# ADAPTATION AND DEVELOPMENT OF CULTURE-INDEPENDENT TECHNIQUES FOR THE INDENTIFICATION AND ENUMERATION OF MICROORGANISMS IN WINE FERMENTATIONS

**Immaculada Andorrà Solsona**

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IMMACULADA ANDORRÀ SOLSONA

# Adaptation and development of culture-independent techniques for the identification and enumeration of microorganisms in wine fermentations

**TESI DOCTORAL**

Dirigida pel Dr. Albert Mas Baron
Dr. José Manuel Guillamón Navarro
Dr. Braulio Esteve Zarzoso

DEPARTAMENT DE BIOQUÍMICA I BIOTECNOLOGIA
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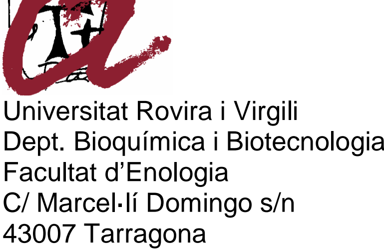

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Els sotasignants, El Dr. Albert Mas Baron, Catedràtic d'Universitat de Nutrició i Bromatologia, Departament de Bioquímica i Biotecnologia de la Universitat Rovira i Virgili, Dr Braulio Esteve Zarzoso, Personal de Suport a la Recerca del Departament de Bioquímica i Biotecnologia de la Universitat Rovira i Virgili i Dr José Manuel Guillamón Navarro, Investigador titular del Departmento de Biotecnología de los Alimentos, Instituto de Agroquímica y Tecnología de los Alimentos (CSIC).

FAN CONSTAR,

Que el present treball, amb títol

**Adaptation and development of culture-independent techniques for the identification and enumeration of microorganisms in wine fermentations**

que presenta la Srta. Immaculada Andorrà Solsona, per optar al

Grau de Doctora per la Universitat Rovira i Virgili ha estat realitzat sota la nostra direcció, i que tots els resultats obtinguts són fruit dels experiments duts a terme per l'esmentada doctoranda. I perquè se'n prengui coneixement i tingui els efectes que correspongui, signem aquesta certificació.

| Dr. Albert Mas Baron | Dr. José Manuel Guillamón Navarro | Dr. Braulio Esteve Zarzoso |
|---|---|---|

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tornarem a lluitar

tornarem a sofrir

tornarem a vèncer.

Lluís Companys

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## Agraïments,

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Als meus pares
al Jordi
i al Pedro

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# OBJECTIVES

# &

# EXPERIMENTAL DESIGN

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## OBJECTIVES

Wine composition and quality are the result of many different variables; some of them are microbiologically mediated. There is a need, then, to undestand and control the microorganisms that conduct the fermentation, as well as all the microorganisms that can be found at any stage during winemaking, ageing or storage.

The Oenological Biotechnology Group of the Rovira i Virgili University focuses on the use of various methods to determine, quickly and efficiently, the microbial population during wine production. When I joined this Group, some studies had already been done to quantify and characterise wine microorganisms by using molecular methods instead of classical physiological techniques. These methods are faster, more reliable, more specific, more sensitive, etc. However, most of them still need the microorganisms to be cultived previously so only the culturable microorganisms were analyzed. The next step was to use culture-independent techniques to detect and quantify wine microorganisms, avoiding the bias associated with culturability, and then go on to use these culture-independent techniques to detect and quantify only living cells (culturable cells, damaged cells, and viable but non culturable cells), not dead cells.

The working hypothesis of the present study is that **culture-independent techniques can detect microorganisms that have been overlooked by classical microbiological methods**. To demonstrate this hypothesis our general aim was to adapt or develop a variety of culture-independent techniques to identify and quantify the microorganisms in winemaking, study the impact of different wine microorganisms on the fermentation, and finally use these culture-independent techniques to detect and quantify live cells and distinguish them from dead cells. This general objective can be divided into the following partial objectives:

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1. To develop culture-independent molecular techniques to identify and quantify wine microorganisms

1.1. Detection and enumeration of wine microorganisms by QPCR, DGGE and direct cloning of a ribosomal fragment

One of the aims was to increase the information obtained with QPCR. Various primers had already been developed for quantifiing general yeast, *Saccharomyces*, *Hanseniaspora*, and lactic and acetic acid bacteria. However, other yeasts are also important in wine productuction. The development of new primers or sequences of relevant microorganisms would be a goal of the studies with this technique, in order to have a more accurate approach to the population dynamics of a given process.

Nevertheless, one drawback of this technique is that it analyzes only one species (or group of species), but it is not appropiate for analysing the whole range of microbiota. Thus, complementary techniques will be applied: DGGE, as a robust technique that had been used previously, and direct cloning of the amplified 5.8S-ITS rDNA region of the yeast into *E. coli*.

1.2. Improvement of the culture-independent techniques in order to differentiate between live and dead cells in wine fermentation

The aim of this part was to prevent the microorganism population from being overestimated because of the dead microorganisms in the wine at different stages of fermentation. Some dead microorganisms maintain DNA for long periods of time and are counted when culture-independent techniques using DNA are applied. The fluorescence *in situ* hybridization (FISH) technique will be directly applied to wine samples. As the target is ribosomal RNA, the presence of this molecule could be directly related with a viable cell with metabolic activity. Another approach for evaluating viability is to use dyes that selectively bind to the DNA of dead cells, thus avoiding the amplification of this DNA, in conjunction with the QPCR technique.

2. To validate the methodologies: Application of culture-independent molecular techniques to monitor different wine fermentations

The aim was to validate these methods in vinification conditions. This application aimed to understanding the effect of different oenological practices on the microbial

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population and the contribution of different wine microorganisms to the final wine product.

3. To study the effect of coinoculation of the main wine yeast species

The objective of this part was to follow the yeast population and differentiate the effects of yeast species by fermenting with a single species and mixed inocula. This is an interesting oenological practice that can improve wine flavour.

## EXPERIMENTAL DESIGN

To reach the objective proposed, we use the following experimental design:

1. Development of culture-independent techniques (DGGE, QPCR and direct cloning of ribosomal fragments) to be applied on wine microorganisms

The culture-independent methods adapted or developed were DGGE, QPCR and cloning of the amplified ribosomal fragment. The first one was adapted to study the microbial diversity of a complex matrix, such as wine. Ribosomal fragments were directly cloned to study the diversity of a sample, and its relative quantification. Finally QPCR is a good tool for quantifying different microorganisms, but it requires specific primers, which makes the technique very specific. We developed specific primers for *C. zemplinina* to quantify one of the main yeasts present in wine fermentations.

The results are reported chapter 1 and 2.

2. Validation of these techniques in wine fermentations

The objective was to validate DGGE and QPCR techniques and understand the effect of yeast inoculation and $SO_2$ on the microbial population when these culture-independent methods are used. These oenological practices have proved to restrict the growth of non-*Saccharomyces* and bacterial species with culture-dependent methods.

The results are presented in chapter 1.

Furthermore, the validation of QPCR and direct cloning of ribosomal fragment were done and compared with DGGE and plating methods. These techniques were applied to analyse microorganisms in wine fermentations conducted at different

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temperatures. A low fermentation temperature (13ºC) was compared with optimal temperature for yeast growth (25ºC).

The results are shown in chapter 2.

3. Effect of coinoculation of the main yeasts in fermentations

Inoculating the main wine yeasts can have interesting effects on wine characteristics and population dynamics, and these effects were monitored by plating and QPCR.

The results are reported in chapter 3 and 4.

4. Development of other culture-independent techniques to differentiate viable yeast populations

The development of the above mentioned techniques, however does not solve a key question relevant in oenology: the possibility that viable but non-culturable cells will spoil wines. Two techniques have been developed to monitor this viable population in wine fermentations. First the FISH technique (using probes against the rRNA) was used with fluorescence microscopy and flow cytometry. And second, the QPCR technique was combined with dyes (EMA and PMA) of different membrane permeabilities.

The results are presented in chapter 5 and 6.

5. Validation of the combination of dyes and QPCR in wine fermentations, storage and ageing wines

The combined EMA-, PMA-QPCR technique was validated by monitoring a spontaneous wine fermentation, and applied to some ageing and stored wines.

The results are shown in chapter 5.

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# INTRODUCTION

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# 1. Wine fermentation

## 1.1. Wine fermentation process

The winemaking process starts in the vineyard and the viticulture practices used. Of particular importance are the cultivar, soil quality, water management and the variety of *Vitis vinifera*. When the grapes are considered to have acquired optimal ripeness, they are collected. The way in which they are harvested may have an influence on the final product. Then, the wine fermentation starts, varying as a function of whether white, rosé or red wines are to be produced. The main steps in this process are represented in Figure 1. And, finally, the wine can be aged and/or stored.



Figure 1. The main steps in winemaking (adapted from Pretorius, 2000)

The must largely consists of sugars, mainly glucose and fructose. Organic acids are also important in must composition: in particular tartaric and malic acid, citric and lactic acid to a lesser extent, and also as traces of succinic and keto acids. Furthermore, it contains nitrogen compounds, other mineral salts and vitamins. Finally, phenolic compounds and aromas contribute to wine aroma, although they do not play an

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essential role in fermentation kinetics. The concentrations of the compounds present in a standard must are listed in Table 1, although they can vary considerably.

Table 1. Average chemical composition of grape must (Henschke & Jiranek, 1993)

| *Component* | *Amount per litre* | *Component* | | *Amount per litre* |
|---|---|---|---|---|
| **Carbon sources**[a] | | **Nitrogen sources**[b] | **% Nitrogen**[c] | |
| Glucose | 200 g | Alanine | 15.7 | 100 mg |
| **Salts** | | Arginine | 32.2 | 750 mg |
| K Tartrate | 5 g | Asparagine | 21.2 | 150 mg |
| L-Malic acid | 3 g | Aspartic acid | 10.5 | 350 mg |
| Citric acid | 0.2 g | Glutamic acid | 9.5 | 500 mg |
| $K_2HPO_4$ | 1.14 g | Glutamine | 19.2 | 200 mg |
| $MgSO_4.7H_20$ | 1.23 g | Glycine | 18.6 | 50 mg |
| $CaCl_2.2H_2O$ | 0.44 g | Histidine | 27.1 | 150 mg |
| **Trace minerals** | | Isoleucine | 10.7 | 200 mg |
| $MnCl_2.4H_2O$ | 198.2 µg | Leucine | 10.7 | 300 mg |
| $ZnCl_2$ | 135.5 µg | Lysine | 19.2 | 250 mg |
| $FeCl_2$ | 32.0 µg | Methionine | 9.4 | 150 mg |
| $CuCl_2$ | 13.6 µg | Phenylalanine | 8.5 | 150 mg |
| $H_3BO_3$ | 5.7 µg | Proline | 12.2 | 500 mg |
| $Co(NO_3)_2.6H_2O$ | 29.1 µg | Serine | 13.3 | 400 mg |
| $NaMoO_4.2H_2O$ | 24.2 µg | Threonine | 11.8 | 350 mg |
| $KIO_3$ | 10.8 µg | Tryptophan | 13.7 | 100 mg |
| **Vitamins** | | Tyrosine | 7.7 | 20 mg |
| Myo-Inositol | 100 mg | Valine | 12.0 | 200 mg |
| Pyridoxine.HCl | 2 mg | Diammonium phosphate[d] | 21.2 | |
| Nicotinic acid | 2 mg | | | |
| Ca Pantothenate | 1 mg | **Lipids**[e] | | |
| Thiamin.HCl | 0.5 mg | Ergosterol | | 10 mg |
| *p*-amino benzoic acid | 0.2 mg | Tween 80® | | 0.5 ml |
| Riboflavin | 0.2 mg | | | |
| Biotin | 0.125 mg | | | |
| Folic acid | 0.2 mg | | | |

a Equimolar concentrations of glucose and fructose

b Medium given contains 4,87g amino acids/l

c Nitrogen content of source

d Ammonium chloride may be used; typically 100mg/l (26,2 mg N/l) for grape juice.

e Optional.

The wine fermentation process transforms the must into wine. This biotransformation consists mainly of two fermentations. First, the alcoholic fermentation and then the malolactic fermentation. The alcoholic fermentation is conducted by yeasts and converts sugars into ethanol, carbon dioxide and other minor metabolites. And the malolactic fermentation converts malic acid into lactic acid and $CO_2$ by lactic acid bacteria (LAB). However, the wine fermentation process is complex and sophisticated, and several pathways are involved in the fermentation.

Yeasts can degrade sugars by two metabolic pathways: fermentative and oxidative. Glycolysis is the common route for both these processes (Figure 2). The sugars in grape juice are metabolized to pyruvate by the enzymes of the glycolytic pathway. Afterwards, in the fermentative one, the pyruvate is decarboxylated to acetaldehyde, which is reduced to ethanol, whereas, in the respirative pathway, the pyruvate that arose from glycolysis undergoes an oxidative decarboxylation in the presence of coenzyme A inside the mitochondria. The respiration can take place at a low sugar concentration and in the presence of oxygen. However, for high glucose concentrations (above 9 g/l approximately), yeasts only metabolise sugars by the fermentative pathway. Even in the presence of oxygen, respiration is blocked. This phenomenon is known as the Crabtree effect, catabolic repression by glucose or the Pasteur contrary effect (Ribéreau-Gayon et al., 2006). The high sugar concentration of the grape must makes the fermentative pathway the main sugar catabolic route. In a standard fermentation, one molecule of sugar (glucose/fructose) yields two molecules of ethanol and carbon dioxide. However, only 90-95% of the sugar is converted into ethanol and carbon dioxide, 1-2% into cellular material and 4-9% into other secondary metabolites such as glycerol, succinic, lactic and acetic acids, fusel alcohols and esters (Boulton et al., 1996). Fermentation activity decreases under stressful fermentation conditions, such as nutrient limitation, low pH, lack of oxygen, extreme temperatures, and the presence of toxic substances.

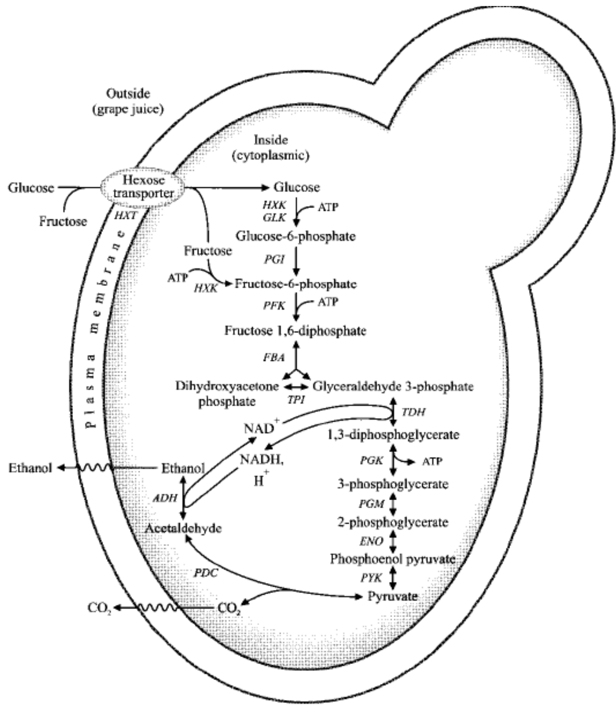


Figure 2. Glycolytic pathway in wine yeast (Pretorius, 2000)

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Quantitatively, assimilable nitrogen is the second nutritive source in the grape must, after carbon, and it is essential for yeast development and fermentation activity. A common cause of poor fermentative performance and sluggish or stuck fermentations, then, are deficiencies in the assimilable nitrogen compounds. The ammonium and amino acid content of grape juice varies as a consequence of different viticultural practices, soil, grape variety, oenological practices, etc (Ribéreau-Gayon et al., 2006) and regions. *Saccharomyces* can use different nitrogen sources for growth, but not all of them support growth equally well. Good nitrogen sources are ammonium, glutamine and asparagine whereas alanine, arginine, proline and urea are poor ones. The presence of good nitrogen sources decreases the level of enzymes and permeases required for the utilization and uptake by poorer nitrogen sources, this phenomenon is known as nitrogen catabolite repression (NCR) (Magasanik et al., 1992). During wine fermentation, yeasts change their metabolism from a nitrogen repressed situation to a nitrogen derepressed situation, as a function of the availability of nitrogen compounds. The nitrogen repressed/derepressed conditions determine the different patterns of ammonium and amino acid consumption. Beltran et al. (2004) found that glutamine and tryptophan are the main amino acids consumed throughout the fermentation. Arginine, alanine, aspartate, glutamate and glycine are the amino acids that are most affected by the NCR, and these amino acids are hardly consumed when there is an excess of nitrogen. Amino acid consumption, then, depend on such factors as the amount of nitrogen, the winemaking practices used in the fermentation and fact that different wine yeasts have very different nitrogen requirements. The consequences of these factors affect the production of higher alcohols and their associated esters. The general representation of the degradation of nitrogenous compounds by wine yeast is shown in Figure 3.

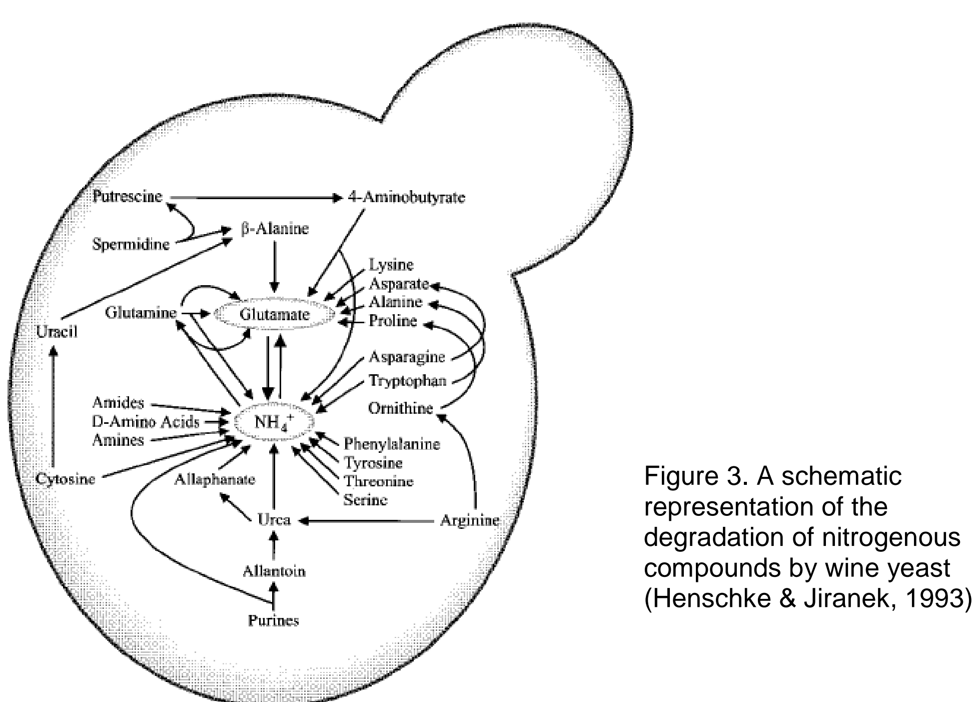


Figure 3. A schematic representation of the degradation of nitrogenous compounds by wine yeast (Henschke & Jiranek, 1993)

### 1.2. Final composition of wine

The chemical composition of the final product determines the sensory appreciation of the wine or wine flavour. The wine flavour includes varietal flavour (coming from the grapes), pre-fermentative flavour (which depends on the extraction operations and the conditioning of must), fermentative flavour (produced by yeasts and bacteria during alcoholic and malolactic fermentations) and post-fermentative flavour (which depends on the ageing process) (Schreier, 1979; Boulton et al., 1996; Rapp, 1998). Thus, the quality of the wine is determined by several factors, including viticultural practices, winemaking techniques and the yeast strains used (Boulton et al., 1996). So, the organoleptic profile is determined by the presence or absence of flavour compounds and metabolites.

