* UniFG 23 (C), *O. oeni* UniFG 24 (D), *O. oeni* UniFG 25 (E), *O. oeni* UniFG 26 (F), *O. oeni* UniFG 27 (G) and *O. oeni* UniFG 49 (H). In red, is reported the concentration of L-malic acid, while in blue, is reported the concentration of L-lactic acid. Continuous line represent the sequential inoculation, while dashed line represent the co-inoculation approach.

In **Fig. 28,** degradation of malic acid and production of lactic acid is reported for esemplificative associations between yeast –bacteria, that need about 30 and 40 days to completely degrade malic acid respectively in co or sequential inoculation.

Strain **UniFG 5** (Fig. 28A) complete MLF in 30 days when used in co-inoculation, in fact residual concentration of malic acid is about 0.10 g/L and the amount of lactic acid produced is about 2 g/L. This strain induce a successful MLF also when used in sequential inoculation, about in 40 days. Strains **UniFG 28, 29, 39** and **43** (**Fig.s 28 B-C-D-E**) shown a similar behavior inducing a successful MLF both in co or sequential inoculation, respectively in 30 and 40 days.

Strain UniFG 20 represents the only exception, in fact these strain induce a more successful and quick fermentation when used in sequential inoculation, in fact it need about 30 days to completely degrade malic acid in sequential inoculation, while need about 40 days in co-inoculation.

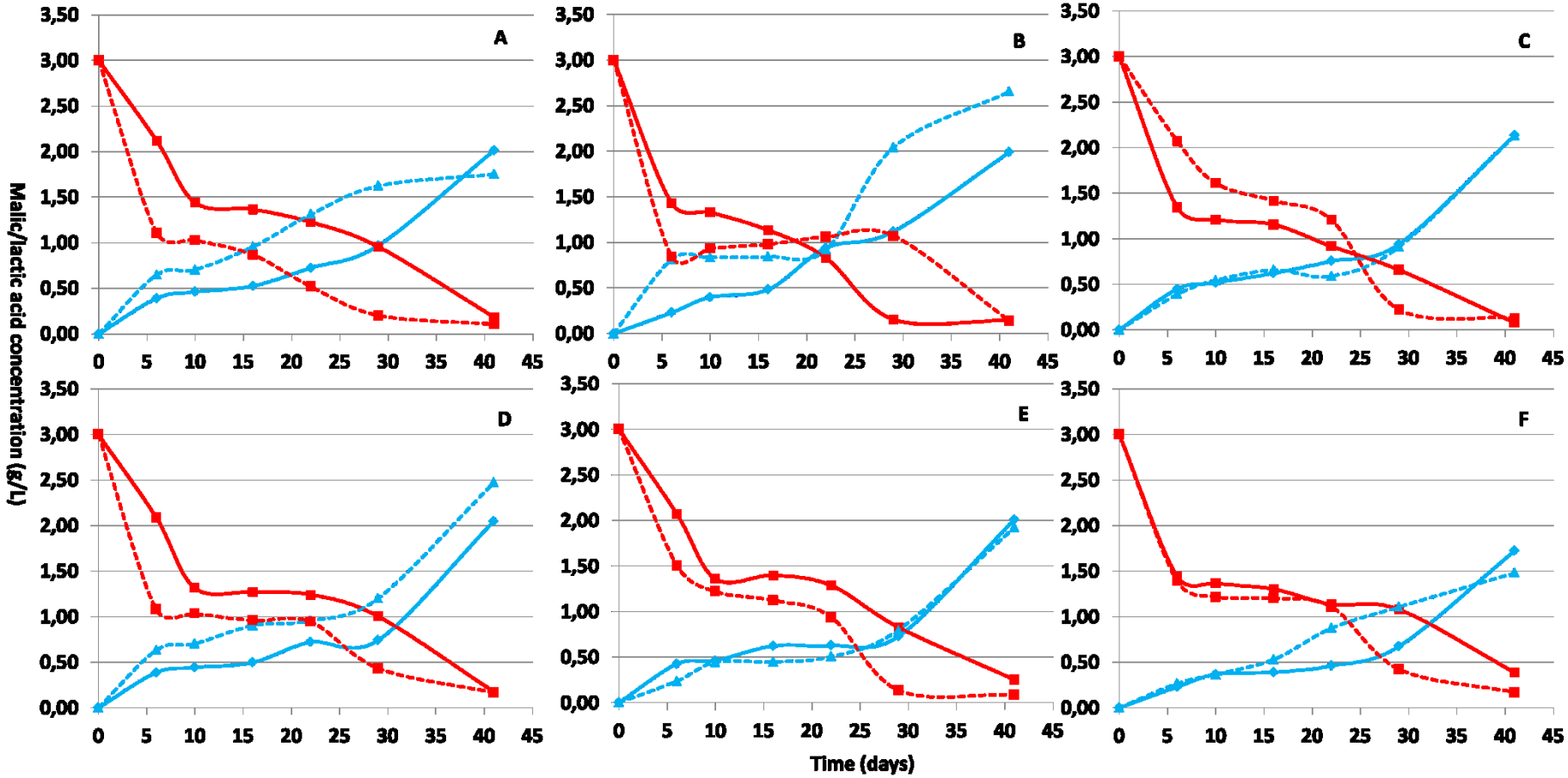


Figure 28. Monitoring of malolactic fermentation with co-inoculation and sequential inoculation of yeast *S. cerevisiae* I6 and *O. oeni* UniFG 5(A), *O. oeni* UniFG 20 (B), *O. oeni* UniFG 28 (C), *O. oeni* UniFG 29 (D), *O. oeni* UniFG 39 (E) and *O. oeni* UniFG 43 (F). In red, is reported the concentration of L-malic acid, while in blue, is reported the concentration of L-lactic acid. Continuous line represent the sequential inoculation, while dashed line represent the co-inoculation approach.

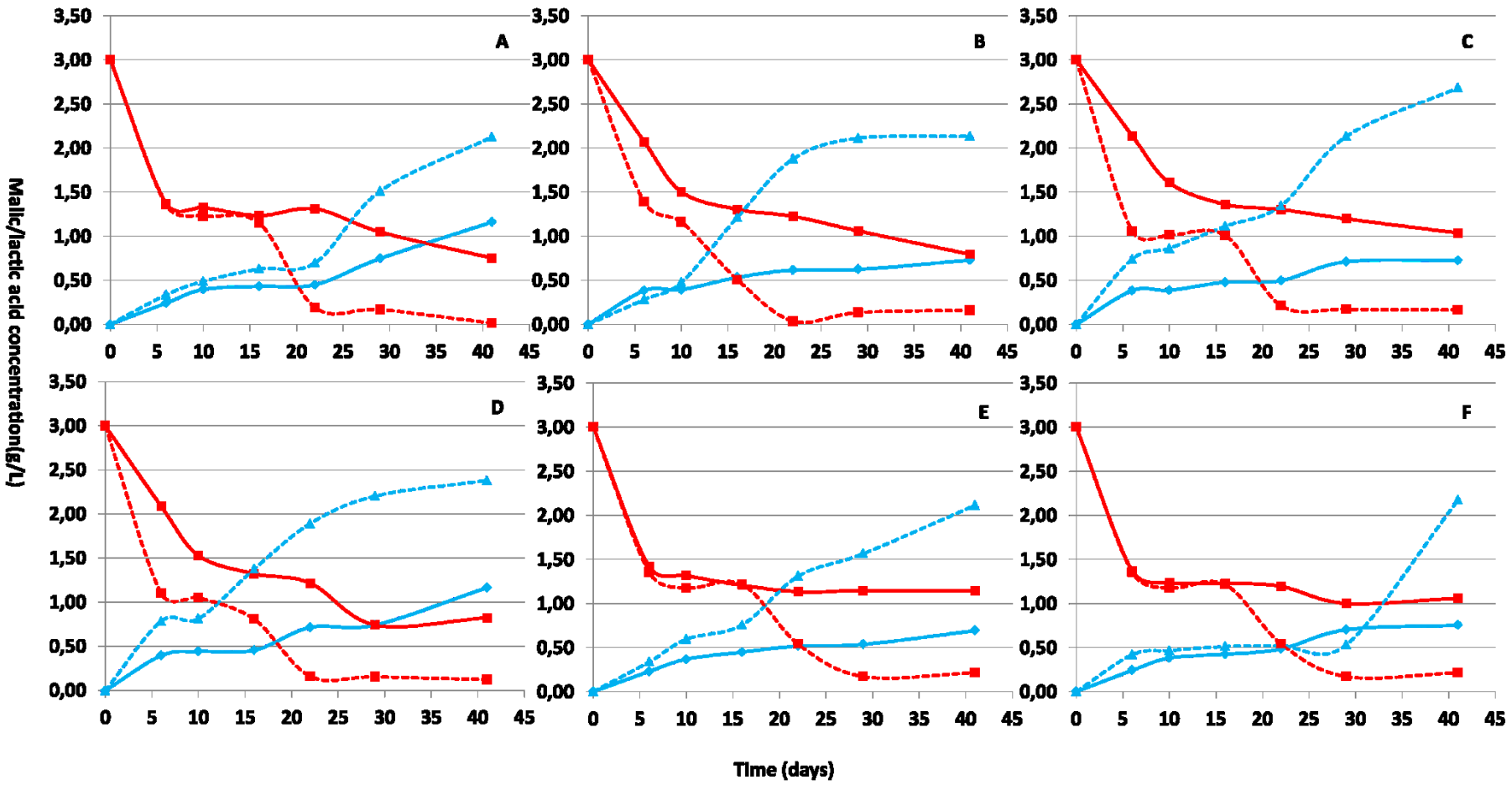


Figure 29. Monitoring of malolactic fermentation with co-inoculation and sequential inoculation of yeast *S. cerevisiae* I6 and *O. oeni* UniFG 6(A), *O. oeni* UniFG 9 (B), *O. oeni* UniFG 12 (C), *O. oeni* UniFG 14 (D), *O. oeni* UniFG 47 (E) and *O. oeni* UniFG 48 (F). In red, is reported the concentration of L-malic acid, while in blue, is reported the concentration of L-lactic acid. Continuous line represent the sequential inoculation, while dashed line represent the co-inoculation approach

In **Fig. 29,** degradation of malic acid and production of lactic acid is reported for esemplificative associations between yeast –bacteria, that need about 20-25 days to completely degrade malic acid in co-inoculation.

All the strains reported in **Fig. 29** induce a successful and quick MLF when used in co-inoculation, while in sequential inoculation ML strains aren't able to completely degrade malic acid (see **Fig.s 29 A-B-C-D-E-F**). In all assays obtained with simultaneous inoculation residual concentration of malic acid is low, about 0.10-0.20 g/L, and lactic acid production higher (about 2-2.5 g/L). Furthermore sequential inoculation lead to a partial degradation of malic acid, its residual concentration is higher than those obtained with co-inoculation (about 0.8-1 g/L), also lactic acid production is lower (about 0.7 g/L).