The main fermentative products are ethanol, carbon dioxide and glycerol. However, wine contains numerous volatile and non-volatile end-products that contribute to the aroma and flavour characteristics of the wine. The synthesis of these compounds is detailed in Figure 4 (Swiegers et al., 2005). Ethanol is the main volatile product of yeasts metabolism, followed by diols, higher alcohols and esters. Ethanol determines the viscosity of the wine and acts as a fixer of aroma. However, in some given conditions, where acetaldehyde is not available to be reduced to ethanol, the dihydroxyacetone, formed during glycolysis, is reduced to glycerol by glycerol-3-

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phospate. Meanwhile, pyruvic acid participates in the formation of secondary products such as diacetyl, keto acids, succinic acid, butanediol, etc. (Ribéreau-Gayon et al., 2006). Succinic acid and glycerol are two of the most important by-products affecting the "body" of the wine. The main non-volatile acids are similar to those in the must, but in different proportions because of the fermentation (Radler, 1993; Boulton et al., 1996). Among these non-volatile acids, tartaric acid varies little or not at all during fermentation. The concentration of malic acid, on the other hand, usually decreases initially as a consequence of yeast metabolism (limited decrease) but later, during the malolactic fermentation, as a consequence of LAB metabolism. Succinic acid is the main acid produced by yeasts and its formation is strain dependent (Radler, 1993; Coulter et al., 2004)

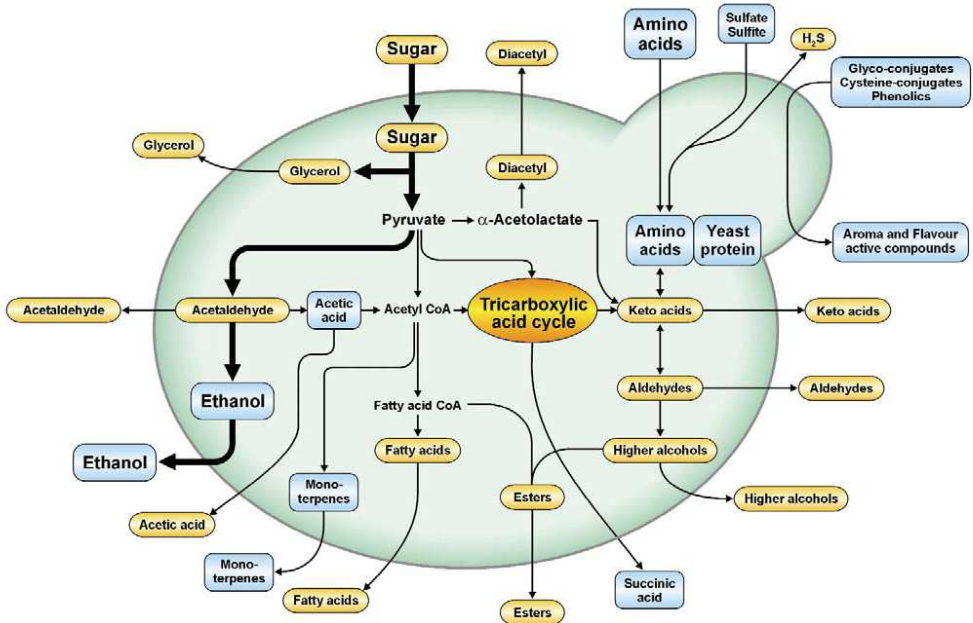


Figure 4. A schematic representation of derivation and synthesis of flavour-active compounds from sugar, amino acids and sulfur metabolism by wine yeast (Swiegers et al., 2005)

Of the volatile acids, acetic acid is the most important, this represents more than 90% of the volatile acid of wine (Henschke & Jiranek, 1993; Radler, 1993) and it is one of the most important by-products that negatively affect the analytical profile of wine. It is synthetised by yeasts and acetic acid bacteria, its concentration limit in wine may not be higher than 1.0-1.5 g/l. The rest of the volatile acids, principally propionic and hexanoic acids, are produced as the result of fatty acid metabolism by yeast and bacteria (Swiegers et al., 2005).

Another important, but not always desirable, secondary compound of wine fermentation is acetaldehyde. This compound is the product of the decarboxylation of pyruvate during the alcoholic fermentation. Wines containing amounts of 500 mg/l are considered unmarketable (Romano et al., 2006).

Higher alcohols represent another group of secondary products influencing the analytical profile of the wine. The concentrations of higher alcohols are influenced by such factors as the yeast strains, the concentration of amino acids (the precursors for higher alcohols), ethanol concentration, fermentation temperature, pH, composition of grape must, aeration, etc. (Swiegers et al., 2005). The first step in the synthesis of higher alcohols involves the synthesis of α-keto acids derived from branched-chain amino acids that are transaminated to the respective α-keto acids (leucine to α-ketoisocaproic acid, valine to α- ketoisovaleric acid, and isoleucine to α-keto-β-methylvaleric acid). The pyruvate decarboxylase converts the resulting α-keto acid to the corresponding branched-chain aldehyde with one carbon-less atom and the alcohol dehydrogenase catalyses the NADH-dependent reduction of this aldehyde to the corresponding fusel alcohol. Alternatively, the aldehyde might be oxidised to an acid (Derrick & Large, 1993). Higher alcohols are also important precursors for ester formation. Higher alcohols and their esters are associated with a pleasant aroma; although at high concentrations they can be undesirable.

Apart from the many biosynthetic pathways of yeast, LAB are also involved in the formation of wine aroma. LAB development and aroma production is affected by such factors as the composition and pH of grape must and, of course, the fermentation temperature. LAB play an important role in winemaking and modulates the chemistry, aroma and flavour of wine by modifying its components and sensory properties (Figure 5). LAB decrease the wine acidity with the decarboxylation of malic acid to lactic acid, this is the basis of malolactic fermentation, (Laurent et al., 1994; Bartowsky et al., 2002). But LAB also contribute to the aroma of wine by metabolising other acids such as citric acid. The metabolisation of citric acid produces acetic acid and diacetyl, both of which have an important effect on wine flavour. Other metabolites affected by LAB metabolism and which have an impact on wine flavour, are alcohols such as glycerol and mannitol, or carbonyls as acetaldehyde and diacetyl. Finally, esters can also be modified for LAB species, esterase activity of wine-associated bacterial species is not well understood, although some researchers have observed the changes in ethyl ester

concentration after malolactic fermentation including ethyl acetate, ethyl hexanoate, ethyl lactate, and ethyl octanoate, as well as decreases in some esters (Laurent et al., 1994; de Revel et al., 1999; Delaquis et al., 2000; Gambaro et al., 2001).

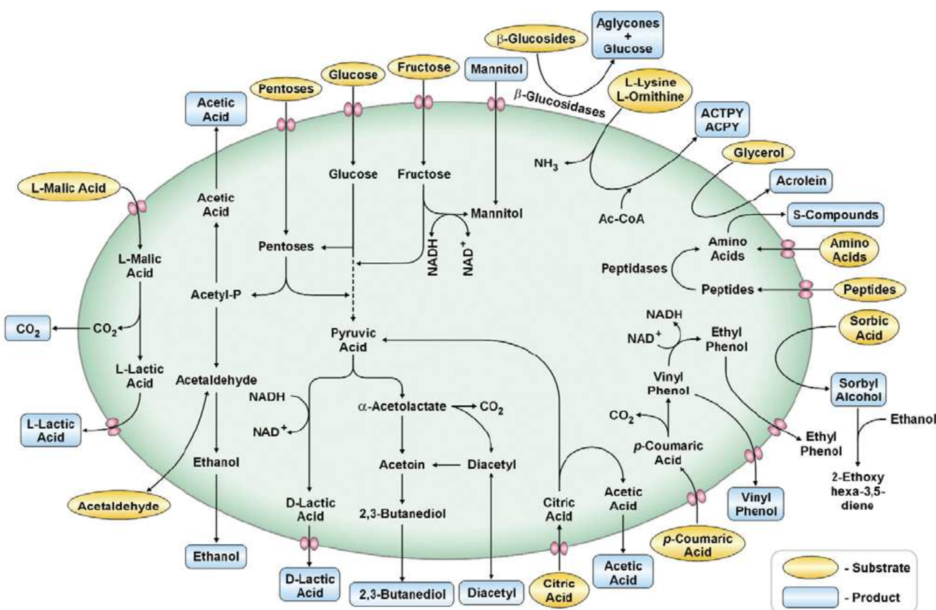


Figure 5. Biosynthesis and modulation of flavour-active compounds by lactic acid bacteria (Swiegers et al., 2005)

Meanwhile, acetic acid bacteria (AAB) are only spoilage microorganisms in winemaking, because they lead to the formation of such major oxidised products, as acetaldehyde, acetic acid and ethyl acetate (Drysdale & Fleet, 1989; Henick-kling, 1993; Bartowsky et al., 2002; Matthews et al., 2004).

Some esters, higher alcohols and other aroma and flavour compounds commonly found in wines are listed in Table 2, which also shows the wide concentration range of these compounds in wine and their perception threshold.

Table 2. Esters, higher alcohols, other aroma and flavour compounds (Swiegers et al., 2005)

| Compound | Concentration in wine (mg/L) | Aroma threshold (mg/L) | Aroma descriptor |
|---|---|---|---|
| Ethyl acetate | 22.5–63.5 | 7.5* | VA, nail polish, fruity |
| Isoamyl acetate | 0.1–3.4 | 0.03* | Banana, pear |
| 2-Phenylethyl acetate | 0–18.5 | 0.25* | Flowery, rose, fruity |
| Isobutyl acetate | 0.01–1.6 | 1.6**** | Banana, fruity |
| Hexyl acetate | 0–4.8 | 0.7** | Sweet, perfume |
| Ethyl butanoate | 0.01–1.8 | 0.02* | Floral, fruity |
| Ethyl hexanoate | 0.03–3.4 | 0.05* | Green apple |
| Ethyl octanoate | 0.05–3.8 | 0.02* | Sweet soap |
| Ethyl decanoate | 0–2.1 | 0.2***** | Floral, soap |
| Propanol | 9.0–68 | 500** | Pungent, harsh |
| Butanol | 0.5–8.5 | 150* | Fusel, spiritous |
| Isobutanol | 9.0–174 | 40* | Fusel, spiritous |
| Isoamyl alcohol | 6.0–490 | 30* | Harsh, nail polish |
| Hexanol | 0.3–12.0 | 4** | Green, grass |
| 2-Phenylethyl alcohol | 4.0–197 | 10* | Floral, rose |
| Acetic acid | 100–1150 | 280* | VA, vinegar |
| Acetaldehyde | 10–75 | 100** | Sherry, nutty, bruised apple |
| Diacetyl | <5 | 0.2** / 2.8*** | Buttery |
| Glycerol | 5–14 g/L | 5.2 g/L** | Odourless (slightly sweet taste) |
| Linalool | 0.0017–0.010 | 0.0015******/0.025***** | Rose |
| Geraniol | 0.001–0.044 | 5******/30* | Rose-like |
| Citronellol | 0.015–0.042 | 8******/100* | Citronella |
| 2-acetyl-1-pyrroline (ACPY) | Trace | 0.0001****** | Mousy |
| 2-acetyltetrahydropyridine (ACPTY) | 0.0048–0.1 | 0.0016****** | Mousy |
| 4-ethylphenol | 0.012–6.5 | 0.14*/0.6*** | Medicinal, barnyard |
| 4-ethyl guaiacol | 0.001–0.44 | 0.033*/0.11*** | Phenolic, sweet |
| 4-vinyl phenol | 0.04–0.45 | 0.02****** | phamaceutical |
| 4-vinyl guaiacol | 0.0014–0.71 | 10****** | Clove-like, phenolic |

* 10% etanol, ** wine, *** red wine, **** beer, ***** synthetic wine, ****** water

## 1.3. Microorganisms associated with wine fermentation

The biotransformation of grape must is a complex ecological and biochemical process involving the sequential development of such microbial species as fungi, yeast, LAB and AAB.

### 1.3.1. Yeasts

Yeasts are the most simple of eukaryotes. The yeast cell contains two cell envelopes: the cell wall and the membrane, separated by the periplasmic space. The cell wall essentially consists of polysaccharides, mainly β-glucans and mannoproteins. Its first function is to protect the cell. The plasma membrane is a highly selective barrier controlling exchanges between the living cell and its external environment. It is principally made up of lipids and proteins. The lipids of the membrane are essentially phospholipids and sterols; they are amphiphilic molecules (with a hydrophilic and a hydrophobic part). The principal phospholipids are phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidylinositol (PI), and to a lesser extent,

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phosphatidylserine (PS) and diphosphatidylglycerol (PG) or cardiolipin (CL). The subcellular compartmentalization of wine yeast is shown in Figure 6.

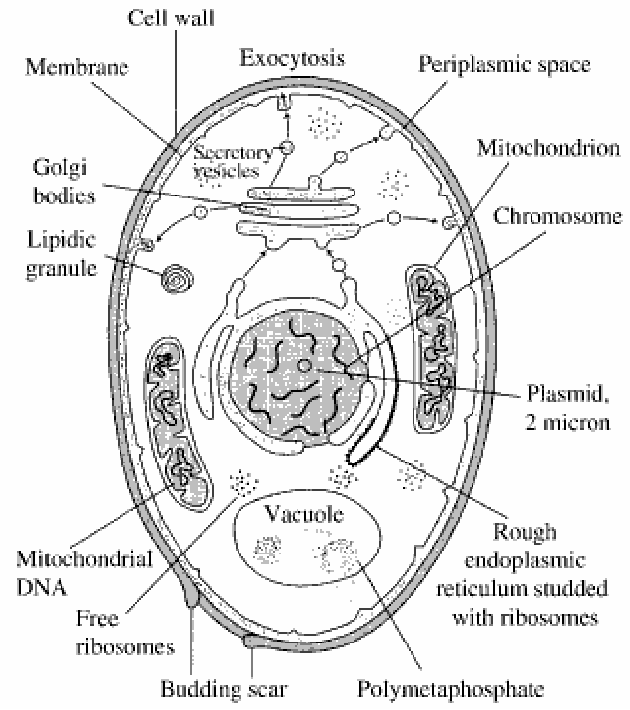


Figure 6. Yeast cell (Ribéreau-Gayon et al., 2006)

Yeasts are unicellular fungi and can be classified into two phylogenetic groups, teleomorphic and anamorphic ascomyceteous yeasts and teleomorphic and anamorphic basidiomycetous yeasts (Kurtzman & Fell, 1998). Yeasts can multiply either asexually by vegetative multiplication (budding or fission) or sexually by forming spores (sporulation). The absence of sexual spores during the life cycle means that this isolate is classified as an anamorph or asexual form (imperfect fungi). The presence of sexual spores indicates a teleomorph or sexual form (perfect fungi). The germination of the sexual spore produces a new vegetative cell, which may reproduce again by budding or fission. Yeasts belonging to the imperfect fungi can only reproduce by vegetative multiplication (Boekhout & Kurtzman, 1996).

Yeasts are the most important group of microorganisms for winemakers. *Saccharomyces* is the main yeast responsible for the alcoholic fermentation, although there are other genera and species present during winemaking.

The *Saccharomyces* genus is the most commonly used in industry. Species associated with industrial fermentation process are, *S. bayanus*, *S. cerevisiae*, and *S. pastorianus* and species isolated from natural habitats are, S. cariocanus, *S.*

*Kudriavzevii*, *S. mikatae* and *S. paradoxus* (Barrio et al., 2006). The *Saccharomyces* species cannot be well differentiated by physiological tests, only by their DNA sequences (Ribéreau-Gayon et al., 2006). *Saccharomyces* genera have a range of singular characteristics that are not found in other genera and their capacity to ferment sugars is considerable. This ability allows them to colonize sugar-rich media and compete with other yeasts, which are not so tolerant to alcohol (Barrio et al., 2006).

Non-*Saccharomyces* yeast are commonly known as wild yeast, because they are mostly present in grapes and at the beginning of the fermentation (Fugelsang & Edwards, 2007). There are around 15 non-*Saccharomyces* genera involved in wine fermentation. These are: *Dekkera* (anamorph *Brettanomyces*), *Candida*, *Cryptococcus*, *Debaryomyces*, *Hanseniaspora* (anamorph *Kloeckera*), *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Rhodotorula*, *Saccharomycodes*, *Schizosaccharomyces*, *Torulaspora* and *Zygosaccharomyces* (Pretorius et al., 1999). Most of the non-*Saccharomyces* wine-related species have low fermentation activity and a low $SO_2$ resistance (Ciani et al., 2010). However, they play play an important role in the metabolic impact and aroma complexity of the final product and also contribute to the enzymatic activities described for some non-*Saccharomyces* species (protease, β-glucosidase, esterase, pectinase and lipase) (Esteve-Zarzoso et al., 1998).

#### 1.3.2. Bacteria

The cellular structure of bacteria consists of a cell wall, a plasma membrane, the cytoplasm containing genetic material (chromosome and plasmids), ribosomes, and all the enzymatic equipment. The main differences between LAB and AAB are in the cell wall composition. Peptidoglycan is the principal constituent of Gram-positive cell walls (LAB), but it is less present in Gram-negative cells (AAB) in which the wall is composed of phospholipids, lipoproteins and lipopolysaccharides, like the plasma membrane.

The cell wall provides protection and shape to the cells, the plasma membrane has the classical structure of a lipid bilayer, and their fluidity depends on lipid-protein interactions. The principal genome of bacteria consists of a single circular chromosome of double stranded DNA suspended in the cytoplasm without any separation. Different species have different sizes of chromosome. Furthermore, these cells have plasmids to conduct different functions.

### 1.3.2.1. Lactic acid bacteria

LAB are Gram positive cells. This means that the cell wall is essentially composed of a peptidoglycan. The LAB cells tend to be round for cocci and elongated for bacilli. LAB are aerotoleant and mesophilics, and their optimal growth temperature is between 15-30ºC. The primary product of their glucose metabolism is lactic acid. The LAB of grape must and wine belong to the genera *Lactobacillus*, *Leuconostoc*, *Oenococcus* and *Pediococcus* (Ribéreau-Gayon et al., 2006). LAB can be homofermentative (producing exclusively lactic acid from glucose and/or fructose) and heterofermentative (producing carbon dioxide, ethanol, acetic acid and lactic acid from the same carbohydrates). *O. oeni* present a heterofermentative metabolism whereas *P. pentosaceus* and *P. damnosus* are homofermenative. *L. casei* and *L. plantarum* are described as facultative heterofermentative. Finally, *L. brevis* and *L. hilgardii* are strictly heterofermentative. *O. oeni* is the main LAB that conducts the malolactic fermentation (Ribéreau-Gayon et al., 2006).

### 1.3.2.2. Acetic acid bacteria

Generally AAB are catalase positive and Gram negative. They are very prevalent in nature and are well adapted to grow in sugar- and alcohol-rich media. They have an ellipsoidal or rod-like form, with small dimensions of 0.6-0.8 by 1-4 µm. They have locomotive organs that give them a mobility that is visible under the microscope. Their metabolism is strictly aerobic. Their principal property is that they oxidize ethanol into acetic acid. This is done in two steps: first to acetaldehyde and then to acetic acid.

AAB belong to the *Acetobacteraceae* family. They are separated into the genera: *Acetobacter*, *Gluconobacter*, *Gluconacetobacter*, *Acidomonas*, *Asaia, Kozakia*, *Swaminathania, Saccharibacter, Neoasaia, Granulibacter* and *Tanticharoenia* (Yukphan et al., 2009). The genus *Acetobacter* comprises the main oenological species: *A. aceti, A. pasterianus, A. malorum, A. pomorum, A. cerevisiae and A. oeni*, these species have all been isolated at some point during the winemaking process. The only species of the genus *Gluconobacter* that is important in winemaking is *G. oxydans*. And the species of the *Gluconacetobacter* genus that have been described in wine are: *Ga. liquefaciens*, *Ga. hansenii*, Ga*. xylinus*, *Ga. europaeus*, *Ga. oboediens* and *Ga. intermedius* (Guillamón & Mas, 2009).

### 1.3.3. Fungi

A wide range of fungi can infect grapes prior to harvest: for example, species of *Botryotinia*, *Uncinula*, *Alternaria*, *Plasmapara*, *Aspergillus*, *Penicillum*, *Rhizopus*, *Oidium* and *Cladosporum* (Fugelsang, 1997; Fleet, 2001). Some studies found that *Botryotinia fuckeliana* (or its anamorph *Botrytis cinerea)*, *Aspergillus* spp. and *Penicillum* spp. produce metabolites that retard the growth of yeasts during fermentation. Furthermore, fungal growth on grapes may contribute to the growth of some AAB on the grape surface (Ribéreau-Gayon et al., 2006).

## 1.4. Population dynamics of wine microorganisms

The microorganisms present in the berry surfaces are mainly yeasts. The microflora of grapes varies constantly in response to grape variety, climatic conditions, viticultural practices, stage of ripening, physical damage (caused by mould, insects and birds) and fungicides applied to vineyards (Pretorius et al., 1999). Although, grape must is relatively complete in nutrient content, the low pH and high sugar content leads to a selective media in which only a few bacteria and yeast species can grow. Furthermore, the addition of sulphur dioxide, a common winery technique, as an antioxidant and antimicrobial preservative imposes an additional selection, mainly against undesirable oxidative microbes. Another important factor is the restriction created by the anaerobic conditions when the fermentation starts (Henschke, 1997). The fermentation of grape juice to wine can be regarded as a heterogenous microbial process. The number of yeasts on the grape berry just before the harvest varies from $10^3$ to $10^6$ cells/ml (Romano et al., 2006) depending on different factors above mentioned. The predominant species on the surface of the grape berries are *Candida*, *Hanseniaspora, Hansenula, Metschnikowia* and *Pichia*. The population of the main wine yeasts *Saccharomyces cerevisiae* in grape juice is very low (Torija et al., 2001) (Figure 7). The non-*Saccharomyces* yeasts present in the grape juice could proliferate to final populations about $10^6$-$10^7$ cells/ml, and started to decline by mid-fermentation. During the latter stages of natural wine fermentation the strongly fermentative strains of *S. cerevisiae* become predominant and complete the fermentation. *S. cerevisiae* species are the most alcohol tolerant yeast and can reach populations up to $10^7$-$10^8$ cells/ml (Romano et al., 2006). Besides, some species of *Brettanomyces*, *Kluyveromyces*, *Schizosaccharomyces*, *Torulaspora* and *Zygosaccharomyces* may also be present

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during fermentation and in wine. Some of these yeast species are spoilage microorganisms because they produce metabolites with an undesirable impact (Pretorius, 2000).

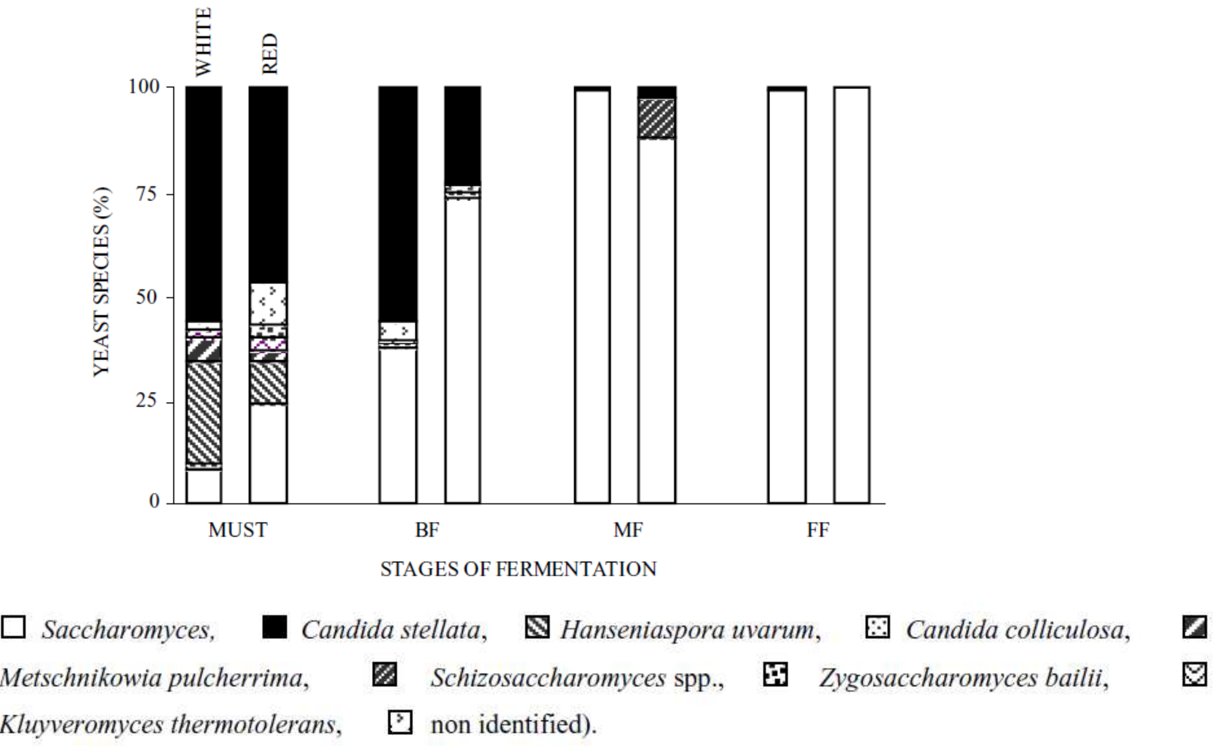


Figure 7. Biodiversity of yeast species during two alcoholic fermentations (white and red wine) analysed as a function of the vinification type. BF: Beginning of fermentation, MF: Mid fermentation, FF: Final of fermentation (Torija et al., 2001)

In the *Candida* genus Sipiczki (2004) described *Candida zemplinina* as a species that is very closely related to *C. stellata*. *C. zemplinina* grew much faster than the *C. stellata* strains at high sugar concentrations, at low temperatures and in the presence of ethanol, suggesting that they could easily overgrow *C. stellata*. Subsequently, Csoma & Sipiczki (2008) found that *C. zemplinina* was the most abundant species of the *Candida* genus in wine and must samples, although *C. stellata* has been mentioned in some studies (Torija et al., 2001; Di Maro et al., 2007; Xufre et al., 2006). According to Csoma & Sipiczki (2008), most of the wine yeasts identified and described in the literature as *C. stellata* might be *C. zemplinina*. The same authors proposed that response to 1% acetic acid is an easy physiological test that can be used in routine taxonomic differentiation of the strains of these species. *Candida zemplinina* grows in the presence of 1% acetic acid, which is inhibitory to *C. stellata*. However, their unequivocal identification requires

molecular techniques, either PCR-RFLP of the ITS-5.8 rRNA region or sequencing of the D1/D2 domain of the LSU rRNA gene. Csoma & Sipiczki (2008) proposed using the enzyme *Dra*I to obtain different species-specific patterns with the PCR-RFLP of the ITS-5.8 rRNA region.

Different types of LAB have been described during wine fermentation. In grapes, the main LAB belong to the species *Lactobacillus plantarum*, *L. hilgardii* and *L. casei*. The species *O. oeni*, which dominates the malolactic fermentation, is rarely detected at the beginning of the fermentation. During the first stages of alcoholic fermentation, the most common LAB are: *L. plantarum*, *L. casei*, *L. hilgardii*, *L. brevis*, *P. damnosus*, *P. pentosaceus*, *L. mesenteroides* and *O. oeni* (Figure 8). Must, after crushing, generally contains LAB population of $10^2$ to $10^4$ CFU/ml. Their behaviour at this time depends on the pH of the medium and the concentration of the $SO_2$ added to the must. The levels of sulphur dioxide used in winemaking do not completely inhibit the LAB growth but they do limit it. After the alcoholic fermentation, LAB remain in a latent phase for some time. The beginning of the malolactic fermentation depends on temperature, pH, ethanol, etc. The growth phase takes several days and increases the population to $10^7$CFU/ml. As soon as the malic acid is completely transformed to lactic acid, the bacterial population begins to decline. If the wine is not sulphited after malolactic fermentation, the bacterium remain for months (Lonvaud-Funel, 1999).

The AAB may also be present in winemaking. *Gluconobacter oxydans*, *Acetobacter aceti* and *A. pasteurianus* are mostly found in the course of winemaking and to a lesser extent *Gluconacetobacter liquefaciens* and *Ga. hansenii* (González et al., 2005). *G. oxydans* is present on grapes and disappears to give way to species of the genus *Acetobacter*, which subsists in wine (Lafon-Lafourcade & Joyeux, 1981).

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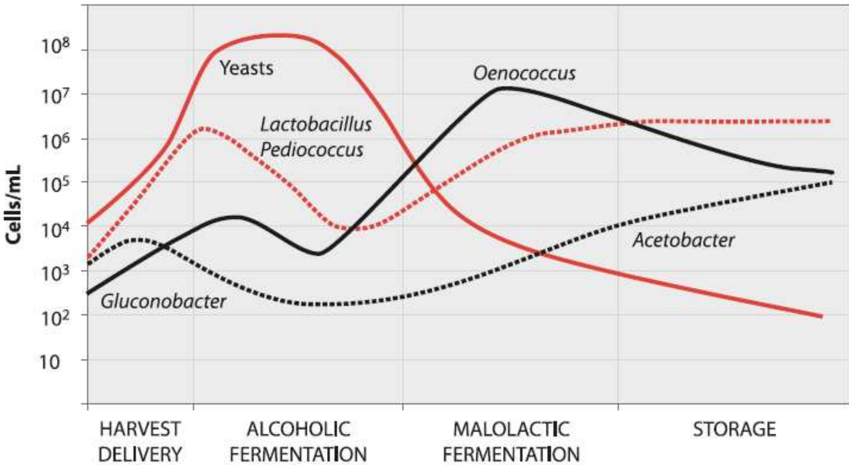


Figure 8. Microorganism evolution during wine fermentation process and storage (Krieger, 2005)

### 1.5. Principal factors affecting the growth of wine microorganisms

- pH

The low pH of grape must and wine is one of the main restrictions on selecting the microorganisms present in these media. This pH can oscillate between 2.8 and 4.2 (Heard & Fleet, 1988). This pH does not restrict wine yeasts growth. A pH below 2.8 can raise some problems, because it increases the toxicity of ethanol and sulphur dioxide (Pampulha & Loureiro-Dias, 1989). LAB can conduct the malolactic fermentation at the normal low pHs of wines. In high pH wines (>3.5), *Lactobacillus* spp. dominates, whereas *O. oeni* is present in higher relative populations at lower pH (Henick-kling, 1993). *O. oeni* is the most acid-tolerant LAB and it can survive and keep its metabolism at a pH as low as 2.9 (Bartowsky, 2005). The optimal pH for AAB is around 5.5-6.3 (Holt et al., 1994) although some AAB have been isolated at pH 3.0.

- Sulphur dioxide

Sulphur dioxide is usually used in winemaking because of its properties as an antimicrobial and antioxidant agent. $SO_2$ inhibits the development of most microorganisms although wine microorganisms present a different degree of sensitivity to this compound. Moreover, its antioxidant characteristic protects the compounds responsible for wine colour (polyphenols) against oxidation (Ribéreau-Gayon et al., 2006).

Molecular $SO_2$ is present in solution in a pH-dependent equilibrium with bisulphite ($HSO_3^-$) and sulphite ($SO_3^{2-}$) ions. Only the molecular $SO_2$ has anti-microbial effects. The proportion of molecular $SO_2$ is between 1% and 10% of the free form depending on

the pH of the wine. Therefore, the lower the pH is, the greater the proportion of molecular $SO_2$ and the higher the anti-bacterial effect (Ribéreau-Gayon et al., 2006). In comparison with other species, *Saccharomyces* species are relatively resistant to $SO_2$. Such non-*Saccharomyces* species as apiculate yeast are more sensitive to sulphur dioxide. For some species of LAB, 100 mg/l of total $SO_2$ is needed to hinder their development (Ribéreau-Gayon et al., 2006) although the pH and the strain used might change the levels of $SO_2$ required. The common levels of free $SO_2$ (40-60 mg/l) do not completely inhibit the growth of some AAB (Du Toit et al., 2005). Wantanabe & Lino (1984) found that 100 mg/l of total $SO_2$ was needed to inhibit the growth of *Acetobacter* species in grape must.

- Ethanol

Ethanol acts by modifying the cell active transport systems across the membrane, which reduces the assimilation of nitrogen and decreases yeast growth. It has been assumed that non-*Saccharomyces* die off earlier because they are more sensitive to ethanol than *S. cerevisiae* (Fleet & Heard, 1993; Boulton et al., 1996; Cocolin et al., 2000). However, some studies found that *Hanseniaspora*, *Candida* and *Kluyveromyces* species have ethanol tolerances similar to those of *S. cerevisiae* (Cocolin et al., 2001; Mills et al., 2002; Xufre et al., 2006; Nisiotou et al., 2007). LAB are increasingly inhibited at alcohol concentrations above 14%, and most strains tolerate the upper limit. Ethanol is sometimes the main energy source for AAB, although if the concentration is too high (more than 15%) it may inhibit the growth of AAB. It should be pointed out that it is difficult to generalise about the ethanol tolerance of the various bacteria or yeast species because it often varies among the strains of the same species.

- Temperature

The optimal temperature for *S. cerevisiae* is around 28ºC, although some usual wine non-*Saccharomyces* species have a lower optimal growth temperature of around 25ºC (Heard & Fleet, 1988). Red wine is usually fermented at 25-30ºC, because it is better for the color and phenol extraction. White wine, however, is fermented at a lower temperature (15-20ºC) to avoid the loss of volatile compounds. The toxicity of ethanol, then, increases with temperature (Torija et al., 2003). The higher sensitivity to ethanol of some non-*Saccharomyces* species accounts for their survival at low temperatures at which *Saccharomyces* may have limited growth. Heard & Fleet (1988) showed that *H.*

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*uvarum* and *C. stellata* retained high populations until the end of fermentations at low temperatures. These low temperatures may also enhance the aroma in the final wine because aroma compounds and the contribution of the non-*Saccharomyces* species increase throughout the process (Beltran et al., 2006). LAB are mesophyllic, with an optimal growth temperature between 15 and 30ºC. The rate of LAB growth and malolactic fermentation is strongly inhibited by low temperatures and generally warming up is required for the start of malolactic fermentation (Ribéreau-Gayon et al., 2006). The optimum growth temperature for AAB is around 25-30ºC, but AAB growth has been detected in wine conserved at 10 ºC (Joyeaux et al., 1984).

- Oxygen

Aeration is not detrimental to yeasts. However, this aeration accelerates yeasts growth because it does not limit the synthesis of unsaturated fatty acids and ergosterol. A lack of oxygen during the exponential phase of yeasts may produce sluggish or stuck fermentations (Bisson, 1999). LAB are defined as aerotolerant because oxygen does not limit their growth or can even stimulate it (Christensen et al., 1999). AAB, however, do require oxygen to growth. Their metabolism is strictly aerobic and oxygen limits their growth. Nevertheless, they can survive for prolonged periods under relatively anaerobic conditions in wine (Du Toit et al., 2005).

### 1.6. Impact of microorganisms on wine fermentation

Yeast modulates the wine flavour through the alcoholic fermentation, transforming grape components into flavour active components, producing new metabolites through autolysis, bioadsorbing components of grape juice and influencing the growth of LAB (Ciani et al., 2010).

The fermentation process can be conducted by the flora present on the grapes and in the winery, or inoculated with a commercial strain mostly *S. cerevisiae*. Inoculation will minimize the influence of the grape's microflora, although it is well known that the inoculation does not prevent the growth of the indigenous microorganisms (Fleet, 2008). The persistence of these non-*Saccharomyces* depends on such factors as fermentation temperature, nutrient availability, quantity of sulphur dioxide, etc (Constantí et al., 1998). Furthermore, other factors have been described that can also affect the persistence of non-*Saccharomyces* or the imposition of *Saccharomyces* species. Nissen et al. (2003) found that the death of two non-

*Saccharomyces* yeast was caused by cell-cell interactions (*T. delbrueckii* and *K. thermotolerans*). Pérez-Nevado et al. (2006) hypothesized that *S. cerevisiae* produce some toxic compounds that may cause the death of one specific non-*Saccharomyces* yeast, *H. guilliermondii*. And recently, Albergaria et al. (2010) have postulated that these toxic compounds are antifungal peptides secreted by *S. cerevisiae* against non-*Saccharomyces* species.

All the commercial inocula available on the market are strains belonging to the *Saccharomyces* genus. It is currently postulated that mixed cultures of *Saccharomyces* and non-*Saccharomyces* species might improve fermentation kinetics and the analytical composition of wine, which may lead to a more complex aroma (Bisson & Kunkee, 1993; Heard, 1999; Rojas et al., 2003; Romano et al., 2003; Ciani et al., 2006; Jolly et al., 2006). Non-*Saccharomyces* yeast produces such enzymes as esterases, glycosidases, proteases, etc, which can interact with grape precursor compounds to produce aroma active compounds. For this reason the non-*Saccharomyces* yeasts might play an important role in the aroma (Charoenchai, et al., 1997). However, the grape must and the fermentation process give an adaptative advantage to *S. cerevisiae*, the yeast with the most efficient fermentative catabolism (Swiegers et al., 2005).

Several studies have shown that non-*Saccharomyces* wine yeast makes a positive contribution to wine flavour (Fleet, 2003) or have evaluated the biotechnological interest of their enzymatic activities (Charoenchai et al., 1997; Esteve-Zarzoso et al., 1998; Fernandez et al., 1999). Several studies have analyzed the contributions of various microorganisms to wine flavour (Table 3). Bely et al. (2008) studied mixed culture fermentations and found that simultaneous mixed cultures with a ratio of 20:1 of *T. delbrueckii*-*S. cerevisiae* produced 53% and 60% reductions in volatile acidity and acetaldehyde, respectively. However, if the inoculation was sequential, first *T. delbrueckii* and later *S. cerevisiae*, had less effect on the reduction of these metabolities. Another author, Moreira et al. (2008) reported that the apiculate yeast enhanced the production of desirable compounds, such as esters, without increasing the undesirable heavy sulphur compounds. *H. uvarum* increased the proportion of isoamyl acetate whereas *H. guilliermondii* enhanced that of 2-phenylethyl acetate. Furthermore, the combined use of *S. cerevisiae* and non-*Saccharomyces* wine yeast has been proposed to enhance the glycerol content of wines (Ciani & Ferraro, 1996; Soden et al., 2000).

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Table 3. Mixed fermentation processes that have been proposed in winemaking using *S. cerevisiae* and non-*Saccharomyces* yeast (Ciani et al., 2010)

| Species used | Aim | Process | References |
|---|---|---|---|
| *S. cerevisiae*<br>*T. delbrueckii* | Reduction of acetic acid production | Sequential cultures | Castelli (1969); Herraiz *et al.* (1990); Ciani *et al.* (2006); Salmon *et al.* (2007); Bely *et al.* (2008) |
| *S. cerevisiae*<br>*S. pombe* | Malic acid degradation | Sequential cultures<br>Immobilized cells (batch process)<br>Immobilized cells (continuous process) | Snow & Gallender (1979); Magyar & Panyik (1989); Yokotsuka *et al.* (1993), Ciani (1995) |
| *S. cerevisiae*<br>*C. stellata* | Enhancement of glycerol content | Immobilized cells (pretreatment or sequential cultures) | Ciani & Ferraro (1996); Ciani & Ferraro (1998); Ferraro *et al.* (2000) |
| *S. cerevisiae*<br>*C. cantarellii* | Enhancement of glycerol content | Mixed or sequential cultures | Toro & Vazquez (2002) |
| *S. cerevisiae*<br>*C. stellata* | Improve wine aroma profile | Mixed or sequential cultures | Soden *et al.* (2000) |
| *S. cerevisiae*<br>*H. uvarum (K. apiculata)* | Simulation of natural fermentation (improvement of aroma complexity) | Mixed or sequential cultures | Herraiz *et al.* (1990); Zironi *et al.* (1993); Moreira (2005); Ciani *et al.* (2006); Moreira *et al.* (2008); Mendoza *et al.* (2007) |
| *S. cerevisiae*<br>*K. thermotolerans* | Reduction of acetic acid production<br>Enhancement of titratable acidity | Sequential cultures | Mora *et al.* (1990); Ciani *et al.* (2006); Kapsopoulou *et al.* (2007) |
| *S. cerevisiae*<br>*Issatchenkia orientalis* | Reduction of malic acid content | Mixed fermentation | Kim *et al.* (2008) |
| *S. cerevisiae*<br>*Pichia fermentans* | Increased and more complex aroma | Sequential cultures | Clemente-Jimenez *et al.* (2005) |
| *S. cerevisiae*<br>*Pichia kluyveri* | Increased varietal thiol | Mixed fermentation | Anfang *et al.* (2009) |
| *S. cerevisiae*<br>*Candida pulcherrima* | Improve wine aroma profile | Mixed fermentation | Zohre & Erten (2002); Jolly *et al.* (2003) |
| *S. cerevisiae*<br>*Debaryomyces vanrijі* | Increase in geraniol concentration | Mixed fermentation | Garcia *et al.* (2002) |
| *S. cerevisiae*<br>*Schizosaccharomyces* spp.<br>*Saccharomycodes* spp.<br>*Pichia* spp. | Influence on sensorial and physico-chemical properties of wines | Ageing over the lees during wine maturation | Palomero *et al.* (2009) |

However, pure culture fermentations with non-*Sacchromyces* wine yeast generally increase metabolite contributions to noticeably negative levels and poor fermentation activities mean that they cannot be generally used as starter cultures. The most important spoilage metabolites produced by non-*Saccharomyces* yeast are acetic acid, acetaldehyde, acetoin and ethyl acetate (Ciani et al., 2010).

LAB species also influence the quality of wine. Nowadays, there are a varitety of LAB inoculums although they are mainly different strains of *O. oeni*. Some species of *Lactobacillus* and *Pediococcus* are considered to decrease the quality of the wine. *Pediococcus* is undesirable due to the formation of excessive amounts of diacetyl, biogenic amines, degradation of glycerol, etc. And the growth of *Lactobacillus* in bottled wines may result in haze formation, sediment, excessive volatile acidity, etc. (Fugelsang & Edwards, 2007). Thus, the inoculation of *O. oeni* can also be used to prevent the development of these LAB species.

## 1.7. Spoilage microorganisms in winemaking

In the wine industry, alcoholic fermentation is conducted by many microorganisms, and it is difficult to distinguish between beneficial fermenting activity and spoilage activity. Microorganisms can spoil wines at several stages during production. Any inappropriate growth of microorganisms may produce undesirable flavours. Wine that is exposed to air may develop oxidative yeasts on its surface, usually species of *Candida* and *Pichia* (Fleet, 2003). These species oxidise ethanol, glycerol and acids to give wines with unacceptably high levels of acetaldehyde, esters and acetic acid. Other wines can also be spoiled by fermentative species of *Zygosaccharomcyes*, *Dekkera* (anamorph *Brettanomyces*), *Saccharomyces* and *Saccharomycodes*. In addition to causing excessive carbonation, sediments, and haze, these species produce estery and acid off-flavours (Sponholz, 1993).

The winemaker's most feared spoilage yeast is *Dekkera*. This yeast produces off-flavours as a result of the synthesis of tetrahydropyridines and volatile phenols (4-ethylguaiacol and 4-ethylphenol) (Loureiro & Malfeito-Ferreira, 2003). Generally these phenolic off-odours are described by such descriptors as "barnyard-like, mousy, horsey, leather, pharmaceutical" (Grbin & Henschke, 2000; Du Toit & Pretorius, 2000). Among the species of this genus, *Dekkera bruxellensis* is the most representative in wines (Rodrigues et al., 2001). Other species have also been found to be able to produce volatile phenols. Among these, *Pichia guilliermondii* has the ability to produce 4-ethylphenol with efficiencies as high as those observed in *D. bruxellensis* (Dias et al., 2003).

*Pichia anomala*, *Metschnikowia pulcherrima* and *H. uvarum* are known for producing high levels of ethyl acetate and acetic acid before and during initial fermentation steps, leading to serious wine deterioration (Romano et al., 1992; Plata et al., 2003). However, *H. uvarum* does not produce ethyl acetate when it is present during the fermentation with *S. cerevisiae* (Zohre & Erten, 2002).