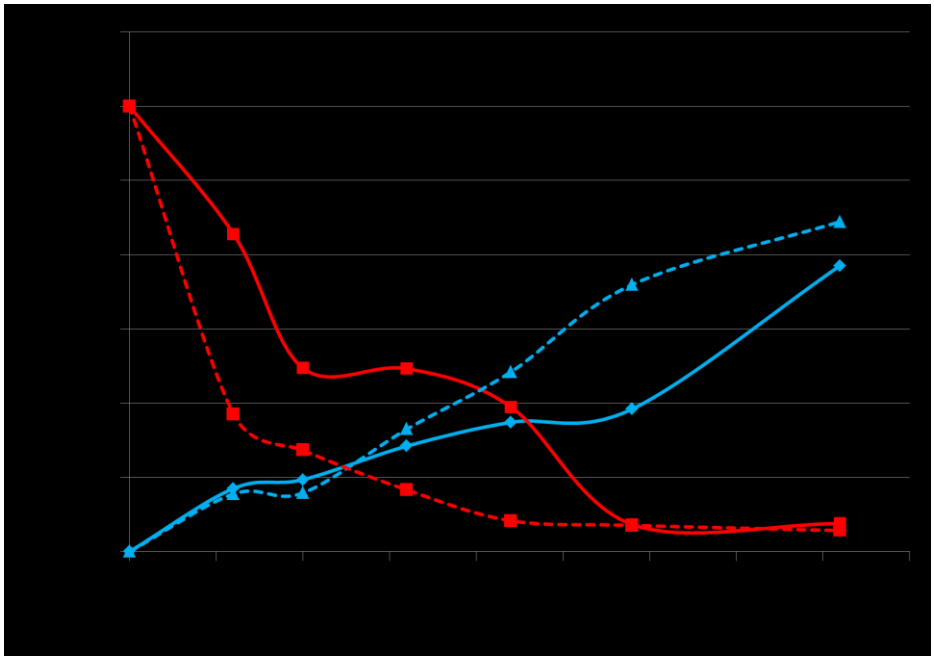


Figure 30. Monitoring of malolactic fermentation with co-inoculation and sequential inoculation of yeast *S. cerevisiae* I6 and *O. oeni* UniFG 22. In red, is reported the concentration of L-malic acid, while in blue, is reported the concentration of L-lactic acid. Continuous line represent the sequential inoculation, while dashed line represent the co-inoculation approach.

In **Fig. 33,** degradation of malic acid and production of lactic acid is reported for the best associations between yeast –bacteria, that need about 15 days to completely degrade malic acid in co-inoculation, the same strain induce a successful MLF also in sequential inoculation (about in 30 days).

Residual concentration of malic acid is low both in co or sequential inoculation (about 0.1-0.2 g/L), also the amount of lactic acid produced is high, 2.2 and 1.9 g/L respectively in co and sequential inoculation.

Results obtained from preliminary microvinification assays suggest the importance of association between yeast-bacteria, in fact several *O. oeni* strains shown different malolactic performances when associated with the two autochthonous *S. cerevisiae* strains. Moreover, another important parameter for a complete and successful MLF is the time of inoculum. Our results confirmed results reported in literature, in fact several authors suggested the benefits of simultaneous inoculation of yeast and bacteria.

Preliminary microvinification allowed to investigate the malolactic performances of the *O. oeni* strains co or sequential inoculated and to selected on the base of their fermentation attitude 6 *O. oeni* strains (nominated as strains 14, 22, 23, 34, 44 and 45) and used for sequential or co-inoculation approaches in industrial scale-up.

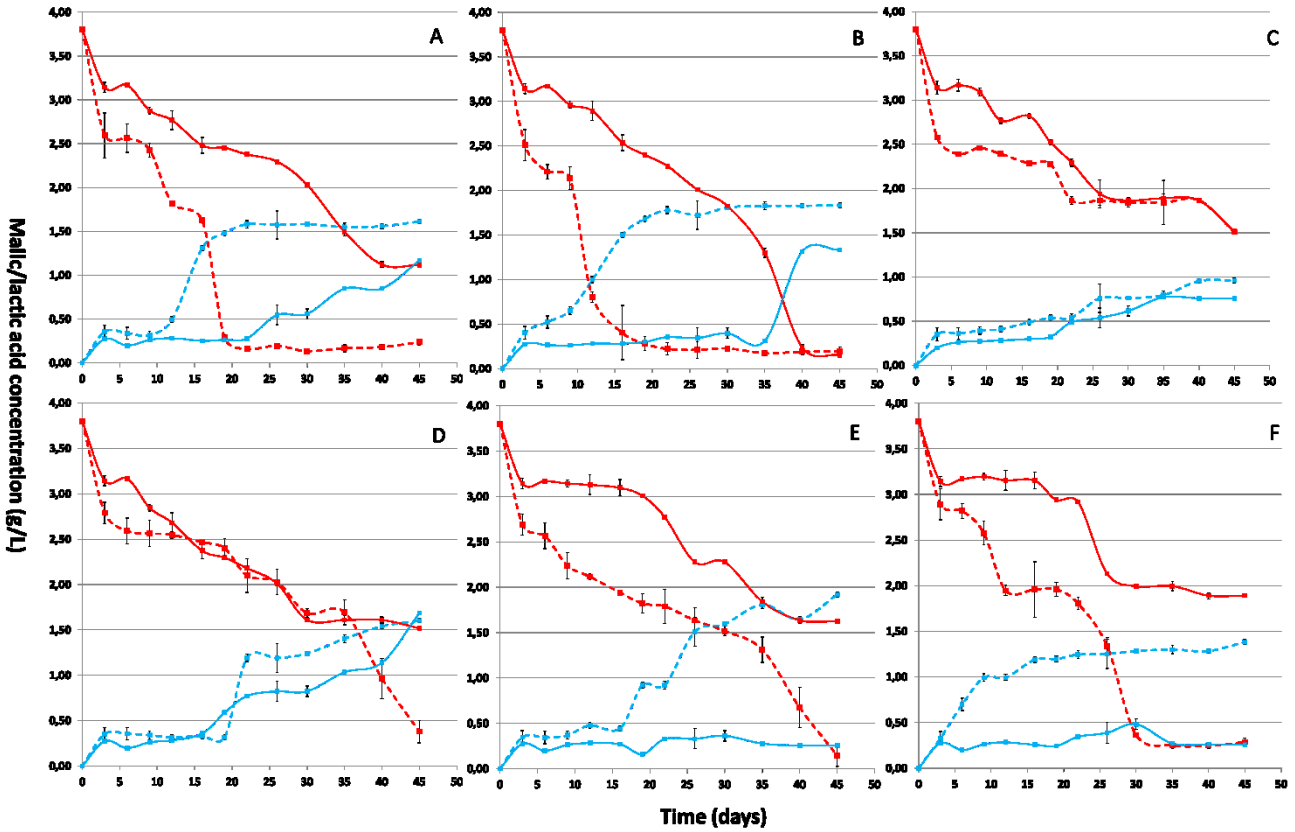


Figure 31. Monitoring of malolactic fermentation with co-inoculation and sequential inoculation of yeast *S. cerevisiae* E4 and *O. oeni* UniFG 14 (A), *O. oeni* UniFG 22 (B), *O. oeni* UniFG 23 (C), *O. oeni* UniFG 34 (D), *O. oeni* UniFG 44 (E) and *O. oeni* UniFG 45 (F). In red, is reported the concentration of L-malic acid, while in blue, is reported the concentration of L-lactic acid. Continuous line represent the sequential inoculation, while dashed line represent the co-inoculation approach.