Spoilage species of LAB and AAB may grow at different stages of winemaking, during storage in the cellar and after bottling (Sponhoz, 1993; Fuselsang, 1997; Fleet, 1998; Du Toit & Pretorius, 2000). LAB can spoil wine during winemaking or during maturation and bottle aging. In the first case, bacteria start performing malolactic fermentation too early, at the end of alcoholic fermentation, but before all the sugars have been consumed by yeasts. The fermentation of these carbohydrates by LAB leads

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to the production of lactic acid as the major metabolite but acetic acid, ethanol and $CO_2$ are also produced. Ideally during wine aging, no yeasts or bacteria should survive in wine. Not all strains spoil wine, most depreciations and diseases are related to lactobacilli and pediococci, but they are normally destroyed during wine production. However, some strains demonstrate abnormal tolerance to the medium, and particularly to ethanol concentration. Other undesirable compounds that are consequence of LAB metabolism are biogenic amines and ethylcarbamate (Lonvaud-Funel, 1999). These metabolites have no impact on the aroma of wine, but they are considered as pernicious to the health of the wine consumer.

AAB can also spoil wines at many stages during the winemaking process. Prevention of AAB proliferation and wine spoilage is based on an understanding that these bacteria are aerobic and require oxygen to grow. However, it is evident that these bacteria may survive and even multiply under semi-anaerobic conditions, such as wine stored in tanks and barrels (Bartowsky & Henschke, 2008). AAB are considered as spoilage organisms because their major metabolites result in disagreeable wine sensory characteristics. AAB produce acetic acid as the main product of ethanol metabolisation. However, acetaldehyde and ethyl acetate are also produced and have a similar negative influence on wine quality. Acetic acid is the main constituent of the volatile acidity of wine and, depending on wine type, it is considered to be undesirable at concentrations exceeding 0.5–1.5 g/L. Sensorially, acetic acid produces a sour flavour with a vinegar-like aroma. The intermediate metabolite, acetaldehyde, can also contribute to the sensory spoilage of wine with distinct aroma descriptors: sherry-like, bruised apple. The ethyl ester of acetic acid, ethyl acetate, has a pungent solvent-like aroma, reminiscent of nail polish remover or nuts (Bartowsky & Henschke, 2008).

Finally, filamentous fungi can also impact on wine production at several stages: grapes spoilage in the vineyard, production of mycotoxins in grapes and their transfer to wines, production of metabolites that enhance or inhibit the growth of wine yeast and malolactic bacteria, and cause earthy, corky taints in wines after growth in grapes, corks and wine barrels (Fleet, 2003).

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## 2. Characterization of wine yeast and bacteria species using traditional methods

The traditional methods for detecting and quantifying different wine microorganisms are based on morphological tests supplemented with physiological tests. Besides, traditional methods require microorganisms to be previously isolated before they are identified or quantified.

### 2.1. Traditional methods for identification

Yeasts can be identified by conducting the tests and following the classification schemes described in Barnett et al. (2000). Numerous tests need to be conducted to reliably identify most yeasts at species level. Consequently, the work is time-consuming and accurate interpretation requires considerable expertise. Gram stain and catalase tests are routine analyses for distinguishing between LAB and AAB in wine although it is quite difficult to distinguish at species level and sometimes physiological tests are not enough.

One of the first microbiological tests is to examine the morphology of the microorganisms using phase-contrast microscopy. This examination will yield information about the shape (cocci, rods, pointed ends, bowling pin, egg, ogival, elongated, lemon, needle-like, etc.), size (dimensions), and arrangements (single, pairs, tetrads, groups or chains) of the cells. This can lead to incorrect interpretations, because the appearance of the cells depends on age and culture conditions. As well as cell morphology the characteristics of the colonies created in a specific medium are also important. Such characteristics include shape (circular, irregular, or rhizoid), size (dimensions), topography (flat, raised, convex, concave, or umbonate), presence of pigments, opacity (transparent, translucent or opaque), surface (smooth, rough, dull or glistening), edge (entire, undulate, lobate, denate or rhizoid) or any changes to the agar (color or opacity changes due to indicators) (Fugelsang & Edwards, 2007).

Once isolated the unknown microorganisms can be characterized using physiological traits. These methods are based on different physiological parameters, such as the assimilation of carbon and nitrogen sources, fermentation of carbohydrates, demonstration of ascospores, the formation of carbon dioxide from sugars, Gram stain, Catalase, test, oxidation of ethanol, oxidation of lactate etc. Numerous tests need to be performed to correctly identify yeast or bacteria (Fugelsang & Edwards, 2007).

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### 2.2. Traditional methods for quantification

Estimating microbiological population density and diversity plays an important role in the winemaking process. Population densities can be measured using many methods, but the two that are most used by oenologists are counts under the microscope and direct plating. Microscope counting techniques are the quickest but require a minimal population of $10^4$ cells/ml; lower populations need to be concentrated by filtration. For low population densities, direct plating methods are normally used. Membrane filtration, followed by direct plating is applied to those wines suspected of having a low viable population (<25 cells/ml).

Microscope counting consists of quantification using a microscope counting chamber, such as the Neubauer chamber, the Thoma chamber or similar. By this procedure all the cells are quantified without distinction. The main drawback is the low detection limit and the fact that it counts viable, non-viable and dead cells. To make the distinction between viable and non-viable cells, various stains and dyes can be used. And to improve the limit detection, a specific volume of wine can be filtered by a membrane with a proper pore size to retain the microorganisms to be counted.

Plate enumeration consists of growing different microorganisms in selected media and finally counting the colonies formed. Some non-selective media allow the growth of all yeasts associated with the fermentation. Although there is some limitation with samples of mixed species, the most prevalent species dominate on the plate and prevent species in lower quantities from being observed. To overcome these limitations, plating media need to be used that will selectively suppress the growth of undesirable or dormant species. Lysine agar is an example of a selective medium which prevents *S. cerevisiae* from growing because this yeast is unable to grow if lysine is the sole nitrogen source (Angelo & Siebert, 1987). This medium, then, is effective at isolating and enumerating non-*Saccharomyces* yeasts. An alternative to selective nutrients is to add of antibiotics to the growing media and inhibit particular microorganisms. Finally, some culture media are optimal for different types of microorganisms, such as MRS (Man, Rogosa Sharpe) for LAB or the glucose yeast extract-calcium carbonate-agar for AAB. This latter medium should also be considered as a differential medium because the calcium carbonate precipitates are dissolved by the acid produced by the acetic acid bacteria, forming a halo surrounding the AAB colony. Other factors than can convert a general medium into a selective one are the pH, temperature, anaerobiosis condition,

etc. Plate enumeration quantifies only the viable and culturable population. The results are represented by CFU/ml (colony-forming units per millilitre). The plating technique takes a long time to show the growth of different microorganisms, which may detract from its usefulness in industry. Yeasts usually need 48 hours to form a colony but LAB and AAB might need 5 to 10 days.

These techniques present many drawbacks. The results are subjective, and depend on the growth capacity of the microorganisms and their physiological stage. They are also laborious and time consuming. Industry, then, needs to detect and quantify the microorganisms present at every stage of the process as soon as possible. Traditional methods, however, do no do this.

## 3. Characterizing wine yeast and bacteria using molecular methods

Molecular methods can genotype, identify and quantify the various wine microorganisms as a function of their variability in the genome. In comparison to traditional methods, these methods are generally faster, more specific, more sensitive and more accurate, making it possible to perform accurate studies of microbial populations and their diversity. They can use either RNA or DNA. The most general RNA used is ribosomal RNA (rRNA). DNA is a stable molecule that survives cell death and rRNA is a valid index of cell viability, as viable cells have high numbers of rRNA molecules (Giraffa & Carminati, 2008). Some methods can use the RNA directly and others need a previous retro transcription to obtain the cDNA, complementary to RNA. Depending on the purpose of the study one approach or the other can be used. Some of these molecular methods can be applied to characterize the sample directly while others need a previous cultivation.

### 3.1. Culture-dependent techniques

Culture-dependent techniques need first to culture the cells and then identify or quantify them with a molecular technique. These methods are more reliable than traditional methods, because the identification and/or quantification is more accurate. Nevertheless, they may often fail to characterize minor populations or microorganisms, for which selective enrichment is necessary, stressed or weakened cells often need specific culture conditions to recover and to become culturable, and all the microorganisms which are not culturable will not be detected.

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### 3.2. Culture-independent techniques

Culture-independent techniques use molecular techniques to identify and/or quantify wine microorganisms and do not require the microorganisms to be cultured previously (Rantsiou et al., 2005). Culture-independent methods provide better information about the population, because they are not biased by the microorganisms that do not grow or do not grow well in a plate. The presence of viable but non-culturable microorganisms has been previously described in wine (Divol & Lonvaud-Funel, 2005; Millet & Lonvaud-Funel, 2000).

Millet & Lonvaud-Funel (2000) studied the behaviour of various wine microorganisms and found a viable population between $10^4$-$10^5$ cells/ml with the DEFT (direct epifluorescence technique) but lower than 1 CFU/ml with colony counts. They also enumerated AAB in the absence of oxygen and nutrients in the medium and the differences between plate counting and DEFT were also clear. As soon as starvation ceased and the medium was aerated, the DEFT counts were the same as colony counts. This clearly indicates that the cells were not dead and that the stress situation prevented them from developing on a plate. This was also observed by Du Toit et al. (2005) who studied the survival of *A. pasteurianus* in wine under anaerobic conditions, and observed that the cell counts by plating were lower than epifluorescence counts. Cocolin & Mills (2003) also found that $SO_2$ completely inhibited the growth of *Hanseniaspora* and *Candida* populations on culture media, although these species persisted for as long as 20 days with culture-independent techniques. Numerous other studies report the differences between culture-dependent and culture-independent techniques.

### 3.3. Strain genotyping

#### 3.3.1. Amplified Fragment Length Polymorphism (AFLP-PCR)

The AFLP technique uses restriction enzymes to digest genomic DNA, and then ligates the adaptors to the sticky ends of the restriction fragments. A subset of the restriction fragments is then selected to be amplified by using primers complementary to the adaptor sequence, the restriction site sequence and a few nucleotides inside the restriction site fragments. Only restriction fragments in which the nucleotides flanking the restriction site match the selective nucleotides are amplified (Vos et al., 1995). The

amplified fragments are visualized on agarose or polyacrylamide gels, or with capillary electrophoresis using sequencing equipments.

The main advantage of this technique is that there is no need to know the genome of the microorganism. Its sensibility and reproducibility are also high. The main drawbacks are that it is is time consuming and the technology used is expensive and complicated.

Some authors have used this technique to genotype wine strains. De Barros Lopes et al. (1999) genotyped different *Saccharomyces* strains and other strains of different species such as *D. bruxellensis*, *D. anomala*, *T. delbrueckii*, *I. orientalis*, *H. uvarum*, *H. guilliermondii*, *M. pulcherrima*, *P. fermentans* and *P. membranifaciens*. Gallego et al. (2005) used this technique to genotype different strains of *S. cerevisiae*, isolated from spontenaous fermentations. More recently, Esteve-Zarzoso et al. (2010) simplified it and applied it to genotype various non-*Saccharomyces* strains during wine fermentation. In LAB, Capello et al. (2008) genotyped different species belonging to *O. oeni*.

### 3.3.2. Amplification of repetitive elements of the genome

These techniques are based on the presence of repetitive elements in the genomes of different microorganisms. Designing oligonucleotides homologous to these repeated sequences enable these regions to be amplified, and each species or strain has a specific banding pattern. Different techniques are used for genotyping yeast and bacteria.

#### 3.3.2.1. Repetitive extragenic palindromic (REPs) and enterobacterial repetitive intergenic consensus (ERICs)

Both have been described as consensus sequences derived from highly conserved palindromic inverted repeat regions found in bacteria (Pooler et al., 1996). These sequences seem to be widely distributed in the genomes of various bacterial groups. These amplifications have generated DNA fingerprints of several bacteria, (Wieser & Busse, 2000; Guinebretier et al., 2001).

González et al. (2004, 2005) used these techniques to genotype acetic acid bacteria in wines, grapes and alcoholic fermentations. Also Hierro et al. (2004) also used these techniques to genotype some yeast strains. The REPs were not useful for genotyping yeast strains and the ERICs technique was found to be able to genotype

some strains of the species *C. boidini*, *C. stellata* and *I. terricola*. However, these authors did not detect these bacteria repetitive sequences in yeasts and the banding pattern had to be the consequence of a random amplification.

#### 3.3.2.2. PCR microsatellites or SSR (simple sequence repeats)

Microsatellites are tandem repeat units of short DNA sequences, 1-10 nucleotides in eukaryotic cells. The number of these repeated sequences is extremely variable, which makes the differences highly polymorphic. This technique consists of amplifying the region genome that contains these microsatellites, which gives an amplification pattern that enables the strains to be differentiated. The common primers used are $(GACA)_4$, $(GAG)_5$, $(GTG)_5$ and others. The ability of these primers to develop polymorphism among strains of *S. cerevisiae* was shown by Lieckfieldt et al. (1993). This technique has recently been used by Maqueda et al. (2010) to genotype different *S. cerevisiae* from spontaneous fermentations. They found a good reproducity detecting different strains, although found that the restriction analysis of the mitochondrial DNA (mtDNA) showed more variability.

This technique using $(GTG)_5$-PCR is a rapid, straightforward, and reproducible tool for differentiating a wide range of food-associated lactobacilli and other LAB species (Gevers et al., 2001). In bacteria, this technique is also known as rep-PCR. Nowadays, both ERIC-PCR and $(GTG)_5$-PCR are extensively used to genotype AAB in wine vinegar production (Hidalgo et al., 2010; Vegas et al., 2010).

Minisatellites also exist, the main difference being the size of the repeat sequences (in this case around 10 to 100 nucleotides) (Marinangeli et al., 2004). This technique was also applied to genotype *S. cerevisiae* species in palm wine, although greater strain diversity was detected using delta sequences (Stringini et al., 2009).

#### 3.3.2.3. Delta sequences amplification

Delta elements are conserved sequences of 330 base pairs that flank transposable Ty elements. As the separation distance between these delta elements does not exceed 1-2kb, oligonoucleotides homologous to these δ-elements are used to amplify the region between them. The size of the bands of this amplification is used to genotype *S. cerevisiae* strains. This method was developed by Ness et al. (1993) and Masneuf & Dubourdieu (1994) to genotype strains of *S. cerevisiae*. Fernández-Espinar

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(2001) used this technique to genotype different *S. cerevisiae* strains, although strain diversity was greater using the mtDNA restriction analysis. More recently, Stringini et al. (2009) successfully used this technique to genotype *S. cerevisiae* strains.

### 3.3.3. Restriction analysis of mitochondrial DNA (mtDNA)

This technique is mainly used to genotype *S. cerevisiae* strains. The mitochondrial DNA of *S. cerevisiae* is extremely polymorphic among different strains and very stable during vegetative multiplication.

The basis of this technique is to use specific restriction endonucleases to fragment the DNA into specific sites, generating fragments of variable sizes. These fragments are separated on agarose gel and are pattern strain-specific.

Aigle et al. (1984) was the first to apply this technique to brewer's yeast and, since 1987, it has been used to characterize the oenological strains of *S. cerevisiae* (Dubourdieu et al., 1987). Querol et al. (1992) simplified this protocol, because the mtDNA has a high proportion of AT. Then, treating the total cell DNA with restriction enzymes with target sequences type GCAT, they will cut more frequently nuclear DNA, with few cuts in the the mtDNA. The different sizes of nuclear and mitochondrial fragments mean that they separate differently in an agarose gel. So far, this is the most used technique to genotype the strains of *S. cerevisiae* (Torija et al., 2001; Beltran et al., 2002; Nikolaou et al., 2007; Maqueda et al., 2010).

This technique has been also applied to non-*Saccharomyces* genotyping such as different strains of *D. bruxellensis* (Ibeas et al., 1996) *Zygosaccharomyces* (Guillamón et al.,1997; Esteve-Zarzoso et al., 2003), *C. stellata*, *M. pulcherrima*, and *T. delbrueckii* (Pramateftaki et al., 2000) and *P. guilliermondii* (Martorell et al., 2006).

### 3.3.4. Random Amplification of Polymorphic DNA (RAPD-PCR)

This technique is based on the random amplification of genomic DNA with a single primer sequence 9 or 10 bases of long. Each strain presents amplification fragments that are different in size and number. The amplification is followed by agarose gel electrophoresis, which yields a band pattern that should be characteristic of a particular strain.

This technique has been used to genotype *Saccharomyces* and non-*Saccharomyces* yeasts (Cocolin et al., 2004; Capece et al., 2005; Martorell et al., 2006).

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However, in some studies comparing different methods, Gallego et al. (2005) found the microsatellites and AFLP techniques were more polymorphic than the RAPD-PCR technique. This technique has also been used to genotype LAB strains of *O. oeni* (Cappello et al., 2008; Ruiz et al., 2010) and AAB strains (Bartowsky et al., 2003).

### 3.3.5. Pulsed-Field Gel Electrophoresis (PFGE)

This technique consists of separating entire chromosomes by a special electrophoresis in which alternating electrical fields are applied. The chromosomes are forced to continually change their direction of migration and they avoid being retained in the lattice of the agarose gel and enable large fragments of DNA to be separated (Fernández-Espinar et al., 2006). The number and size of the chromosomes for different strains is very variable. This technique has been extensively used to genotype strains of *S. cerevisiae* (Guillamón et al., 1996; Martínez et al., 2004a). For non-*Saccharomyces*, Esteve-Zarzoso et al. (2001, 2003) genotyped strains of different *Hanseniaspora* and *Zygosaccharomyces* species. Versavaud et al. (1993) used restriction endonucleases of low restriction frequency to cut the chromosomes and then separate the fragmented chromosomes with pulse field gel electrophoresis. A previous restriction has also been applied for genotyping different strains of *D. bruxellensis* (Oelofse et al. 2009).

This technique has also been used for bacterial genotyping. Rodas et al. (2005) used PFGE with restriction endonucleases to genotype different *Lactobacillus* strains, and Vigentini et al. (2009) used it to genotype *O. oeni* in wine fermentations.

### 3.3.6. DNA array technology

Perhaps the most promising advance towards intraspecific discrimination of wine yeast is the use of whole genome sequences. The number of available full genome sequences is increasing day by day. An increasingly common approach to examining strain evolution and differentiation is to use comparative genomic hybridization with whole or partial genomic arrays: microarray comparative genomic hybridization (array CGH) (Dunn et al., 2005). With this technique, specific oligonucleotides are immobilized on a solid support and hybridize with homologous labelled fragments of the genome. This strategy has proven to be successful for microbial identification, even when species can only be discriminated by a single nucleotide polymorphism. In addition,

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since hybridization signals are proportional to the quantity of target DNA, this technique may also provide quantitative information (Justé et al., 2008).

Arrays enable various sets of microbes to be simultaneously detected and discriminated and, with high throughput methods, would enable comprehensive microbial ecological analysis (Zhou, 2003; Gentry et al., 2006). Molenaar, et al. (2005) published a study using CGH and wine-related bacteria. Salinas et al. (2010) used CGH to demonstrate genome changes in the *S. cerevisiae* strains analyzed that allow them to be discriminated. Furthermore, molecular analysis by amplified fragment length polymorphism (AFLP) and RAPD-PCR was not able to distinguish between these strains.

### 3.4. Species identification

The ribosomal genes of yeasts are grouped in tandem forming transcription units that are repeated in the genome between 100 and 200 times (figure 9). In each transcription unit there are both internal transcriber spacers (ITS) and external ones (ETS), and the encoding units are separated by intergenic spacers (NTS). The gene 5S is adjacent to these tandem repetitions. The ribosomal genes are powerful tools for establishing the phylogenetic relationship and identifying species (Kurtzman & Robnett, 1998).

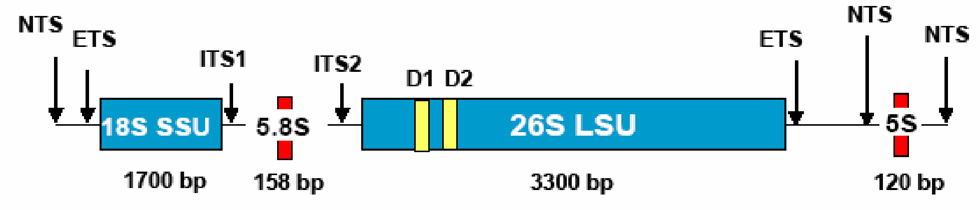


Figure 9. Structure of eukaryotic ribosomal genes (Fernández-Espinar et al., 2006)

The ribosomal genes of LAB and AAB are also grouped in tandem. The 16S rRNA is the macromolecule that is most used to identify bacteria (Figure 10).

Figure 10. Structure of prokaryotic ribosomal genes

Using information from these regions, various methods have been developed to identify species of yeasts and bacteria.

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#### 3.4.1. rRNA Gene Sequencing Analysis

This technique consists of amplifying specific ribosomal regions, followed by sequencing the amplified fragment. Then a comparison can be made between the unknown sequence and existing databases, and the species can be identified. Alignments with homologies equal to or higher than 99% are considered to belong to the same species (Kurztman & Robnett, 1998).

To sequence ribosomal genes of yeast, the main regions are domains D1 and D2 in the 26S gene (Kurtzman & Robnett, 1998). In the case of bacteria, the main gene used is 16S rRNA (Cole et al., 2005). These regions have been used to differentiate between yeast species (Montrocher et al., 1998; Egli & Henick-Kling, 2001; Belloch et al., 2002; Di Maro et al., 2007) and bacteria species (Le Jeune & Lonvaud-Funel, 1997) in wine.

#### 3.4.2. Restriction analysis of ribosomal genes (PCR-RFLPs)

An alternative to sequencing is to use PCR to amplify these or other ribosomal regions and then carry out further restriction analysis with specific endonucleases. Each restriction enzyme gives a specie-specific profile. The region most used for wine yeast identification by PCR-RFLPs is the region between the 18S and 26S rRNA genes, which includes the intergenic spacers (ITS1 and ITS2) and the 5.8S rRNA gene.

Guillamón et al. (1998) and Esteve-Zarzoso et al. (1999) identified different wine yeast species. Since then, several studies have used this technique (Torija et al., 2001; Beltran et al., 2002; Raspor et al., 2006), which has also been used to identify LAB (Rodas et al., 2005) and AAB (Poblet et al., 2000; Ruiz et al., 2000; González et al., 2006; Gullo et al., 2006; Vegas et al., 2010) by amplifying the 16S rRNA gene. Additional species discrimination has been done in the 16S and 23S intergenic spacer region of AAB (Ruiz et al., 2000).

#### 3.4.3. Denaturing Gradient Gel Electrophoresis (PCR-DGGE) and Temperature Gradient Gel Electrophoresis (PCR-TGGE)

Both techniques are based on the denaturing properties of DNA. The fragments of similar length but with different sequences can be separated by their melting properties. Double strain DNA is denatured by applying a denaturing gradient

temperature (PCR-TGGE) or denaturing chemical gradient, such as urea and formamide (PCR-DGGE).

These techniques consist of amplifying of a conserved region of the genome, generally ribosomal genes. One of the primers has a GC clamp to prevent the fragment from completely denaturalizing. The next step is to run the amplicons in a denaturing gradient polyacrylamide gel (PCR-DGGE). Initially the melting process is only partial. Discrete domains become single stranded, which decreases the mobility of the DNA fragment through the gel. Eventually, strand separation stretches over the entire length of the amplicon, and only the GC clamp remains double stranded. Amplicons are all of the same size, mainly around 200-700pb. Different species have different base sequences (or rather a different proportion and distribution of GC bases) and these sequences determine their denaturing behaviours. Therefore, the amplicons from different species have different migration rates in the polyacrylamide gel. This method also has the theoretical potential to detect differences of as little as a few base pairs between species. It is recommendable to excise a band and confirm the identification by sequencing.

Prakitchaiwattana et al. (2004) studied the detection limit of this technique and found that the lower limit of yeast detection was 100 cfu/ml. The detection limit of individual yeast species in mixed populations, however, was also determined by their relative populations. Species can be detected in the mixture when populations are equal or even when some populations are 10-100 fold less than others in the mixture. Detection is not possible, however, when this ratio exceeds 100-fold.

These techniques show which species are present in a complex matrix like wine, and do not require to know the DNA sequence of the species to do so. These techniques, then, have been used to identify different bacteria (López et al., 2003; Renouf et al., 2006; De Vero et al., 2006; De Vero & Giudici, 2008; Ilabaca et al., 2008) and yeasts (Cocolin et al., 2004; Di Maro et al., 2007; Stringini et al., 2009). Possible problems associated with this technique are that different species might have identical electrophoresis mobility, which can lead to results being interpreted incorrectly.

#### 3.4.4. Analysis of clone libraries

This technique consists of amplifying highly or poorly conserved gene regions and the directly cloned and sequenced amplicon, which enables the species of

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individual community members to be identified. A major drawback of the use of this technique is the selective transformation due to amplicon size and sequence. Alternatively, direct digestion of the entire plasmid containing the yeast region may be helpful to initially screen the clone library and distinguish different restriction types which can then be sequenced (Lagacé et al., 2004; Kim & Chun, 2005). This technique was applied to study the evolution of AAB in vinegar fermentation (Ilabaca et al., 2008).

#### 3.4.5. Whole genome analysis

Probably the most accurate way of identifying microorganisms is whole sequence analysis, although its high cost has limited its application. Nowadays, pyrosequencing allows high throughput, is relatively cheap and does not require cloning, which means that there are no aberrant recombinants and cloning-related artefacts. This method has the potential advantages of accuracy, flexibility, parallel processing, and can be easily automated. It also dispenses with the need for labelled primers, labelled nucleotides, and gel-electrophoresis. This technique has not been used for genome sequencing due to the limitation in the read length, but it has been employed for applications such as genotyping (Ronaghi, 2001). Currently, pyrosequencing is being used in the microbial community analysis of soils and mines (Justé et al., 2008). However, the price is still too high for routine analysis.

#### 3.4.6. Other techniques

This section provides brief descriptions of other techniques used to identify different microorganisms but not widely applied to wine microorganisms (Giraffa & Carminati, 2008; Justé et al., 2008).

- Single-strand conformation polymorphism (SSCP-PCR) electrophoretically separates PCR products on the basis of conformational differences in folded single-stranded products. After denaturation, single-stranded DNA fragments are loaded on a non-denaturing polyacrylamide gel. Under non-denaturing conditions a stable secondary structure is formed which is mainly determined by the intramolecular interactions that depend on the nucleotide sequence. On the basis of the migration of these secondary structures in the gel, products of similar molecular weights can be separated and visualized. However, a major limitation is that several stable conformations are formed from one single-stranded DNA fragment, resulting in multiple

bands on gel. The discriminatory ability and reproducibility of SSCP-PCR analysis, which is generally most effective for fragments around 400 pb, also depends on the position of the sequence variations in the gene studied (Vaneechoutte, 1996). This technique has been used to study microbial communities in different matrices. So far, applications in food microbiology have been limited to cheese microbiota (Callon et al., 2006; Delbès et al., 2007).

- Terminal-Restriction Fragment Length Polymorphism (T-RFLP) is based on the restriction endonuclease digestion of fluorescent end-labelled PCR products. This digestion yields fluorescent fragments that are of different sizes for the different species. Depending on the species composition of the microbial community, different fluorescent-labelled fragments are detected. Very similar to this technique is the Length Heterogeneity-PCR (LH-PCR), which distinguishes different organisms on the basis of natural variations in the length of one gene (for example, the 16S rRNA gene). Both techniques have been applied to assess microbial dynamics in cheese (Rademaker et al., 2006; Fornasari et al., 2006).

### 3.5. Quantification techniques

To control the winemaking process, it is useful to know which species are present and in which quantities at each stage. The presence of some microorganisms can be quantified by DNA-based techniques, which correlate the amount of DNA to the amount of biomass.

#### 3.5.1. Direct Epifluorescence Technique (DEFT)

This technique directly counts viable cells through a fluorescence microscope. Specific dyes react with organic material or are concentrated in specific subcellular organelles. This technique is more applicable to routine wine control, particularly since fluorescent dyes are now available. Toxic acridine orange was the first to be used (Froudière et al., 1990; Diaper & Edwards, 1994). Nowadays several different kits are available: LIVE/DEAD® BactLight™ Bacterial Viability Kit and LIVE/DEAD® Yeast Viability Kit. The LIVE/DEAD® BactLight Bacterial Viability assay uses mixtures of SYTO 9 green fluorescent nucleic acid stain and red fluorescent nucleic acid stain propidium iodide. This kit can also be used to assess yeast viability (Zhang & Fang, 2004). The SYTO® 9 stains cells with both intact and damaged membranes. In contrast, propidium

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iodide only penetrates cells with damaged membranes, which are considered to be dead cells. When mixed in the recommended proportions, live cells are coloured green (the SYTO® 9 stain) and dead cells are coloured red (the mixture of SYTO 9 and propidium iodide staining). The LIVE/DEAD® Yeast Viability Kit consists of two stains: FUN® 1 which generates red-fluorescent cylindrical intravacuolar structures (CIVS) only in the metabolically active cells, and Calcofluor White M2R which stains the cell wall with blue-fluorescence, regardless of the yeast metabolic state. The difference between viable and non-viable cells, then, is the presence of red-fluorescent CIVS in the cell cytoplasm for the metabolically active cells. However, it has been observed that the CIVS are not always clearly visible because of their small size, so viability can potentially be underestimated (van Zandycke, 2003).

This is a rapid and reliable technique, which quantifies viable and non-viable cells, although it cannot distinguish between the different genera or species. It has been used to demonstrate significant non-culturable populations of both bacteria and yeast in ageing wines (Millet & Lonvaud-Funel, 2000; Du Toit et al., 2005; Divol & Lonvaud-Funel, 2005).

### 3.5.2. Fluorescence *In Situ* Hybridization (FISH)

This technique consists of the direct hybridization of some labelled probes, mainly fluorescent, to DNA or RNA. First of all, a fixation step is required to maintain the integrity of the cell, usually with paraformaldehyde. Then, the membrane is permeabilized so that the probe can enter the cell. Generally these probes are 15 to 20 nucleotides in length and are covalently labelled at the 5' end with a fluorescent dye. Some washing steps are needed to eliminate the excess of non-hybridized probes and the detection is finally made using a fluorescence microscope. The probes need to be specifically designed, so a considerable amount of knowledge about each community must be available. One modality of FISH is multiplex FISH, which uses several probes labelled with different fluorochromes, thus detecting and quantifying different species in the same analysis (Giraffa & Carminati, 2008).

Xufre et al. (2006) developed 26S rRNA gene probes for the identification of numerous wine-related yeast including *S. cerevisiae*, *C. stellata*, *H. uvarum*, *H. guilliermondii*, *K. thermotolerans*, *K. marxianus*, *T. delbrueckii*, *P.membranifaciens* and *P. anomala*. They have also been used to identity lactic acid bacteria (Sohier &

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Lonvaud-Funel, 1998; Blasco et al., 2003) and acetic acid bacteria (Blasco, 2009: PhD Thesis).

The technique combines the simplicity of microscopy and the specificity of DNA/RNA hybridization. In theory FISH could detect single cells, but in practice the detection limit is often $10^4$ cells/ml. In general, it is less sensitive than PCR-based techniques (Hogardt et al., 2000; Moreno et al., 2003; Poppert et al., 2005). Another limitation is insufficient automation for high sample size throughput (Amann et al., 2001).

### 3.5.3. Flow Cytometry

Flow cytometry simultaneously measures and then analyzes multiple physical characteristics of single particles, such as cells. These cells flow in a fluid stream through a beam of light. The properties measured include a particle's relative size, relative granularity or internal complexity, and relative fluorescence intensity. These characteristics are determined using an optical-to-electronic coupling system that records how the cell or particle scatters incident laser light and emits fluorescence. The characteristics or parameters of each event are based on its light scattering and fluorescent properties. This data can be analyzed to provide information about subpopulations within the sample.

Cell viability can also be directly assessed by using fluorescent dyes to view the metabolic state of yeast and bacteria in wine (Malacrino et al., 2001; Boyd et al., 2003; Chaney et al., 2006; Herrero et al., 2006). Flow cytometry can be combined with FISH to selectively enumerate mixed microbial populations and carry out a high resolution automated analysis (Amann et al., 1990). The main advantage of this technique is its sensitivity (it can detect one cell in a million).

### 3.5.4. Impedance

Impedance analysis is based on the modification of the medium's conductivity, which is measured by applying an electric voltage to electrodes. The molecules in this medium (proteins, carbohydrates, etc.) are electrically neutral and less ionized. The microbial metabolism transformes these molecules into smaller molecules with a higher charge and electric mobility (amino acids, organic acids, etc). The technique can be used for direct (increase in conductivity) or indirect analysis. The indirect analysis

measures the decrease in the conductivity of the highly ionized medium (with KOH, for instance) that reacts with the $CO_2$ produced by the microorganisms and decreases the conductivity of the medium.

This technique is easy and rapid. The main problem is that it cannot individually quantify the different microorganisms in a sample. Martínez et al. (2004b) used it to quantify microorganisms in bottled wines. It can also be used to determine the vitality of wine yeast, as an indicator of fermentative capacity (Redón et al., 2008; Rodríguez-Porrata et al., 2008).

#### 3.5.5. Real time or Quantitative PCR (Q-PCR)

Quantitative PCR uses specific technology to continuously detect the PCR product after each reaction cycle. It detects and quantifies a fluorescent donor, the signal of which increases in direct proportion to the quantity of PCR product obtained. The fluorescence can be obtained through binding agents or probes. SYBR Green is the common binding agent which binds to double stranded DNA. The most used probes are TaqMan, which are characterized by having a donor photochrome together with an acceptor photochrome (quencher). When they are both bound in the probe, the acceptor quenches the fluorescence emitted by the donor. When the *Taq* polymerase releases the acceptor photochrome the donor fluorescence is emitted. This is the same rationale for such other probes as Beacon or Scorpio probes, which both make up a hairpin-forming oligonucleotide probe. Real-time PCR allows sensitive detection of the DNA product, ensures detection during the linear range of amplification, eliminates the need for post-PCR analysis, and incorporates specialized software to simplify data analysis. The information is represented as an amplification curve which provides the cycle number for which the intensity of the donor emission increases compared with the background noise. This cycle number is called the cycle threshold (Ct) and is inversely proportional to the number of copies of the sample. It is used to evaluate the initial quantity of DNA or cells.

The technique has high specificity and sensibility, and is quick. Nevertheless, all these parameters strictly depend on the primer design. It has been widely used to detect and quantify different wine microorganisms, such as acetic acid bacteria (González et al., 2006; Torija et al., 2010), lactic acid bacteria (Neeley et al., 2005), and wine yeast (Martorell et al., 2005; Hierro et al., 2006).

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## 4. "Viable but non-culturable" microorganisms

### 4.1. Definition

Microorganisms in the viable but non-culturable (VBNC) state are those which lose the ability to grow in a culture medium. This does not mean that the microorganisms are dead, as was previously believed. These microorganisms are alive although they are in a new state that prevents them from forming colonies in a culture medium. This VBNC state renews the microorganisms it they are allowed to recover in a medium in which the stress that causes the state is absent (Oliver, 2000b). The typical behaviour of VBNC microorganisms in response to stress is shown in figure 11. In this case, the colony counts showed the usual population dynamics (i.e. a growth phase followed by a stationary phase until day 10, and then a decline phase). From day 16 the population decreased more slowly in the red wine than in the same wine without phenolic compounds. Using DEFT, the results were of the same order at the beginning of the experiment (about 2–3 $10^6$ cells/ml). However, from day 13 to day 53, the viable population estimated by DEFT was 3 or 4 log units higher than the colonies recovered in the red wine. The samples were aerated at day 53 to dissolve the oxygen which is necessary for AAB metabolism and growth. Within the following 3 days, rapid growth occurred and the population able to form colonies was almost as numerous as the population enumerated by DEFT.

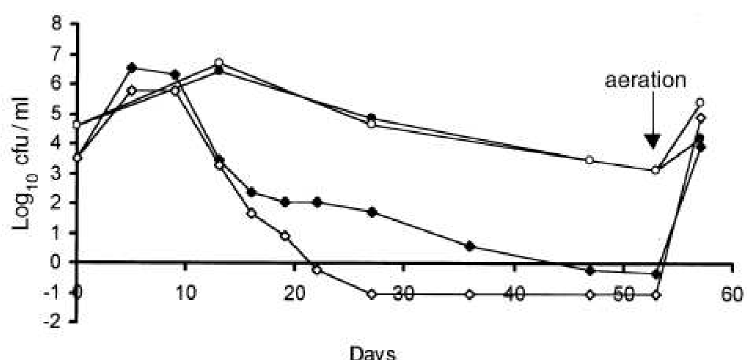


Figure 11. Evolution of acetic acid bacteria determined by colony counts and by DEFT in a red wine (Wine A) and the same wine deprived of phenolic compounds (Wine B). Wine A, colony counts: ♦, DEFT: ●, Wine B, colony counts: ◊, DEFT: ○ (Millet & Lonvaud-Funel, 2000)

### 4.2. What induces this state and why do microorganisms enter this state?

The microorganisms enter this state in response to stress, which could otherwise be lethal. The natural stresses that induce this state are osmotic stress, high or low temperatures, starvation, oxygenation, or exposure to light (Oliver, 2000c). During this period, the VBNC microorganisms decrease their activity as the result of a reduction in nutrient transport, respiration rates, and macromolecular synthesis (Porter et al., 1995;

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Oliver 2000a). However, these microorganisms maintain the basal metabolism so that the main cellular functions keep active. Some studies have demonstrated continued gene expression by VBNC microorganisms (Lleò et al., 2000, 2001; Yaron & Matthews, 2002). Heim et al. (2002) reported that microorganisms entering this state have quite a different protein profile. Furthermore, VBNC microorganisms presented modifications in the fatty acid composition of the plasma membranes; which are essential to enter in this state (Day & Oliver, 2004) and necessary for maintaining the membrane potential (Porter et al., 1995; Tholozan et al., 1999). Nevertheless, VBNC microorganisms have been reported to have a greater autolytic capacity than microorganisms in the exponential phase.

### 4.3. Deviation in monitoring wine microorganisms

First of all, it should be pointed out that live cells, dead cells, and damaged cells in VBNC state can be found in a culture medium. It was previously thought that all the microorganisms that could grow in an optimal medium were alive. Different microorganisms may have such requirements as specific nutrients in the media, growth temperature, growth rate, etc. The best medium for each microorganism and the selective medium for the growth of a given microorganism often have to be defined. However, this is not always the case because different stress conditions may produce the VBNC state. Several states can be defined among the live cells that can support growth and dead cells (Figure 12). The result in both cases will be the same: that is, the lack of growth in general or universal media. Then, a deviation in the determination of the microorganisms will appear when culture-dependent and independent techniques are used. Culture-dependent techniques may underestimate the population, because only the culturable microorganisms are analysed (Millet & Lonvaud-Funel, 2000). Culture-independent techniques, on the other hand, may overestimate the population because they detect of dead cells as live ones (Nocker & Camper, 2006).

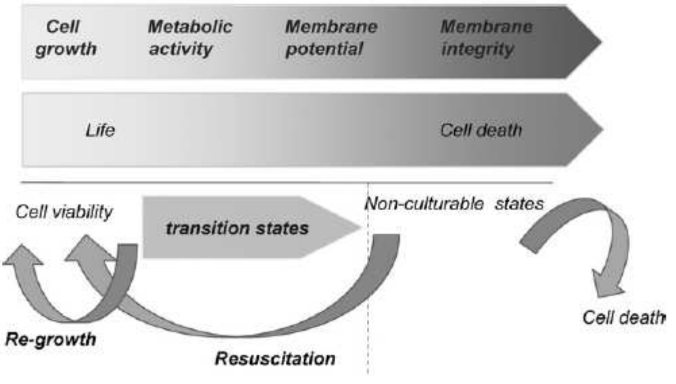


Figure 12: Functional criteria for determining different levels of cell viability and vitality (Diaz et al., 2010)

#### 4.4. Solutions

One solution for differentiating between dead cells, VBNC cells and culturable live cells could be to use culture-independent techniques with some modifications to eliminate the DNA from dead cells or to use RNA. Several studies have used RNA instead of DNA for quantifying or detecting the viable population because it is rapidly degraded in the dead cells (Cocolin & Mills, 2003; Hierro et al., 2006). However, working with RNA can be complex because it is unstable and can be degraded during purification or analysis. Various authors have proposed an alternative to RNA (Rudi et al., 2005; Nocker & Camper, 2006), which consists of applying DNA binding dyes that penetrate only the dead cells (damaged membranes) and do not require the DNA to be amplified. First, Nogva et al. (2003) proposed using ethidium monoazide bromide (EMA) to detect the bacteria viable cells. Subsequently, Nocker et al. (2006) presented propidium monoazide bromide (PMA), a novel chemical that could enter the bacteria dead cells. Both chemical dyes penetrate only dead cells (or, to be more precise, those with compromised membrane integrity) not live cells with intact cell membranes. When they bind to the DNA of dead cells, the photo-inducible azide group allows these dyes to be covalently cross-linked by exposure to bright light. This process renders the DNA insoluble and it is lost during subsequent genomic DNA extraction (Nocker & Camper, 2006; Nocker et al., 2006). Rudi et al. (2005) reported that the unstained DNA from viable cells was PCR amplified, while the DNA from dead cells with bound dyes was not. Thus, only the DNA from live cells is detected and quantified after treatment with the dyes.

Nocker et al. (2006) observed that the application of EMA is hampered by the fact that the chemical can also penetrate the live cells of some bacterial species. Transport pumps actively export EMA out of metabolically active cells, but the remaining

EMA level can lead to substantial loss of DNA. The fact that the PMA charge is higher might be the reason for the greater impermeability of intact cell membranes, which prevents this live cell from losing its DNA.

Several studies have been made using this methodology in a variety of bacteria species, although none in wine microorganisms. Recently, this technique was adapted, first to detect *Zygosaccharomyces bailii* which was causing spoilage in fruit juices (Rawsthorne & Phister, 2009a) and, subsequently, to detect *S. cerevisiae* without detecting the DNA remaining from yeast extract (Rawsthorne & Phister, 2009b).

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## Chapter 1

# Effect of oenological practices on microbial populations using culture-independent techniques

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**Abstract**

Sulphur dioxide ($SO_2$) addition and yeast inoculation are well-established practices in winemaking for restricting the growth of indigenous yeasts and bacterial populations. The effect of these oenological practices on wine microbial populations has been evaluated using culture-independent methods. These are quantitative PCR (qPCR) for the enumeration of yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB), and PCR-DGGE to determine the yeast and bacteria species diversity. The PCR-DGGE method detected a low yeast and bacteria species diversity. On the contrary, the specificity of the primers designed for the qPCR allowed that minor microbial groups such as *Hanseniaspora* were accurately quantified regardless of a large presence of other microbial groups such as *Saccharomyces*. From an oenological point of view, inoculation increased the proportion of *Saccharomyces* vs. non-*Saccharomyces* in a shorter time. *Hanseniaspora* increased during the first phase and decreased during the latter phases of the process, especially in the sulphited fermentations. Both yeast inoculation and $SO_2$ kept the LAB populations at very low level, while the AAB populations were hardly affected by these two practices.