In **Figures 31** and **32** degradation of malic acid and production of lactic acid is reported for the associations the two yeast strains (*S. cereviasiae* E4 and I6) and 6 different *O. oeni* strains, choosed for they different behavior and malolactic performances in association with the two different yeast strains selected as starter cultures.

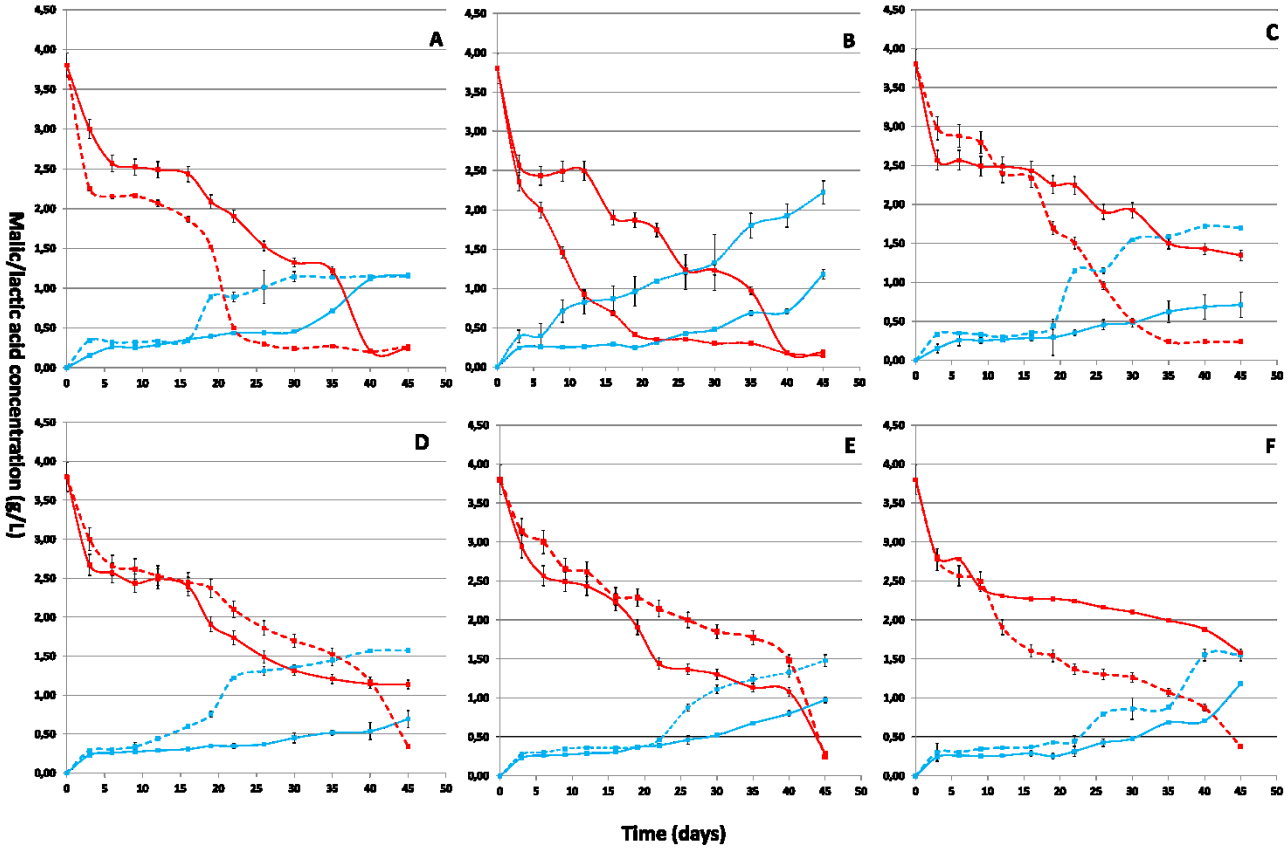


Figure 32. Monitoring of malolactic fermentation with co-inoculation and sequential inoculation of yeast *S. cerevisiae* I6 and *O. oeni* UniFG 14 (A), *O. oeni* UniFG 22 (B), *O. oeni* UniFG 23 (C), *O. oeni* UniFG 34 (D), *O. oeni* UniFG 44 (E) and *O. oeni* UniFG 45 (F). In red, is reported the concentration of L-malic acid, while in blue, is reported the concentration of L-lactic acid. Continuous line represent the sequential inoculation, while dashed line represent the co-inoculation approach.

Samples were analyzed at 0, 3, 6, 9, 12, 16, 19, 22, 26, 30, 35, 40 and 45 days after the beginning of AF. AF finished in all assays about 15 days after the yeast inoculation, independently of the timing of LAB inoculations. No differences in duration of AF were observed between the two bacteria inoculation times in any of the yeast/bacteria associations evaluated (see **Tab. 36**).

Viable yeast population was not significantly influenced by *O. oeni* addition in simultaneous or sequential treatments. Bacterial viabilities were highly similar among simultaneous and sequential treatments, while different rates of L-malic acid degradation were recorded. *O.oeni* populations stayed constant or increased and reached peak populations above 10^6 CFU/mL. *O. oeni* population decreased during the first days after inoculation, followed by a slow increase of bacterial population and consequently a slow rate of L-malic acid degradation (data not shown).

Yeast strain	*O. oeni* strain	Inoculation time	MLF time after LAB inoculation (day)	Total fermentation time-AF + MLF- (day)	pH	L-Malic acid (g/L)	L-Lactic acid (g/L)
E4	UniFG 14	SIM	19	20	3.25	0.20	1.61
		SEQ	NR	NR	3.14	1.11	1.11
E4	UniFG 22	SIM	15	15	3.27	0.15	1.83
		SEQ	24	40	3.14	0.15	1.33
E4	UniFG 23	SIM	NR	NR	3.14	1.51	0.96
		SEQ	NR	NR	3.13	1.52	0.75
E4	UniFG 34	SIM	45	45	3.18	0.14	1.91
		SEQ	NR	NR	3.1	1.62	0.25
E4	UniFG 44	SIM	45	45	3.16	0.37	1.51
		SEQ	NR	NR	3.1	1.51	1.68
E4	UniFG 45	SIM	30	30	3.15	0.26	1.38
		SEQ	NR	NR	3.16	1.89	0.26
I6	UniFG 14	SIM	25	25	3.15	0.24	1.16
		SEQ	24	40	3.13	0.24	1.16
I6	UniFG 22	SIM	30	20	3.15	0.18	2.22
		SEQ	24	40	3.13	0.19	1.18
I6	UniFG 23	SIM	35	35	3.10	0.23	1.70
		SEQ	NR	NR	3.07	1.34	0.71
I6	UniFG 34	SIM	45	45	3.11	0.34	1.57
		SEQ	NR	NR	3.10	1.34	0.69
I6	UniFG 44	SIM	45	45	3.11	0,24	1.48
		SEQ	28	45	3.11	0,24	0.97
I6	UniFG 45	SIM	25	25	3.12	0.37	1.58
		SEQ	24	40	3.10	1.58	1.18

Table 36. Time required to complete MLF (L-malic acid concentration below 0,5 g/L) and average of chemical analysis data recorded after MLF in Nero di Troia must fermented with two ***S. cerevisiae*** **(E4-I6)** and with 6 *O. oeni* strains (**UniFG14-22-23-34-44-45**), comparing two inoculation times (SIM: simultaneous, SEQ: sequential). NR: Not reached.

Different results related to the efficiency of MLF were observed when different association of yeast and bacteria were analyzed, industrial scale up confirmed preliminary results obtained with microvinification assays.

Several *O. oeni* strains performed an improved MFL when associated with *S.cerevisiae* I6, while an improved MFL was observed when others *O. oeni* strains were associated with *S. cerevisiae* E4 (see Figs. 31 and 32 for details). In wines with simultaneous treatments (co-inoculation), MLF was

completed in 15 to 45 days (table 36), and the pH incresead by 0.1-0.3 units compared with the initial values.

All the *O. oeni* strains analyzed in association with *S. cerevisiae* E4 used in sequential inoculation failed to reduce L-malic acid below 0.5 g/L, the level generally recognized as the threshold for a complete MLF, only UniFG 22 strain in sequential inoculation completely degrade malic acid, in fact residual concentration of malic acid in sequential treatment was 0. 15 g/L and the amount of lactic acid produced was 1,33 g/L, value lower than those obtained with simultaneous treatment (1.83 g/L). This result was also confirmed by pH, 3.27 and 3.14, respectively in simultaneous and sequential inoculation. While the same *O. oeni* strains in association with *S. cerevisiae* I6 induced a successful MLF in 40-45 days, exceptions to this trend were the *O. oeni* strains UniFG 23 and 34, that weren't able to completely degrade malic acid.

Treatments with simultaneous inoculation showed a reduced total fermentation time (AF+MLF) compared to sequential inoculations.

In the major part of sequential treatments, 30–45 % of L-malic acid had already been metabolized at inoculation time, usually 20% of L- malic acid reduction is the maximum percentage that could be attributed to yeast metabolism (Redzepovic *et al*., 2003; Radler *et al*., 1993).

For the associations with the autochthonous yeast E4, 2 of the 6 simultaneous treatments completely degraded L-malic acid in 15 and 20 days (See Figs.31 A-B), other 3 assays shown a slower MLF and need respectively 30 and 45 days to complete MLF (see Figs. 31D-E-F), while for 1 simultaneous treatments *O. oeni* strain co-inoculated weren't able to completely degraded L-malic acid, which residual concentration was 1.51 g/L (see Fig 31C). In addition, slow rates of L-malic acid degradation were also observed in their respective sequential inoculation treatments and *O. oeni* strains used in sequential treatments failed to completely degraded L-malic acid (See Figs. 31A-C-D-E-F), only 1 strains in sequential treatment complete malic acid degradation in 24 days after LAB inoculation (see Fig.31B).

Instead, the associations with the autochthonous yeast I6 generally shown a slower MLF than the same *O. oeni* strains associated with E4. In simultaneous treatments, all *O. oeni* strains investigated completely degraded L-malic acid in 20-45 days (see Fig.s 32A-B-C-D-E-F).

Two strains, UniFG 34 and UniFG 44, need about 45 days to complete malic acid degradation in simultaneous inoculation (see Fig.s 32 D-F), their residual concentrations of malic acid were 0,34 and 0.24 g/L, respectively for the association I6-UniFG 34 and I6-UniFG44, while lactic acid produced during MLF was 1.57 and 1.48 g/L. Other two *O. oeni* strains, UniFG 14 and 45, in simultaneous inoculation completely degrade malic acid in 25 days, while UniFG 23 complete malic acid degradation about in 35 days.

UniFG 22 in co-inoculation induced a successful and rapid MLF, in fact need about 20 days to completely degrade malic acid. Residual concentration of malic acid was 0.18 g/L and lactic acid produced was 2.22 g/L. This result was also confirmed by increased pH, from 3.00 of grape juice to 3.15 of wine.

Generally, sequential treatments need more time to complete MLF, about 40-45 days (see Fig.s 32A-B-E-F) and slow rates of malic acid degradation were also observed in their respective sequential inoculation treatments.

Only two *O. oeni* strains (UniFG 23 and 34) in sequential treatments failed to completely degrade L-malic acid (see Fig.s 32C-D).

Generally simultaneous inoculation was more suitable than sequential inoculation and lead to a rapid and efficient MLF, both for association with *S. cerevisiae* I6 and E4.

The exception to this trend was the pair *S. cerevisiae* I6-*O. oeni* UniFG 44, that induce complete MLF about in 45 days both in simultaneous or sequential inoculation (see Fig. 32E).

Industrial scale up confirmed results obtained with preliminary microvinification, in particular allows selecting two *O. oeni* strains for their malolactic performances.