**Keywords:** Wine; *Acetobacter aceti*; *Gluconacetobacter hansenii*; *Candida zemplinina*; *Oenococcus oeni*

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## 1. Introduction

The conversion of grape must to wine is a complex biochemical process involving interactions between yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB). The metabolism of these microorganisms contributes to the quality of the wine by releasing metabolites which contribute to the flavour and aroma of the wines (Swiegers et al., 2005). Yeasts and LAB are the main contributors to wine aroma-enrichment during the alcoholic and malolactic fermentation, respectively. Yeasts with low fermentation activity, such as *Candida* spp., *Hanseniaspora* spp., *Kluyveromyces* spp., *Pichia* spp., *Rhodotorula* spp. are predominant in grape musts and during the early stages of fermentation. Subsequently, *Saccharomyces cerevisiae* proliferates, dominating and completing the wine fermentation (Fleet and Heard, 1993; Beltran et al., 2002). *Oenococcus oeni* is the major species during malolactic fermentation but other LAB genera such as *Lactobacillus*, *Leuconostoc* and *Pediococcus* can grow in the wine. LAB may enhance wine flavour and complexity but their growth has been also related with the production of off-flavours. Likewise, AAB growth generates off-flavours in the wine such as acetic acid and ethyl acetate, which have a negative effect on wine quality (Drysdale and Fleet, 1988).

Most studies on wine microbial ecology have invariably been done after the culture of the different microorganisms in different media. Nowadays we are in a new era of microbiology due to the development of molecular biology techniques that allow us to identify and enumerate microorganisms using culture-independent methods. Avoiding the selective cultivation and isolation of microorganisms from natural samples is justified, considering the biases related to traditional culture-dependent methods (Rantsiou et al., 2005). The presence of viable but non-culturable microorganisms has been described in wine samples (Millet and Lonvaud-Funel, 2000; Divol and Lonvaud-Funel, 2005). These microorganisms are unable to grow in a plate but may justify the differences reported by various authors between isolated and naturally occurring species in wine samples (Mills et al., 2002; Cocolin and Mills, 2003; Hierro et al., 2006b).

Wine microbiota is influenced by multiple factors, which can be grouped into viticultural and oenological practices. We have previously analysed the effect of two of the most common oenological practices (yeast inoculation and $SO_2$ addition) on yeast (Constantí et al., 1998) and on AAB (González et al., 2005) populations. In these

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previous studies, microbes were analysed using culture-dependent methods. Microbial counts were done by plating in different media and colonies were identified by different molecular methods (RFLPs of rDNA and mtDNA, ERIC-PCR, etc.). The aim of the present study was to analyse the microbial content in different wine fermentation conditions using only culture-independent techniques. PCR-DGGE and qPCR are two of the most widely used techniques for independent-culture microbial analysis. PCR-DGGE has been widely used to study the ecology of wine fermentation (Di Maro et al., 2007; Renouf et al., 2007; Mills et al., 2002; Cocolin et al., 2000) and qPCR assays have been developed to detect and enumerate bacteria and yeasts in wine (Phister and Mills, 2003; Delaherche et al., 2004; Pinzani et al., 2004; Martorell et al., 2005; González et al., 2006; Hierro et al., 2006a; Rawsthorne and Phister, 2006; Hierro et al., 2007). Thus, we chose the real-time quantitative PCR (qPCR) to enumerate the main wine microbial groups: yeast, LAB and AAB. We also enumerated the two main wine yeast genera: *Saccharomyces* and *Hanseniaspora*. The diversity of yeast and bacteria species was monitored by PCR-DGGE throughout different wine fermentations.

## 2. Materials and methods

### 2.1. Reference strains

The type and reference strains used in this study were obtained from CECT (Spanish Type Culture Collection), DSM (Deutsche Sammlung von Mikroorganismen und Zelkulturen) and LMG (Culture Collection of the Laboratorium voor Microbiologie, Gent). The yeasts used were: *Candida boidinii* CECT10029, *Candida mesenterica* CECT1025, *Candida sake* CECT10034, *Candida stellata* CECT11109, *Dekkera anomala* CECT11162$^T$, *Hanseniaspora guilliermondii* CECT11029$^T$, *Hanseniaspora uvarum* CECT11107, *Issatchenkia terricola* CECT11139 and CECT11176$^T$, *Torulaspora delbrueckii* CECT1880 and CECT10558 and *Zygosaccharomyces rouxii* CECT1230 and CECT1232. We also used the Active Dry Wine Yeast (ADWY) commercial strain *Saccharomyces cerevisiae* QA23 (Lallemand, Inc. Canada). The LAB used were: *Lactobacillus brevis* CECT4121, *Lactobacillus buchneri* CECT4111$^T$, *Lactobacillus hilgardii* CECT4786$^T$, *Lactobacillus plantarum* CECT220, *Leuconostoc mesenteroides* CECT219, *Oenococcus oeni* CECT217$^T$, *Pediococcus parvulus* CECT813 and *Pediococcus pentosaceus* CECT4695. AAB: *Acetobacter aceti* DSM2002 and DSM3508, *Acetobacter oeni* LMG21952$^T$, *Acetobacter pasterianus* DSM3509 and

DSM46617, *Gluconacetobacter hansenii* LMG1529 and DSM5602, *Gluconobacter oxydans* DSM2343 and DSM7145.

Yeasts were grown in YPD (2% glucose, 2% peptone, 1% yeast extract), LAB were grown in MRS (Oxoid, Hampshire, United Kingdom) and AAB were grown in GY (10% glucose, 10% yeast extract).

### 2.2. Wine fermentations and sampling

This study was done in the experimental cellar of the Faculty of Oenology in Tarragona (Spain) during the 2006 vintage. Cariñena was the grape variety we chose for the vinifications. The same grape must was separated into four 80-l tanks after destemming and crushing. When $SO_2$ was added, the concentration was 60 mg $l^{-1}$ and the inoculum used was the commercial strain *S. cerevisiae* QA23 added as rehydrated yeast at a final population of $2\times10^6$ cells $ml^{-1}$. Fermentation tanks were identified as: +I+S (inoculum and $SO_2$); +I–S (inoculum and no $SO_2$), –I+S (no inoculum and $SO_2$) and –I–S (no inoculum and no $SO_2$). Temperature fermentation was controlled at 25–28 ºC. Samples were taken from grape must on day 0 and on several fermentation days (1, 2, 5, 8 and 12). Sugar consumption was daily monitored by measuring the density (g $l^{-1}$) of the fermenting must. Fermentations were considered to be finished when the level of reducing sugars, measured by enzymatic assay (Roche Applied Science; Germany), was below 2 g $l^{-1}$.

### 2.3. DNA extraction

DNA was isolated from cultures of all the reference strains and from fermentation samples (1 ml) as described by Hierro et al. (2006a). Yeast cell suspensions were washed with sterile water and the pellets were resuspended in 700 μl of AP1 buffer (DNeasy Plant minikit, Qiagen, Valencia, California) and transferred to a 2 ml conical-bottom microcentrifuge tube containing 1 g of 0.5 mm diameter glass beads. The tubes were shaken in a mini bead-beater (Biospec Products Inc., Bartlesville, Oklahoma) for 3 min at the maximum rate and then centrifuged at 10.000 rpm for 1 min. The DNA in the supernatant was transferred to a sterile microfuge tube and purified using the DNeasy Plant minikit (Qiagen, Valencia, California) according to the manufacturer's instructions. The same DNA extraction was used for both PCR-DGGE and qPCR analyses.

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### 2.4. PCR-DGGE

The primers we used to amplify the specific ribosomal region of each microbial group were: $U1^{GC}$/U2 (Meroth et al., 2003a) for yeasts and $L1^{GC}$/HDA2 (Meroth et al., 2003b) for LAB (Table 1). So far, no specific primers for AAB have been reported, thus, we used the $WBAC1^{GC}$/WBAC2 primer pair (Lopez et al., 2003) described for the common amplification of both LAB and AAB populations found in wines (Table 1). This primer pair has been already used to monitor AAB diversity in balsamic vinegar (De Vero et al., 2006). For LAB, the fragments of the 16S rRNA gene were amplified and separated by DGGE as described by Meroth et al. (2003a). For AAB, the PCR amplification was prepared as in Lopez et al. (2003), although the electrophoretic run was kept at a constant 170 V for 4 h at a constant temperature of 60 ℃ in TAE buffer 0.5×, and in a denaturing gradient from 30% to 60% of urea and formamide. For the yeasts, amplification of the fragments and denaturing electrophoresis was done according to Meroth et al. (2003a). All PCR amplifications were done in a Gene Amp PCR System 2700 (Applied Biosystems, Fosters City, USA), using EcoTaq DNA Polimerase (Ecogen, Spain). The Dcode universal mutation detection system (Bio-Rad, Hercules, California) was used to run the PCR-DGGE analysis.

Table 1. Primers used for qPCR and PCR-DGGE assays

| Technique | Microbial group | Primer | Sequence 5'-3' | PCR product size (bp) |
|---|---|---|---|---|
| q-PCR | Total Yeasts | YEASTF | GAGTCGAGTTGTTTGGGAATGC | 124 |
| | | YEASTR | TCTCTTTCCAAAGTTCTTTTCATCTTT | |
| | *S. cerevisiae* | SCER-R | CGCAGAGAAACCTCTCTTTGGA | 175 |
| | | CESP-F | ATCGAATTTTTGAACGCACATTG | |
| | *H. uvarum* | HUV-R | AACCCTGAGTATCGCCCACA | 121 |
| | | CESP-F | ATCGAATTTTTGAACGCACATTG | |
| | LAB[1] | WLAB1 | TCCGGATTTATTGGGCGTAAAGCGA | 407 |
| | | WLAB2 | TCGAATTAAACCACATGCTCCA | |
| | AAB[2] | AQ1F | TCAAGTCCTCATGGCCCTTATG | 55 |
| | | AQ2R | CGCCATTGTAGCACGTGTGTA | |
| PCR-DGGE | Total Yeasts | $U1^{GC*}$ | GTGAAATTGTTGAAAGGGAA | 260 |
| | | U2 | GACTCCTTGGTCCGTGTT | |
| | LAB[1] | $L1^{GC*}$ | CAGCAGTAGGGAATCTTCC | 185 |
| | | HDA2 | GTATTACCGCGGCTGCTGGCAC | |
| | AAB[2] | $WBAC1^{GC*}$ | GTCGTCAGCTCGTGTCGTGAGA | 320 |
| | | WBAC2 | CCCGGGAACGTATTCACCGCG | |

LAB[1]: lactic acid bacteria; AAB[2]: acetic acid bacteria.

* GC-rich sequence (5′-GCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC-3′).

The DNA fagments from the PCR-DGGE gels were excised and purified according to Omar and Ampe (2000). Each excised band was then transferred into 50 µl of sterile water and incubated overnight at 4 ℃ to allow diffusion of the DNA. One microlitre of the eluted DNA was used for re-amplification with primers without the GC clamp. The PCR products were purified and sequenced by Macrogen Inc. facilities (Seoul, South Korea) using an ABI3730 XL automatic DNA sequencer.

### 2.5. qPCR

In all cases qPCR was done on an ABI Prism 5700 Sequence Detection System (Applied Biosystems). Power SyberGreen master mix was used according to the manufacturer's instructions (Applied Biosystems, California). An ABI PRISM 96 well optical plate was used for the reaction. The instrument automatically determined the $C_T$. Samples and cultures for standard curves were analyzed in triplicate.

We quantified the total yeast, *Saccharomyces* and *Hanseniaspora* using the primers YEASTF/YEASTR (Hierro et al., 2006a), CESP-F/SCER-R and CESP-F/HUV-R (Hierro et al., 2007), respectively (Table 1). We quantified the LAB using the primers WLAB1/WLAB2 (Neeley et al., 2005). The reaction mixture was made up of 5 µl of DNA template, 0.2 µM of primer, and 12.5 µl of PowerSybrGreen Master Mix (Applied Biosystems). The amplification programme was as follows: 50 ℃ for 2 min, 95 ℃ 10 min, 40 cycles at 95 ℃ for 15 s and 60 ℃ for 1 mi n. To quantify the AAB we followed the protocol proposed by González et al. (2006) and used the primers AQ1F/AQ2R (Table 1).

Standard curves were created by plotting de Ct (Cycle Threshold) values of the qPCR performed on dilution series of cells against the log input cells $ml^{-1}$. Three of these curves were made for each type of microorganism. All of these curves were made from a fresh culture.

## 3. Results

### 3.1. Wine fermentations

Four semi-industrial fermentations were done under different conditions of SO2 addition (+S) and yeast inoculation (+I) (see Material and Methods, where the conditions are designated as +I+S, +I–S, –I+S and –I–S). Grape must (density=1095 g l–1) was divided into four tanks and then yeast inoculum and $SO_2$ were added to the necessary fermentations. Slight differences were observed between the different

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fermentation conditions (Fig. 1). Yeast inoculation slightly speeded up the beginning of the fermentations, especially when $SO_2$ was absent. The fermentation without inoculum and without $SO_2$ (–I–S) showed the slowest fermentation rate. In any case, all the fermentations finished in 12 days.

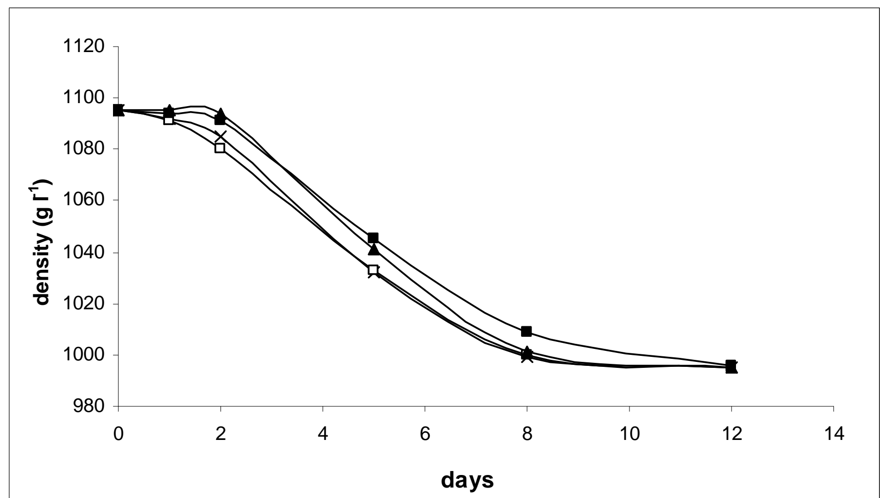


Fig. 1. Fermentation kinetics (as density g $l^{-1}$) of the four experimental fermentations: +I+S (—X—), +I-S (—□—), –I+S (—▲—) and –I-S (—■—).

### 3.2. Experimental set-up for microbial analysis

We used qPCR to enumerate the following main wine microbial groups: total yeasts, *Saccharomyces*, *Hanseniaspora*, LAB and AAB. Standard curves were performed with each pair of primers and Table 2 shows the correlation coefficient ($R^2$), slope and intercept for each of these standard curves.

Table 2.Correlation coefficients, slopes and intercept of the standard curves conducted with the different microorganisms used

| Microrganisms | Slope | Intercept | $R^2$ |
|---|---|---|---|
| Yeast | -4,15±0,03 | 45,65±0,07 | 0,983±0,003 |
| *Saccharomyces* | -2,59±0,05 | 32,57±0,30 | 0,98±0,006 |
| *Hanseniaspora* | -2,87±0,04 | 36,37±0,25 | 0,99±0,003 |
| Acetic acid bacteria | -2,77± 0,50 | 35,35±3 | 0,978±0,015 |
| Lactic acid bacteria | -3,945±0,27 | 45,33±1,75 | 0,987±0,006 |

To identify or detect species diversity during wine fermentations, we used the PCR-DGGE technique. Reference strains of the most usual wine microbial species were amplified with their corresponding primers and were then DGGE-resolved to obtain the relative mobility for each species, and to construct internal markers (Fig. 2). However, we failed to obtain a unique migration distance for each species. In the yeast PCR-DGGE patterns, we were unable to separate *S. cerevisiae*, *C. sake*, *D. anomala* and *Z. rouxii* in the regular denaturing gradient used in this study (from 32.5% to 50% of urea and formamide) (Fig. 2A). A narrower denaturing gradient (from 40% to 45%) was needed to resolve these four species (data not shown). Similar problems were found when trying to separate *Ac. aceti* from *Ac. pasteurianus* in the regular denaturing gradient. The remaining AAB species were easily differentiated under these conditions (Fig. 2C). In the case of the LAB PCR-DGGE pattern, all the species used were clearly distinguished in these standard denaturing conditions (Fig. 2B). The PCR-DGGE patterns of these reference strains should allow the tentative identification of the unknown amplicons. To confirm correct identification, all the unknown amplicons were excised from the gel, reamplified using the same primers but without the GC clamp and identified by sequencing and blast against data bases.

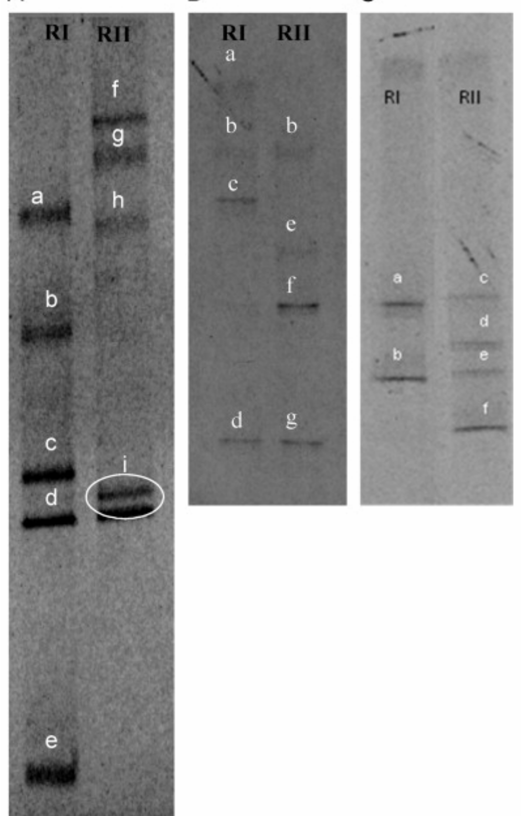


Fig. 2. DGGE patterns of yeasts, lactic and acetic acid bacteria used in this study. Patterns were constructed from pure cultures of different reference strains. (A) Yeasts, Lane **RI**: **a,** *H. guilliermondii*; **b**, *C. stellata*; **c**, *T. delbrueckii*; **d**, *S. cerevisiae*/*C. sake*/*D. anomala*/*Z. rouxii*; **e**, *I. terricola*. Lane **RII**: **f**, *C. mesenterica*; **g**, *H. uvarum*; **h**, *C. boidinii*; **i**, QA23 (inoculated strain of *S. cerevisiae*). (B) Lactic acid bacteria, Lane **RI**: **a**, *Lb. plantarum*; **b**, *O. oeni*; **c**, *Leu. mesenteroides*; **d**, *P. parvulus*. Lane **RII**: **b**, *O. oeni;* **e**, *Lb. brevis*; **f**, *P. pentosaceus*; **g**, *Lb. buchneri*. (C) Acetic acid bacteria, Lane **RI**: **a**, *G. oxydans*; **b**, *Ac. aceti/Ac. pasteurianus*. Lane **RII**: **c**, *G. oxydans*; **d**, *Ac. oeni*; **e**, *Ac. aceti*; **f**, *Ga. hansenii*.

We determined the detection limit of yeasts by PCR-DGGE in a similar experiment to that performed by Prakitchaiwattana et al. (2004). The reference strains of *S. cerevisiae*, *H. uvarum* and *C. stellata* were serially diluted from $10^7$ to $10^1$ cells $ml^{-1}$ to determine the minimum number of cells detected by PCR-DGGE. Since no bands were detected for populations lower than $10^3$ cells $ml^{-1}$, this was considered the lower limit of detection in a pure culture. However, we also examined the presence of a competitive target DNA, represented by the presence of $10^6$ *S. cerevisiae* cells $ml^{-1}$ in the dilutions and the presence of a non-target bacterial DNA, represented by the presence of $10^6$ *O. oeni* cells $ml^{-1}$ in the yeast dilutions. The presence of a large population of *S. cerevisiae* affected the detection threshold of *C. stellata* and *H. uvarum* because no bands were detected for populations lower than $10^4$ cells $ml^{-1}$. Conversely the presence of a non-target DNA did not modify the detection level.

We also examined the minimum bacterial population detected using the total bacteria primers $WBAC1^{GC}$/WBAC2 (Lopez et al., 2003). Serial dilutions of the reference strain of *O. oeni*, *P. parvulus*, *Ac. aceti* and *G. oxydans* were amplified in a pure culture and in the presence of a competitive target (represented by the presence of $10^6$ *O. oeni* cells $ml^{-1}$ in all the dilutions) and a non-competitive target (represented by $10^6$ *S. cerevisiae* cells $ml^{-1}$ in the dilutions). The results were very similar to those obtained with the yeasts. The detection threshold was around $10^3$ cells $ml^{-1}$ in a pure culture which increased to $10^4$ cells $ml^{-1}$ in the presence of a competitive target, but which was unaffected by the presence of a non-target DNA. Conversely, the $L1^{GC}$/HDA2 (Meroth et al., 2003a) showed a higher detection level because we only detected amplicons with a population of $10^6$ *O. oeni* cells $ml^{-1}$ in a pure culture.

### 3.3. Enumeration and identification of yeasts

The total yeast counts of the must were approximately $10^6$ cells $ml^{-1}$. Regardless of the fermentation condition, these populations peaked the fifth day of fermentation and, at the end of the process, the –I–S fermentation had the lowest population (approximately $5\times10^7$ cells $ml^{-1}$) and the inoculated fermentations had the highest (approximately $10^8$ cells $ml^{-1}$) (Fig. 3A).

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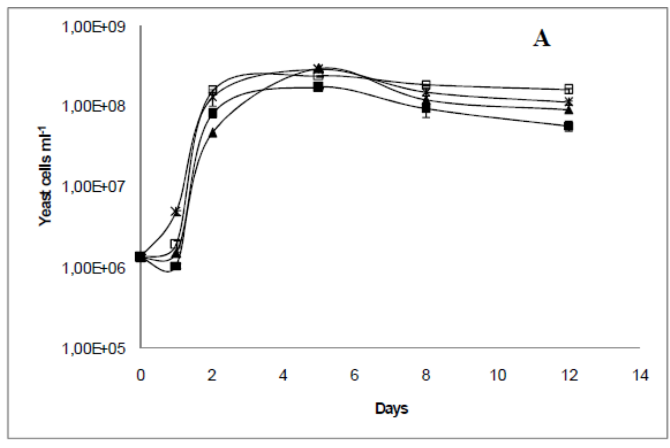


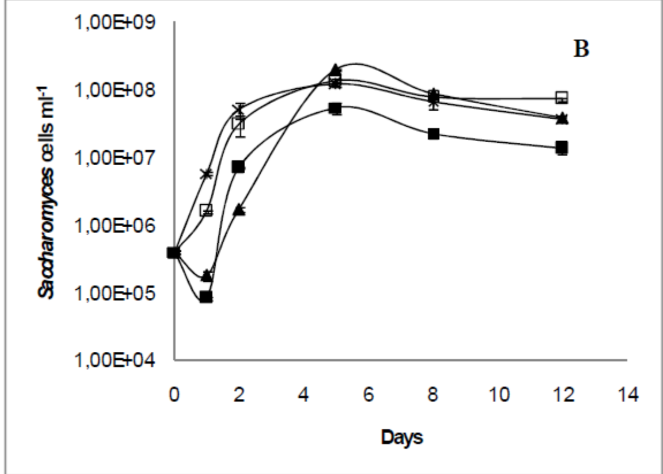


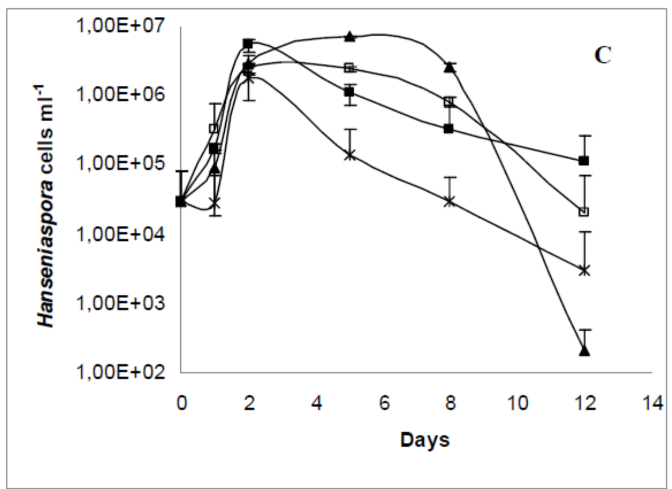


Fig. 3. Evolution of (A) Total yeasts, (B) *Saccharomyces* and (C) *Hanseniaspora* analysed by qPCR during the four fermentations: +I+S (—X—), +I–S (—□—), –I+S (—▲—) and –I–S (—■—)

The counts of *Saccharomyces* and *Hanseniaspora* in the grape must were contrary to the usual distribution of these wine yeast groups. *Hanseniaspora* population (approximately $5\times10^{4}$ cells $ml^{-1}$) was 10 times smaller than the *Saccharomyces* population ($5\times10^{5}$ cells $ml^{-1}$) (Fig. 3B and C). This is unexpected because fermentative species of *Saccharomyces* occur at very low numbers in musts and are rarely isolated (Fleet and Heard, 1993; Pretorius, 2000).

In all the fermentations, *Saccharomyces* populations grew to around $10^8$ cells $ml^{-1}$. As expected, this growth was quicker in the inoculated fermentations. The lowest counts were mostly detected in the –I–S fermentation despite the fact that the fermentation rate was not substantially affected (Fig. 3B). The *Hanseniaspora* population increased to $10^7$ cells $ml^{-1}$ in the first 2 days and, later on, declines fairly sharply depending on the fermentation conditions (Fig. 3C). Therefore, significant differences in the *Hanseniaspora* population were detected at the end of the different fermentations. Sulphited fermentations showed the lowest population counts whereas those fermentations without yeast inoculum or $SO_2$ showed higher values.

PCR-DGGE analysis showed a common band in all samples with the same relative mobility as the *S. cerevisiae* reference strain (Table 3). Moreover, in the inoculated fermentations, this band co-migrated with another band (a doublet). We confirmed that this double band belonged to the inoculated strain by making a PCR-DGGE analysis of a pure culture of this strain (Fig. 2A). We only detected more bands in the sample taken on day 1 from the non-inoculated fermentations. These bands were identified as *H. uvarum*, *H. guilliermondii* and *C. zemplinina*. This latter species showed a relative mobility similar to *C. stellata*, however, its sequence presented a higher homology with the *C. zemplinina* sequences deposited in the GenBank database (97% for *C. zemplinina* vs. 96% for *C. stellata*).

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Table 3. Evolution of different yeast, lactic acid bacteria and acetic acid bacteria species during the four wine fermentations detected by PCR-DGGE

| | Fermentation conditions | | | |
|---|---|---|---|---|
| Day | +I+S | +I-S | -I+S | -I-S |
| 0 | *S. cerevisiae* | *S. cerevisiae* | *S. cerevisiae* | *S. cerevisiae* |
| | *Ga. hansenii* | *Ga. hansenii* | *Ga. hansenii* | *Ga. hansenii* |
| | *Ac. aceti* | *Ac. aceti* | *Ac. aceti* | *Ac. aceti* |
| 1 | *S. cerevisiae* | *S. cerevisiae* | *S. cerevisiae* | *S. cerevisiae* |
| | | | *H.uvarum* | *H.uvarum* |
| | | | *H.guilliermondii* | *C.zemplinina* |
| | | | *C.zemplinina* | |
| | *Ga. hansenii* | *Ga. hansenii* | *Ga. hansenii* | *Ga. hansenii* |
| | *Ac. aceti* | *Ac. aceti* | *Ac. aceti* | *Ac. aceti* |
| 2 | *S. cerevisiae* | *S. cerevisiae* | *S. cerevisiae* | *S. cerevisiae* |
| | *Ga. hansenii* | *Ga. hansenii* | *Ga. hansenii* | *Ga. hansenii* |
| | *Ac. aceti* | *Ac. aceti* | *Ac. aceti* | *Ac. aceti* |
| 5 | *S. cerevisiae* | *S. cerevisiae* | *S. cerevisiae* | *S. cerevisiae* |
| | *Ga. hansenii* | *Ga. hansenii* | *Ga. hansenii* | *Ga. hansenii* |
| | *Ac. aceti* | *Ac. aceti* | *Ac. aceti* | *Ac. aceti* |
| 8 | *S. cerevisiae* | *S. cerevisiae* | *S. cerevisiae* | *S. cerevisiae* |
| | *Ga. hansenii* | *Ga. hansenii* | *Ga. hansenii* | *Ga. hansenii* |
| | *Ac. aceti* | *Ac. aceti* | *Ac. aceti* | *Ac. aceti* |
| 12 | *S. cerevisiae* | *S. cerevisiae* | *S. cerevisiae* | *S. cerevisiae* |
| | | | | *O. oeni* |
| | *Ga. hansenii* | *Ga. hansenii* | *Ga. hansenii* | *Ga. hansenii* |
| | *Ac. aceti* | *Ac. aceti* | *Ac. aceti* | *Ac. aceti* |

+I+S: inoculated and sulphited; +I–S: inoculated and non-sulphited; –I+S: non-inoculated and sulphited; –I–S: non-inoculated and non-sulphited.

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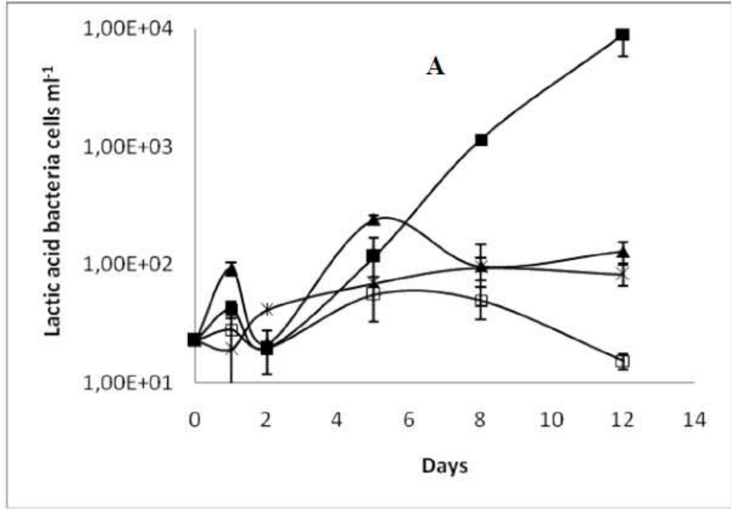
### 3.4. Enumeration and identification of LAB

Inoculation or $SO_2$ (or both) were effective in maintaining low levels of LAB throughout fermentations (ranging from $10^1$ to $10^2$ cells $ml^{-1}$) (Fig. 4A). Only the non-inoculated and $SO_2$-free fermentation (–I–S) showed an increase in the LAB population close to $10^4$ cells $ml^{-1}$ at the end of the alcoholic fermentation. In fact, L-malic acid analysis proved that malolactic fermentation had started during the –I–S fermentation (data not shown).



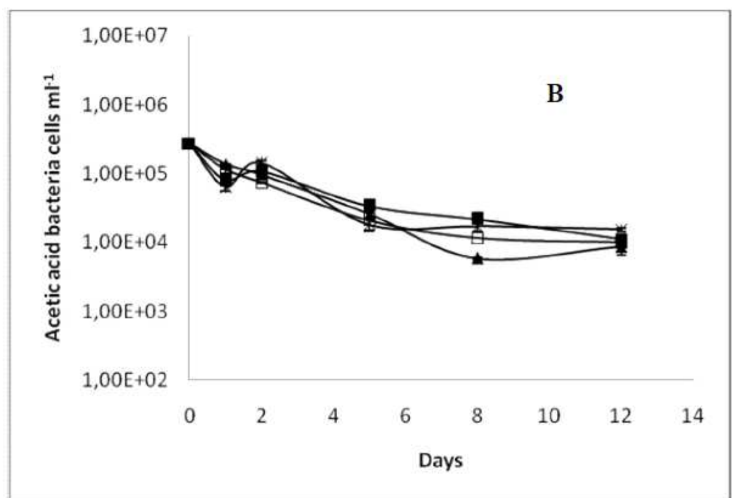




Fig. 4. Evolution of (A) lactic acid bacteria and (B) acetic acid bacteria analysed by qPCR during the four fermentations: +I+S (—X—), +I–S (—□—), –I+S (—▲—) and –I–S (—■—).

Regarding PCR-DGGE analysis, we were not able to amplify any band with the LAB-specific primers L1$^{GC}$/HDA2 (Meroth et al., 2003a). However, we detected a band belonging to *O. oeni* the last day of the –I–S fermentation with the primers WBAC1$^{GC}$/WBAC2. As mentioned above, a population of $10^4$ cells ml$^{-1}$ was enumerated by qPCR which matched the minimum detection threshold established for PCR-DGGE primers. This result proved a lower detection threshold of the common primers for AAB and LAB (Lopez et al., 2003) than the specific LAB primers (Meroth et al., 2003a).

### 3.5. Enumeration and identification of AAB

In contrast to the LAB populations, AAB development was not affected by inoculation or $SO_2$ addition (Fig. 4B). AAB counts in the must were higher than LAB population and these values decreased as fermentation proceeded, although this was regardless of the fermentation conditions. As fermentation rates were similar in all the fermentations, this anoxic environment, which impairs AAB development, was established at the same stage in all the fermentations.

In the PCR-DGGE analysis, all the samples showed two bands regardless of the fermentation condition. These bands were identified as *Ac. aceti* and *Ga. hansenii* by their electrophoretic mobility and further confirmed by sequencing analysis (Fig. 5).

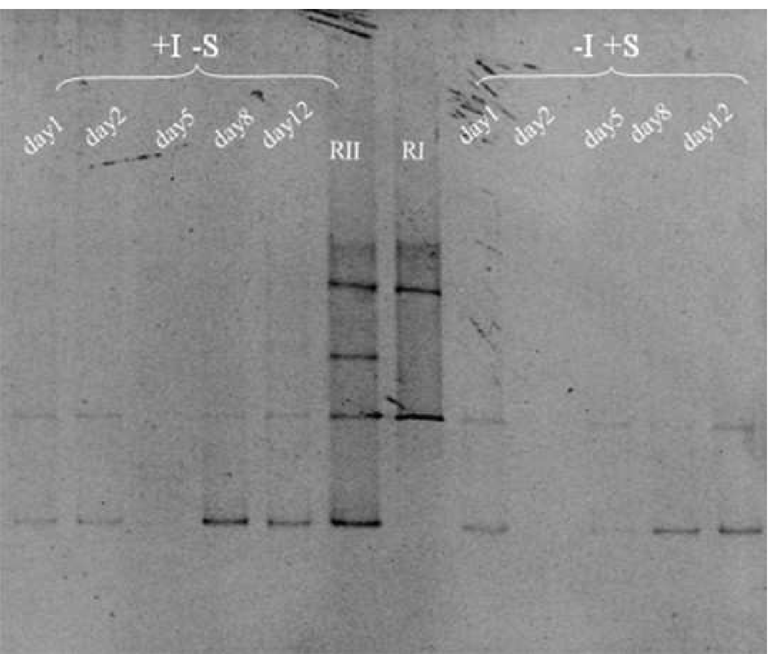



Fig. 5. PCR-DGGE analysis of acetic acid bacteria throughout different wine fermentation conditions. RI is a standard pattern constructed from pure cultures of: *G. oxydans* and *Ac. pasterianus*/*Ac. aceti* from top to bottom. RII is a standard pattern constructed from pure cultures of: *G. oxydans*, *Ac. oeni*, *Ac. aceti*, and *Ga. hansenii* from top to bottom.

## 4. Discussion

The aim of this study was to analyse the population evolution of wine microorganisms during different wine fermentations using only culture-independent methods. To achieve this, we have used qPCR and PCR-DGGE to enumerate and identify the main wine microbial groups: yeasts (total, *Saccharomyces* spp. and *Hanseniaspora* spp.), LAB and AAB. The primers used for the qPCR were described in previous studies by our group (González et al., 2006; Hierro et al., 2006a; Hierro et al., 2007) or other groups (Neeley et al., 2005) and showed good specificity for the intended group of microorganisms. The results of these primers in terms of linearity and detection limit were very similar to those previously reported. PCR-DGGE has been used to study microbial diversity and evolution in wine (Cocolin et al., 2000; Mills et al., 2002; Renouf et al., 2007). These studies have already reported different problems of this technique to detect total diversity (species with different cell lysis or hybridization efficiencies, primer specificity, etc.). However, in our opinion, the main drawback of this technique is that minor species were hardly detected, especially when the best adapted species constituted an overwhelming majority (Renouf et al., 2007). Similar to other studies (Cocolin et al., 2000; Mills et al., 2002), we determined the detection limit for individual yeast species at $10^3$ cells $ml^{-1}$ in a pure culture. However, in a mixed culture, populations represented by less than 1% of the major species were not detected by PCR-DGGE (Muyzer and Smalla, 1998; Prakitchaiwattana et al., 2004). The limit on minor species detection was more relevant in the fermentation samples where populations which represented 10% of the total population were not detected. Some major compounds of wine, such as polyphenols, are known to inhibit the PCR, probably affecting the amplification of the numerically smaller species in the sample. This study also showed the high specificity of the primers $WBAC1^{GC}$/WBAC2 (Lopez et al., 2003) which detected two AAB species in all the fermentation samples. The presence of a high yeast population (~$10^8$ cells $ml^{-1}$) did not affect to the detection of these AAB populations, which ranged from $10^4$ to $5\times10^5$ cells $ml^{-1}$. The bacterial-specific primers $WBAC1^{GC}$/WBAC2 also proved to be more sensitive than the LAB-specific primers $L1^{GC}$/HDA2 (Meroth et al., 2003b) when amplifying LAB species. According to the qPCR results, a population of $10^4$ *O. oeni* cells $ml^{-1}$ was needed to be detected by PCR-DGGE.

Sulphur dioxide ($SO_2$) addition and yeast inoculation are both well established practices in winemaking for restricting the growth of indigenous yeasts and bacterial populations and so another aim of this study was to determine the effect of these oenological practices on wine microbial populations. Although grape must harboured an important population of indigenous *Saccharomyces*, inoculated fermentations showed a high *Saccharomyces* population from the beginning, which prevented the detection of other minor yeast species by PCR-DGGE. As mentioned before, the inoculated *S. cerevisiae* strain could be detected by the doublet. We confirmed that this doublet belonged to the inoculated strain by making a PCR-DGGE analysis of a pure culture of this strain whereas the other *S. cerevisiae* strains did not show the doublet. Obviously in this case we cannot rule out the presence of other *S. cerevisiae* strains as the lower band is common for the inoculated and the other strains. Both bands of the doublet were confirmed using sequencing as belonging to *S. cerevisiae* but with a divergence of 2% between them. Therefore this result confirmed the presence of copies with different rDNA sequences in the same strain, as previously described by other authors (Meroth et al., 2003b; Rantsiou et al., 2005).

Large populations of non-*Saccharomyces* yeasts were only detected in the first day of non-inoculated fermentations when the *Saccharomyces* population size was not so overwhelming. Regarding the *H. uvarum* population, this species had traditionally represented the major species together with *C. stellata* in the grape musts of this cellar (Constantí et al., 1998; Beltran et al., 2002; Hierro et al., 2006b). However, it was under-represented in the present study. The evolution of *Hanseniaspora* population throughout fermentations showed a similar trend to these previous studies, which was an increase at the beginning of fermentation and a decline towards the end. However this decrease was faster in the sulphited fermentations. Other non-*Saccharomyces* species have been detected by PCR-DGGE such as *H. guilliermondii* and *C. zemplinina*. So far we had never before isolated these species, with *C. stellata* together with *H. uvarum*, being the most predominant non-*Saccharomyces* yeasts. Both *Hanseniaspora* species (*H. uvarum* and *H. guilliermondii*) are very closely phylogenetically related species with a very low number of nucleotide substitutions in the D1/D2 (Boekhout et al., 1994), an identical chromosomal profile (Esteve-Zarzoso et al., 2001) and similar ITS-RFLP patterns for most of the restriction enzymes used (Esteve-Zarzoso et al., 1999;Capece et al., 2005). We had mostly used these molecular markers to identify wine yeast

species and, therefore, assumed that we had not been able to separate both species. Similar conclusions could be drawn from the presence of *C. zemplinina*. This species was recently described by Sipiczki (2003) as a new species, closely related to *C. stellata*. Both species can be distinguished by a restriction analysis of the ITS-5.8S sequence with the endonucleases *Dra*I and *Mbo*I (Sipiczki, 2004).

The inoculum and $SO_2$ affected differently the AAB and LAB populations, respectively. AAB population size was considerably higher than LAB population in the must. In all fermentative conditions, the production of carbon dioxide by yeasts during alcoholic fermentation substantially reduced the population of AAB. However, very limited differences were observed when $SO_2$ or yeast inoculum were present. The anaerobic conditions, which arise during alcoholic fermentation are apparently the most important factor in reducing this AAB population. On the other hand, the presence of $SO_2$ and inoculum kept the LAB population at very low level, proving that this population is much more sensitive to both oenological factors than the AAB population. Spontaneous and non-sulphited winemaking processes facilitated the beginning of the malolactic fermentation during alcoholic fermentation. Furthermore, inoculation and/or sulphite addition did not influence the AAB species distribution, being *Ac. aceti* and *Ga. hansenii* the predominant species throughout the fermentations regardless of the different conditions.

In conclusion, the microbial analysis during different wine fermentations was completed using two culture-independent techniques, qPCR for enumerating the yeasts, LAB and AAB, and PCR-DGGE to determine the species diversity. In our opinion, PCR-DGGE is ideal for detecting species diversity in a mixed population with similar relative proportions, although the massive presence of a species did decrease the chances of detecting other minor species. Conversely, the specificity of the primers designed for the qPCR allowed the accurate quantification of a minor microbial group as *Hanseniaspora* regardless of the major presence of other microbial groups such as *Saccharomyces*. From an oenological point of view, the effect of inoculation and $SO_2$ on microbial populations confirmed most of the results previously obtained by culture-dependent techniques (Constantí et al., 1998; González et al., 2005). Inoculation increased the proportion of *Saccharomyces* vs. non-*Saccharomyces* in a shorter time. *Hanseniaspora* increased in the first days, decreasing during the last phases of the process, especially in the sulphited fermentations. Both yeast inoculum and $SO_2$ kept

the LAB populations at very low levels while AAB populations were relatively unaffected by these two widespread oenological practices. However, we also detected some controversial results regarding the widely described general trends of yeast evolution during wine fermentation. An interesting result is that *Saccharomyces* population was one order of magnitude higher than the *Hanseniaspora* population in the grape must. Therefore the most usual distribution of wine yeast species during alcoholic fermentation might be modified by different factors such as grape variety, climatic conditions and viticultural practices (Fleet and Heard, 1993). But, in our opinion, in this case, the most important determining factor is the high degree of contamination with *Saccharomyces* in the winery environment. We came up this conclusion because the grape variety was harvested and processed at the end of the vintage when equipments, tanks and environment were fully contaminated after several weeks of wine processing. During the same vintage and at the same winery, both populations in white grape must were also enumerated by qPCR and the *Hanseniaspora* population was 100 times higher than *Saccharomyces* population (Hierro et al., 2007). However, the grape variety used in our former study was one of the earliest harvested and fermented.