The best *O. oeni* strains analyzed were UniFG 14 and UniFG 22, both induce a successful and rapid MLF. In fact, these strains completely degrade malic acid present in grape juice of Nero di Troia, in

20-25 (UniFG 14) and 15-20 (UniFG 22) days, respectively in association with *S. cerevisiae* E4 and I6. The same strains induced a complete MLF also in sequential treatments, but need more time to complete MLF and slow rate of malic acid degradation was observed. Co-inocultation treatments allow a rapid and complete MLF with positive consequence on final wine obtained, in fact several authors suggested that wines obtained with simultaneous AF/MLF usually are less buttery, retain more fruitiness and are more complex and better structured, levels of acetic acid are higher but sensorial insignificant (Henick- Kling, 1993; Bartowsky *et al*., 2002b; Jussier *et al*., 2006; Krieger, 2006).

Other benefits of simultaneous inoculation is a more efficient MLF in 'difficult' wines (e.g. low pH) due to low levels of ethanol and higher nutrient concentrations. Wines are also immediately available for racking, fining and SO_2 additions (Davis *et al*., 1985; Jussier *et al*., 2006).

5. CONCLUSION

The intellectual property law defines "geographical indications" (GIs) as a sign used on goods that have a specific geographical origin and possess qualities, reputation, or characteristics that are essentially attributable to that place of origin" (World Intellectual Property Organization (WIPO), 2011). GIs has been more and more important, in fact their global impact is clearly demonstrated thanks to the scientific, social, and economic importance of traditional foods. For wines and fermented beverages this concept is presented by Bisson *et al*. (2002), they suggested that consumers expect wine from a particular region to possess unique qualities that differentiate it from other wines of the same varietal from other regions." The GI system is based upon the concept of "terroir," a French word used to describe all geographical aspects of the environment, including the climate, geology, cultivar, human, technical, and cultural practices (and the interactions of these factors) that can influence local production (Capozzi & Spano, 2011).

The importance of fermented foods in the context of GIs is particularly relevant, due to the historic, cultural, and traditional significance (Holtzman, 2006).

Originally, fermented food were obtained by naturally occurring microorganisms. In modern wineries is well diffused the use of commercial starter culture to induce fermentation to ensure consistency, safety, and quality of the final product. Nevertheless, there are disputes about their use, due to the deficiency of some desirable traits, provided instead by spontaneous fermentation (Fleet and Heard, 1993). Is well recognized that a given microbiota in a food matrix influences the global characteristics of the final product. In this context microbial ecology of fermented food play a decisive, crucial and complex role; in fact in literature are still reported several studies on microbial biodiversity related to food GIs (Benito *et al*., 2007; De Angelis *et al.,* 2008; Ercolini *et al*., 2008; Gala *et al*., 2008; Gullo and Giudici, 2008; Capozzi *et al*., 2010; Csoma *et al*., 2010; Valmorri *et al*., 2010; Cocolin *et al*., 2011; Cordero-Bueso *et al*., 2011; Tristezza *et al*., 2011).

In the context of GIs product specification microbial attributes are identified as geographical (territorial) traits, but also as a specific characteristics of product (list of autochthonous species and

strains, preparation of the natural starter culture, use of specific commercial starter cultures, the chemico-physical foodstuff factors responsible of microbial development). There are conflicting opinion on the use of starter culture, in fact commercial starter cultures use might lead to losses in "unique qualities," while the pursuit of wild natural fermentations (natural starter culture) can result in fermentation arrests, slugghish fermentation and production of undesirable metabolites responsible for food depreciation or human toxicity (such as biogenic amines, volatile phenols, ect...).

During last decades the phenomenon of organic products, such as "organic wine", show an increasing demand. In this context, there is an increasing demand for autochthonous yeast, well selected with the aim to produce a specific kind of wine. The use of autochthonous yeast has been proposed as a tool to take advantage of spontaneous fermentation, to avoid the risks of stuck or sluggish fermentations and to increase the sensory properties of wine. Several studies offer new organic-friendly solution, able to reconcile organic viewpoint with safe food fermentations (Suzzi *et al*., 2012; Mendoza *et al*., 2011; Settani *et al*., 2012; Capece *et al*., 2010).

The aim of this study was to enhance quality of the finished product via an improve management of microbial resources. The first objective of this study was to design a microbial starter for the production of typical Apulian wines, coherent with the status of 'Geographical Indication' (GI).

Pretorius (2000) suggested that the preservation of spontaneous micro flora is essential to obtain starter cultures able to develop the typical flavor and aroma of wines originating from different grapevine cultivars and to ensure the conservation of gene pools of technological importance, in addition Capece *et al*. (2010) demonstrated that exploring the biodiversity of indigenous fermentative strains can be an important contribution towards the understanding and selection of strains with specific phenotypes.

In this work, we studied the yeast microflora present on grapes surfaces of "Uva di Troia", isolated in according to Prakitchaiwattana *et al*. (2004).

Yeast microflora from grape berries surfaces shown a great biodiversity of yeast strains of oenological interest. Indeed, strains belong to *H. uvarum, H. guilliermondii, C. zemplinina, S. cerevisiae* and *M.*

pulcherrima were identified**.** The majority of the strains isolated belong to *M. pulcherrima*, a species of oenological interest. *M. pulcherrima* is common on wine grapes at the time of harvest and in grape must during the early stages of wine fermentation; occurs more frequently on damaged berries, on berries used to produce ice wine, and in botrytized (noble-rotted) wines.

Several authors have investigated the potentiality of *M. pulcherrima* for wine fermentation. However, results were discrepant and the absence of relevant changes in fermentation rate and chemical composition often observed (Jolly *et al*., 2003; Comitini *et al*., 2010). Furthermore Comitini et al. (2010) noted a significant decrease in volatile acidity and in total acidity of the final wines.

Other yeast of oenological interest isolated from grape surfaces of Uva di Troia belonged to *Hanseniaspora* spp., mainly *H. guilliermondii* and *H. uvarum*. Our results confirmed results previously reported on literature, in fact Cadež et al. (2010) demonstrated that the apiculate *H. uvarum/K. apiculata* appears to be the most common grape berry species worldwide, this results in other is consistent with its predominance in the beginning of spontaneous must fermentations.

In other was also investigated the biodiversity of several apulian grape cultivars, during spontaneous fermentation at the beginning and at the end of AF. The majority of the strains isolated at the beginning of AF belong to *Hanseniaspora spp.,* in particular *H. guilliermondii* and *H. uvarum*. Other yeast well represent on grape juice at the beginning of AF are *Candida spp.*. Among *Candida spp.* the species most important identified are *C. stellata* and *C. zemplinina.* Several yeast ecology studies demonstrated the frequent presence of this species in wine fermentations (Brezna et al., 2010; Li et al. 2010; Magyar & Bene, 2006; Nisiotou & Nychas, 2007; Tofalo et al., 2009; Tofalo et al., 2012; Urso et al., 2008; Zott et al., 2008), is a typical contaminant of botrytised juice fermentations but its dissemination is also spread to sound grapes (Barata et al., 2008). While all yeast isolated at the endo of AF belonged to *S. cerevisiae* strains, confirmed by RFLP-PCR analys and specie-specific PCR. Amplification of interdelta region revealed an high biodiversity among strains collected, in fact this method, reported in literature as the most suitable tool to differenziate at strain level *S. cerevisiae*

strains (Schuller *et al*., 2004; Capece *et al*., 2012), allows to distinguish 86 different profiles among 90 strains analyzed.

Technological characterization of selected *Saccharomyces cerevisiae* and non-*Saccharomyces* strains was perform with the aim to design multi-strain autochthonous starter cultures and increase the 'unique' qualities of apulian wines. The first step of technological characterization (e.g., killer activity, H_2S production, fermentation kinetics in synthetic medium and in must, cytofluorometric analysis) led us to select the most promising *S. cerevisiae* and non-*Saccharomyces* strains, mainly belong to *Hanseniaspora* and *Candida* species, and to assess the performance of possible co-inoculation approaches. However, using two classical strategies of inoculum and planned to promote the non-*Saccharomyces* 'expression', a strong competition took place with some of the non-*Saccharomyces* strains, compromising an efficient alcoholic fermentation. An effective development of the *S. cerevisiae* strains was only reached when the concentration of the non-*Saccharomyces* strains in the co-inoculation approaches was strongly reduced. Our observations suggest the presence of "robust" competitors between non-*Saccharomyces* strains of oenological importance that may negatively affect wine fermentation.

50 *O. oeni* strains isolated from spontaneous MLF wines were identified by specie-specific PCR and genotipically characterized by VNTR and MLST analysis. The VNTR method is highly discriminating, easy to interpret and facilitates the rapid and reliable typing of *O. oeni* strains, using only five tandem repeat regions. Our results confirmed those reported by Claisse *et al*. (2012) that demonstrated that the VNTR technique is the most discriminanting method used for *O. oeni* typing, better both than PFGE and MLST techniques. In fact VNTR technique allow us to distinguish 30 different VNTR profiles among 50 isolates analyzed, while MLST allows distinguishing only 8 different STs among 20 strains analyzed. Nevertheless is important to inderline that MLST tecniques allows to found six new STs and two new alleles, respectively for gene *rpoB* (ST 5) and *purK* (ST 8).

Future prospective will provide genome sequencing of several *O. oeni* strains isolated from apulian wines, with the aim to increase knowledge on genetic biodiversity of autochthonous *O. oeni* strains.

Finally, we investigated malolactic performances of all *O. oeni* strains in association with two *S. cerevisiae* strains, named I6 and E4, selected for their fermentative behavior, technological features and stress tolerance to design autochthonous microbial resources for typical wines.

O. oeni strains shown different malolactic performances, differences can be due both to different yeast-bacteria associations or timing of inoculum. For instance, some *O. oeni* strains performed an improved MFL when associated with *S. cerevisiae* I6, while an improved MFL was observed when others *O. oeni* strains were associated with *S. cerevisiae* E4. Simultaneous inoculation (co-inoculation) allowed *O. oeni* strains to degrade completely malic acid in 10 or 20 days, while for sequential inoculation the behavior in terms of efficiency of MLF was quite different for each strain. Indeed, for some strains, the complete degradation of malic acid failed, while other strains complete MLF in about 35-40 days. Co-inocultation treatments allow a rapid and complete MLF with positive consequence on final wine obtained, in fact several authors suggested that wines obtained with simultaneous AF/MLF usually are less buttery, retain more fruitiness and are more complex and better structured, levels of acetic acid are higher but sensorial insignificant (Henick- Kling, 1993; Bartowsky *et al*., 2002b; Jussier *et al*., 2006; Krieger, 2006). Other benefits of simultaneous inoculation is a more efficient MLF in 'difficult' wines (e.g. low pH) due to low levels of ethanol and higher nutrient concentrations. Wines are also immediately available for racking, fining and SO2 additions (Davis *et al*., 1985; Jussier *et al*., 2006).

Industrial scale up confirmed preliminary results obtained with microvinification assays and allow us to select two *O. oeni* strains, choosed for their malolactic performances, as potential starter cultures. In addition best microbial resoureces (*S. cerevevisiae*, non-*Saccharomyces* and *O. oeni* spp.) identified and characterized in this work will be tested to improve organoleptic properties of final wine, in particular with the aim to inibihite various spoilage microrganism, mainly yeast and bacteria, that can impart negative off-odors to wine during several steps of winemaking.

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7. APPENDIX

Publications

Capozzi V., **Garofalo C**., Di Toro M.R, Acconciaioco A., Massa S., Beneduce L., Spano G., (2012). Management of microbial resource for autochthonous wine grape varieties. III Convegno Nazionale Società Italiana di Microbiologia Agraria, Alimentare e Ambientale (SIMTREA), Bari, 26-28 June 2012.

Garofalo C., (2012). Management of microbial resources for the production of typical Apulian wines. XVII Workshop on the *Developments in the Italian PhD Research on Food Science Technology and Biotechnology*, University of Bologna, Cesena, 19-21 September, 2012.

Garofalo C., El Khoury M., Patrick L., Beneduce L., Grieco F., Spano G., and Capozzi V. (2013). Biodiversity of *Oenococcus oeni* strains isolated from Apulian wines. *International Conference on Microbial Interactions in Complex Ecosystems*, Torino, October 23-25, 2013.

Garofalo C., Bely M., Grieco F., Spano G. and Capozzi V. (2013). Yeast microflora during spontaneous fermentations of 'Nero di Troia' wines. *International Conference on Microbial Interactions in Complex Ecosystems*, Torino, October 23-25, 2013.

C. Garofalo, M. Bely, M.R. Di Toro, F. Grieco, P. Lucas, G. Spano, V.Capozzi *(2013)* Autochthonous microbial resources for Apulian Nero di Troia wines. CBL 2013 -*19ème édition du Club des Bactéries Lactiques, Bordeaux October* 16-18, 2013.

National and international courses, congress and seminars

Course: Applied Statistic. Dr. Antonio Bevilacqua. University of Foggia, Department of food, agriculture and environmental sciences.

Seminar: Proteomica applicata ai batteri lattici e agli alimenti. Prof.ssa Maria De Angelis Università di Bari

Seminar: Il processo di produzione della birra. Prof. Stefano Buiatti - Università di Udine

Seminar: Dairy bacteriophages: How to turn a problem into a tool. Dr. Miguel Alvarez -Head of the Department of Biotechnology of Dairy Products, IPLA, Spanish National Research Council (CSIC), Oviedo (SPA)

Seminar: Nuove frontiere della scienza degli alimenti: applicazioni della spettroscopia di risonanza nucleare. Prof. F. Paolo Fanizzi - Università di Lecce

Seminar: Adaptative response of bacteria to stress: the case of *Oenococcus oeni*, the wine bacterium. Prof. Jean Guzzo - Equipe de Recherche en vigne et vin(REVV) Institut Jules Guyot Université de Bourgogne ,Dijon (FRA)

Seminar: Aspetti e problematiche della produzione di olive da tavola. Prof. Marco Poiana -Università di Reggio Calabria

Seminar: Tecniche molecolari per l'identificazione e la caratterizzazione di lieviti di interesse alimentare. Prof.ssa Angela Capece - Università della Basilicata

Seminar: *Oenococcus oeni*, the bacteria of wine malolactic fermentation. Prof. Patrick M Lucas - Università di Bordeaux (FRA)

Seminar: Workshop c/o Fiera Internazionale dell'Agricoltura, Foggia. Soluzioni innovative per lo sviluppo delle imprese pugliesi.

Seminar:"Selection of probiotic functional starter cultures for controlled food fermentation: case study on fermentation of African locust beans*"*.Dr. Irene Ouoba- London Metropolitan University**.**

I Workshop "*Collezioni microbiche e Biotecnologia"*. Prof.ssa Esperanza Garay, Direttrice della *Colección Española de Cultivos Tipos*(CECT, Valencia, Spagna), dott.ssa Cristina Varese, (Responsabile scientifico della *Mycotheca Universitatis Turinensis*-MUT, Torino).

Seminar: "*Ricerca interdisciplinare:caso o necessità*?" Prof. Claudio Tuniz ICTP Trieste.

Course: English course. Dr. Claire Moore (University of Foggia).

Course: Food Packaging Dr. Amalia Conte (University of Foggia).

Workshop: XVII Workshop on the *Developments in the Italian PhD Research on Food Science Technology and Biotechnology*, University of Bologna, Cesena, 19-21 September, 2012.

Course: Laboratory safety. Dr. Roberto Di Caterina. University of the Study of Foggia. March-April 2013.

Seminar: STAR Agro Energy Scientific and Technological Advancement in Research on Agro-Energy. "The fate of tar after biomass pyrolysis: a microbiological point of view". Dr. Lorenzo Brusetti, Faculty of Science and Technology Free University of Bozen, Bolzano. Auditorium, University of the Study of Foggia, Via Gramsci 79, Foggia, 5 April 2013

Seminar: Slow Biothecnologies slow food – "Made in Italy" landscape. "Alimenti, R&S e Territorio". Dr. Franco Biasioli. Fondazione Edmund Mach di San Michele all'Adige. University of the Study of Foggia, Aula Magna, Department of Agriculture, Food and Environment Sciences, Via Napoli 25, Foggia, 12 June 2013

Seminar: Slow Biothecnologies slow food – "Made in Italy" landscape. "System Design: progettazione e sviluppo sostenibile". Prof. Luigi Bistagnino. Politecnico di Torino. University of the Study of Foggia, Aula Magna, Department of Agriculture, Food and Environment Sciences, Via Napoli 25, Foggia, 12 June 2013

Seminar: Slow Biothecnologies slow food – “Made in Italy” landscape. “Indicazioni Geografiche: buono, pulito, giusto e in fermento.” Prof. Cinzia Scaffidi. Università di Scienze Gastronomiche di Pollenzo e Centro Studi Slow Food. University of the Study of Foggia, Aula Magna, Department of Agriculture, Food and Environment Sciences, Via Napoli 25, Foggia, 20 June 2013

Seminario: The New Frontier of Consumer Research Welfare Effects of Europe’s Nutrition and Health Claims Regulation: the Italian Yogurt Market. Alessandro Bonanno (Wageningen University). University of the Study of Foggia, Aula Magna, Department of Agriculture, Food and Environment Sciences, Via Napoli 25, Foggia, 1 July 2013

Seminar: The New Frontier of Consumer Research Consumer Perception of Health Claims: an application of the Protection Motivation Theory Giuseppe Nocella (Reading University). University of the Study of Foggia, Aula Magna, Department of Agriculture, Food and Environment Sciences, Via Napoli 25, Foggia, 1 July 2013

Seminar: The New Frontier of Consumer Research Consumers' Attitudes and Acceptance of new products: the new frontier of research methodologies Rosalba Riolo (Hypatia Marketing and Digital Research). University of the Study of Foggia, Aula Magna, Department of Agriculture, Food and Environment Sciences, Via Napoli 25, Foggia, 1 July 2013

Visiting PhD student

ISVV (Institut des Sciences de la Vigne et du Vin) de l'Université de Bordeaux, (Bordeaux, France), on genotyping characterization of *O. oeni* strains isolated from typical Apulian wine. Oenology Research unit, Prof. Patrick Lucas, October

Other pubblications

Bove P., Capozzi V., **Garofalo C**., Rieu A., Spano G., Fiocco D. (2011). Inactivation of the *ftsH* gene of *Lactobacillus plantarum* WCFS1: Effects on growth, stress tolerance, cell surface properties and biofilm formation. *Microbiol. Res.* 167, 187-93.

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