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## Chapter 2

# Effect of fermentation temperature on microbial population evolution using culture-independent and dependent techniques

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**Abstract**

The population dynamics of micro-organisms during grape-must fermentation has been thoroughly studied. However, the main approach has relied on microbiological methods based on plating. This approach may overlook micro-organisms that (i) grow slowly or do not grow well on artificial media or (ii) whose population size is small enough to be detected by regular sampling. Culture-independent methods have been used and compared with the traditional plating method during wine fermentations performed at two different temperatures (13 ℃ and 25 ℃). These methods include a qualitative technique, the DGGE; a semi-quantitative technique, the direct cloning of amplified DNA; and a quantitative technique, the QPCR. The biodiversity observed in the must and at the beginning of fermentation was much higher when DGGE or direct cloning were used. Quantification of the most frequent non-*Saccharomyces* yeast, *Hanseniaspora uvarum* and *Candida zemplinina,* showed that they survived throughout the fermentation process and, specifically, it revealed the quantitatively relevant presence of *C. zemplinina* until the end of fermentation.

**Keywords:** DGGE; Cloning; QPCR; Wine; Grape; *Acetobacter aceti*

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## 1. Introduction

The conversion of grape-must to wine is a complex biochemical process involving interactions between yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB). The metabolism of these micro-organisms contributes to the quality of the wine by releasing metabolites which are constituents of the flavour and aroma (Swiegers, Bartowsky, & Henschke, 2005). Wine microbiota is influenced by multiple factors which can be grouped into viticultural and oenological practices (Pretorius, 1999). The temperature of fermentation is an oenological factor which influences the evolution of wine populations (Fleet, 2003): the lower the temperature of fermentation, the higher the chance of survival of the non-*Saccharomyces* yeasts during alcoholic fermentation (Heard & Fleet, 1988; Sharf & Margalith, 1983). Likewise, Ribéreau-Gayon, Dubourdieu, Donèche, and Lonvaud (2000) reported that low temperature notably reduced the growth of acetic and lactic acid bacteria. Low-temperature fermentations (below 15 ℃) are considered to improve the wine's aromatic profile. The increase in aroma may be related to a higher retention of volatile compounds. However, Beltran, Novo, Guillamón, Mas, and Rozès (2008) observed that this increase in flavour and aroma was not only related to primary aroma retention. The evolution of yeast and bacteria species and their metabolism may also be involved in this improvement in the organoleptical characteristic of wines fermented at low temperature.

Most previous studies on wine microbial ecology have invariably been conducted after the culture of the different micro-organisms in different media. Today, new culture-independent methods allow to identify and enumerate micro-organisms, avoiding the biases associated with traditional culture-dependent methods (Rantsiou et al., 2005). The presence of viable but non-culturable micro-organisms in wine samples has been described (Divol & Lonvaud-Funel, 2005; Millet & Lonvaud-Funel, 2000). These micro-organisms are unable to grow on standard solid media within the laboratory but may justify the differences reported by various authors between isolated and naturally occurring species in wine samples (Cocolin & Mills, 2003; Hierro, Esteve-Zarzoso, González, Mas, & Guillamón, 2006; Mills, Johansen, & Cocolin, 2002).

The aim of this study was to analyse the evolution of wine microbial population during the fermentation of the same grape-must at low (13 ℃) and optimum temperature for wine yeasts during fermentation (25 ℃). Microbial populations were evaluated by using three culture-independent techniques: a qualitative technique

(DGGE), a semi-quantitative technique (the direct cloning of amplified DNA) and a quantitative technique (the QPCR). DGGE and QPCR are two of the most widely used techniques for culture-independent microbial analysis. In a previous study (Andorrà, Landi, Mas, Guillamón, & Esteve-Zarzoso, 2008), we enumerated the main wine microbial groups (yeast, lactic acid bacteria and acetic acid bacteria) using QPCR. In addition, we employed specific primers for the enumeration of two of the main yeast genera, *Saccharomyces* and *Hanseniaspora*. In the present study, we have also designed a new pair of primers for the enumeration of what is probably the third main wine yeast *Candida stellata*, or its current classification as *Candida zemplinina* (Sipiczki, Ciani, & Csoma, 2005). Moreover, in parallel to the analysis of species diversity by DGGE, we have evaluated the richness in yeast species through a direct amplification of DNA purified from wine samples and further cloning and identification of the amplicons. This technique has the additional advantage of making it possible to detect the relative abundance of the different species. To our knowledge, this is the first time that yeast diversity has been analysed using this strategy, thus avoiding some of the problems of cultivability of wine micro-organisms.

## 2. Materials and methods

### 2.1. Reference strains and culture conditions

The reference strains used in this study are listed in Table 1. Yeast were grown in YPD (2% glucose, 2% peptone, 1% yeast extract), lactic acid bacteria were grown in MRS (Oxoid, Hampshire, UK) and acetic acid bacteria were grown in Glucose media (5% glucose, 1% yeast extract).

Table 1. Reference strains used in this study. Sources of strains are abbreviated as: Spanish Type Culture Collection (CECT), Deutsche Sammlung von Mikroorganismen und Zelkulturen (DSM) and Culture Collection of the Laboratorium voor Microbiologie, Gent (LMG).

| | |
|---|---|
| Lactic Acid Bacteria | |
| *Lactobacillus brevis* | CECT 4121 |
| *Lactobacillus buchneri* | CECT 4111$^{T}$ |
| *Lactobacillus plantarum* | CECT 220 |
| *Leuconostoc mesenteroides* | CECT 219 |
| *Oenococcus oeni* | CECT 217$^{T}$ |
| *Pediococcus parvulus* | CECT 813 |
| *Pediococcus pentosaceus* | CECT 4695 |
| Acetic Acid Bacteria | |
| *Acetobacter aceti* | DSM 2002, DSM 3508 |
| *Acetobacter oeni* | LMG 21952$^{T}$ |
| *Acetobacter pasteurianus* | DSM 3509, DSM 46617 |
| *Gluconacetobacter hansenii* | LMG 1529, DSM 5602 |
| *Gluconobacter oxydans* | DSM 2343, DSM 7145 |
| Yeast | |
| *Candida boidinii* | CECT 10029 |
| *Candida mesenterica* | CECT 1025 |
| *Candida sake* | CECT 10034 |
| *Candida stellata* | CECT 11109 |
| *Dekkera anomala* | CECT 11162$^{T}$ |
| *Hanseniaspora guilliermondii* | CECT 11029$^{T}$ |
| *Hanseniaspora uvarum* | CECT 11107 |
| *Issatchenkia terricola* | CECT 11139, CECT 11176$^{T}$ |
| *Saccharomyces cerevisiae* | CECT 1942$^{NT}$ |
| *Torulaspora delbrueckii* | CECT 1880, CECT 10558 |
| *Zygosaccharomyces rouxii* | CECT 1230, CECT 1232 |

### 2.2. Wine fermentations and sampling

This study was conducted in the experimental cellar of the Faculty of Oenology in Tarragona (Spain) during the 2007 vintage in semi-industrial conditions. Macabeo was the grape variety chosen for the vinifications. After destemming and crushing the grapes, $SO_2$ was added (60 mg/L) and the must settled at 10 ºC to separate the particles by density. Afterwards, the clear must was transferred to two 80-L tanks and fermented at 25 ºC and at 13 ºC. The fermentation t emperature was continuously monitored and refrigerated by circulating cool water in a double-jacket stainless steel vat. Both fermentations were conducted by spontaneous microbiota (without yeast inoculation). After settling, the must had 180 g/L reducing sugar concentration, 4.8 g/L of total acidity (expressed as tartaric acid) and a pH of 3.2. The final ethanol concentrations were 10.3 and 10.5 for the wines fermented at 25 ºC and 13 ºC respectively.

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Samples were taken from the must and the settled must at the beginning of fermentation (density of 1080 g/L), middle fermentation (density of 1050 g/L), at the middle-late fermentation stage (density of 1020 g/L) and at the end of fermentation (density of 990 g/L). Must and settled must were common for both fermentations. Samples were taken after homogenization by pumping-over. Several dilutions of each sample were plated on YPD-agar medium. Fifty colonies from each fermentation point were randomly isolated and purified for further identification.

### 2.3. DNA extraction

DNA from reference strains or wine samples was extracted according to Hierro et al. (2006) and diluted to 1–50 ng/µL. The concentration and purity of DNA was determined using a GenQuant spectrophotometer (Pharmacia, Cambridge, UK). Sample DNA was extracted from 1 mL of must or wine. The same DNA was used for DGGE, QPCR and PCR amplification of the ribosomal region.

### 2.4. PCR and restriction analysis

The ITS region and the 5.8S rRNA gene were amplified as described previously (Guillamón, Sabaté, Barrio, Cano, & Querol, 1998). All the amplifications were performed in a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA, USA). Five microliters of the ITS/5.8S rRNA gene amplified product were digested with the restriction endonuclease *Hinf*I according to the supplier's instructions. *Cfo*I, *Dra*I or *Hae*III were also used for further identification when needed. All the restriction enzymes were from Roche Diagnostics GmBh (Mannheim, Germany).

### 2.5. Direct cloning of ribosomal fragment of yeasts

The amplicons of the ITS region and the 5.8S rRNA gene, which used the DNA extracted from wine samples as template, were cloned using pGEM®-T Easy Vector (Promega Corporation, Madison, WI) according to the manufacturer's protocol. Fifty transformed *Escherichia coli* colonies from each sample were purified and their plasmids isolated. Standard procedures for bacterial transformation and plasmid isolation from *E. coli* were performed (Sambrook, Frisch, & Maniatis, 1989). Five microlitres of the isolated plasmid were digested with the restriction endonuclease *Hae*III (Roche Diagnostics GmBh, Mannheim, Germany) according to the supplier's instructions.

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### 2.6. DGGE

The primers we used to amplify the specific ribosomal region of each microbial group were: $U1^{GC}$/U2 (Meroth, Walter, Hertel, Brandt, & Hammes, 2003) for yeasts, $L1^{GC}$/HDA2 (Meroth, Hammes, & Hertel, 2003) for LAB and $WBAC1^{GC}$/WBAC2 (Lopez et al., 2003) for AAB. For lactic acid bacteria, a fragment of the 16S rRNA gene was amplified and separated by DGGE as described by Meroth, Walter, et al. (2003). For acetic acid bacteria, the PCR amplification was prepared as in Lopez et al. (2003) although the electrophoretic run was kept at a constant 170 V for 4 h at a constant temperature of 60 ℃ in TAE buffer 0.5 X, and in a denaturing gradient from 30% to 60% of urea and formamide. For yeasts, amplification of the fragments and denaturing electrophoresis was performed according to Meroth, Walter, et al. (2003). All PCR amplifications were performed in a Gene Amp PCR System 2700 (Applied Biosystems, Fosters City, USA), using EcoTaq DNA Polimerase (Ecogen, Spain). The Dcode universal mutation detection system (Bio-Rad, Hercules, Calif.) was used to run the DGGE analysis.

### 2.7. Sequencing

The DNA fragments from the DGGE gels were excised according to Omar and Ampe (2000). Each excised band was then transferred into 50 µL of sterile water and incubated overnight at 4 ℃ to allow diffusion of the DNA. One microliter of the eluted DNA was used for re-amplification with primers without the GC clamp. The PCR products were purified and sequenced by Macrogen Inc. facilities (Seoul, South Korea) using an ABI3730 XL automatic DNA sequencer. The primers ITS1 and ITS4 were used for sequencing the ribosomal region inserted in the pGEM plasmid. The BLAST search (Basic Alignment Search Tool, Internet address: http://www.ebi.ac.uk/blastall/nucleotide.html) was used to compare the sequences obtained with databases of the European Molecular Biology Laboratory (EMBL). We considered identification to be correct when gene sequences showed identities of 98% or higher.

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### 2.8. QPCR

In all cases QPCR was performed on an ABI Prism 5700 Sequence Detection System (Applied Biosystems). Power SyberGreen master mix was used according to the manufacturer's instructions (Applied Biosystems, CA). An ABI PRISM 96 well optical plate was used for the reaction. This instrument automatically determined the $C_T$.

Yeast and bacteria quantification was performed by using the primers: YEASTF/YEASTR (Hierro et al., 2006) for total yeast, CESP-F/SCER-R for *Saccharomyces* and CESP-F/HUV-R for *Hanseniaspora* (Hierro, Esteve-Zarzoso, Mas, & Guillamón, 2007), WLAB1/WLAB2 for lactic acid bacteria (Neeley, 2005) and AQ1F/AQ2R for acetic acid bacteria (González, 2006), as described by Andorrà et al. (2008). In the case of *C. zemplinina*, the new pair of primers, AF (5′-CTAGCATTGACCTCATATAGG-3′) and 200R (5′-GCATTCCCAAACAACTCGACTC-3′), were designed from the D1/D2 domain of the 26S rRNA gene. AF primer is specific for *C. zemplinina* while the 200R primer is homologous to a conserved region for all the yeasts used for the alignment. Therefore, the specificity was determined by the AF primer. Standard curves were created by plotting the Ct (Cycle Threshold) values of the QPCR performed on dilution series of cells against the log input cells/mL. Samples and cultures for standard curves were analysed in triplicate.

## 3. Results

Microbial populations (yeast, lactic acid bacteria and acetic acid bacteria) were monitored by qualitative (DGGE), semi-quantitative (direct cloning of amplified ribosomal DNA) and quantitative (real-time PCR) culture-independent techniques. In order to evaluate the effect of the temperature of fermentation on the dynamics and diversity of these populations, the same grape-must was divided into two tanks and fermented at 25 ℃ (optimum temperature) and 13 ℃ (restrictive temperature). Both fermentations proceeded spontaneously (non-inoculated) and the low-temperature fermentation took longer (double time) to complete (Fig. 1).

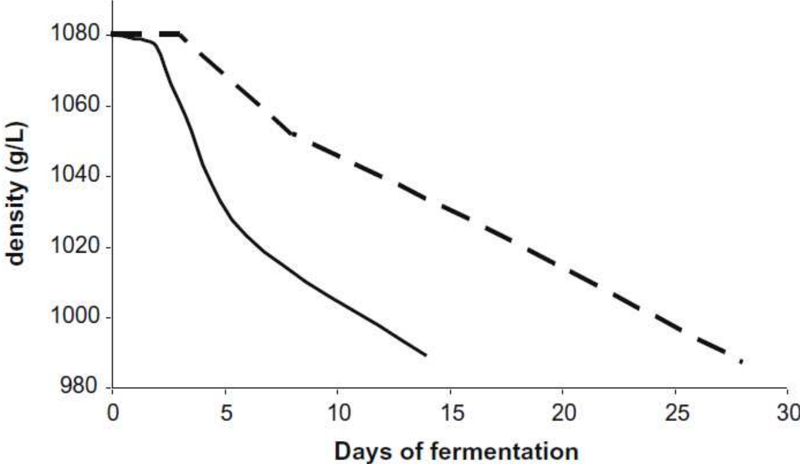


Fig. 1. Evolution of the wine fermentations at 25 °C (—) and 13 ℃ (- -) measured as density (g/L).

### 3.1. Species detection by DGGE

The application of this technique to the different samples taken throughout both wine fermentations allowed us to detect the species of filamentous fungi *Aspergillus niger* and *Botriotinya fuckeliana* (teleomorph of *Botrytis cinerea*), the species of yeasts *C. zemplinina*, *Hanseniaspora uvarum* and *Saccharomyces cerevisiae*, and the species of acetic acid bacteria *Acetobacter aceti*. No species of lactic acid bacteria were detected at any point of the fermentations. The correct identification of these species was confirmed by excision from the gel, purification and sequencing of the different bands. The distribution of these species in the different fermentation stages is shown in Table 2. Filamentous fungi were only detected in the grape-must and they quickly disappeared with the fermentation process. *H. uvarum* was also detected in the grape-must and at the beginning of the alcoholic fermentation. However, *C. zemplinina* and *S. cerevisiae* were detected in all the samples analysed. Likewise, the species *A. aceti* was also ubiquitous throughout the process. The fermentation temperature did not modify the diversity and presence of these species during the alcoholic fermentation. The only difference can be attributed to a faster disappearance of *H. uvarum* in the control fermentation (25 ℃).

Table 2. Microbial population analysed using DGGE during alcoholic fermentation at both temperatures (13 ºC and 25 ºC). *A.: Aspergillus, B.: Botriotinya, C.: Candida, H.: Hanseniaspora, S.: Saccharomyces, Ac.: Acetobacter.*

<table>
<tr><th></th><th>13 ºC</th><th>25 ºC</th></tr>
<tr><td>Must</td><td colspan="2">A. niger<br>B. fuckeliana<br>C. zemplinina<br>H. uvarum<br>S. cerevisiae<br>Ac. aceti</td></tr>
<tr><td>Settled must</td><td colspan="2">A. niger<br>B. fuckeliana<br>C. zemplinina<br>H. uvarum<br>S. cerevisiae<br>Ac. aceti</td></tr>
<tr><td>Beginning</td><td>C. zemplinina<br>H. uvarum<br>S. cerevisiae<br>Ac. aceti</td><td>C. zemplinina<br>S. cerevisiae<br><br>Ac. aceti</td></tr>
<tr><td>Middle</td><td>C. zemplinina<br>S. cerevisiae<br>Ac. aceti</td><td>C. zemplinina<br>S. cerevisiae<br>Ac. aceti</td></tr>
<tr><td>Middle-late</td><td>C. zemplinina<br>S. cerevisiae<br>Ac. aceti</td><td>C. zemplinina<br>S. cerevisiae<br>Ac. aceti</td></tr>
<tr><td>End</td><td>C. zemplinina<br>S. cerevisiae<br>Ac. aceti</td><td>C. zemplinina<br>S. cerevisiae<br>Ac. aceti</td></tr>
</table>

### 3.2. Species identification by direct cloning of amplified ribosomal DNA

The ribosomal region, which spans the 5.8S gene and the ITS region, was amplified by using as template the DNA directly extracted from must and wine samples. The PCR product was ligated into a plasmid and cloned in an *E. coli* strain. Fifty *E. coli* colonies per sample were analysed by plasmid purification and restriction analysis of this plasmid. We had cloned the same PCR product of *C. zemplinina*, *H. uvarum* and *S. cerevisiae* reference strains, as the major species found in the process, and obtained the restriction pattern of the plasmid with ribosomal region inserted. Only clones giving restriction patterns different from those of the major species were identified by sequencing. We also cultured the must and wine samples in a plate and the same number of yeast colonies were randomly analysed by amplification and restriction of the same ribosomal region (Esteve-Zarzoso, Belloch, Uruburu, & Querol, 1999; Guillamón et al., 1998). The percentage of the different yeast species detected throughout

UNIVERSITAT ROVIRA I VIRGILI
ADAPTATION AND DEVELOPMENT OF CULTURE-INDEPENDENT TECHNIQUES FOR THE INDENTIFICATION AND ENUMERATION
OF MICROORGANISMS IN WINE FERMENTATIONS
Immaculada Andorrà Solsona
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fermentations by both culture-independent and culture-dependent methods is shown in Table 3.

Table 3. Microbial population analysed using cloning and plating techniques during alcoholic fermentation at both temperatures (13 and 25 ºC). Values indicate the % of the colonies analysed on each fermentation point. *A.*: *Aspergillus*, *C.*: *Candida, H.*: *Hanseniaspora*, *S.*: *Saccharomyces*, *Smycopsis.*: *Saccharomycopsis*, *Z.*: *Zygosaccharomyces.*

| | | | Must | Settled Must | Beginning | Middle | Middle-late | End |
|---|---|---|---|---|---|---|---|---|
| **13 ºC** | *A. niger* | Plate | - | - | - | - | - | - |
| | | Cloning | 2 | 2 | - | - | - | - |
| | *C. zemplinina* | Plate | 94 | 96 | 96 | 12 | 2 | - |
| | | Cloning | 68 | 66 | 52 | 22 | 24 | 32 |
| | *H. uvarum* | Plate | - | 4 | 4 | 2 | - | - |
| | | Cloning | 6 | 4 | 8 | | - | - |
| | *H. vineae* | Plate | - | - | - | - | - | - |
| | | Cloning | 2 | - | 2 | 4 | - | - |
| | *S. cerevisiae* | Plate | 6 | - | - | 86 | 98 | 100 |
| | | Cloning | 16 | 28 | 38 | 74 | 76 | 68 |
| | *Smycopsis. vini* | Plate | - | - | - | - | - | - |
| | | Cloning | 4 | - | - | - | - | - |
| | | Plate | - | - | - | - | - | - |
| | *Z. bailii* | Cloning | 2 | - | - | - | - | - |
| **25 ºC** | *A. niger* | Plate | - | - | - | - | - | - |
| | | Cloning | 2 | 2 | - | - | - | - |
| | *C. zemplinina* | Plate | 94 | 96 | 78 | 34 | 8 | - |
| | | Cloning | 68 | 66 | 82 | 30 | 16 | 18 |
| | *H. osmophila* | Plate | - | - | 10 | - | - | - |
| | | Cloning | - | - | - | - | - | - |
| | *H. uvarum* | Plate | - | 4 | 2 | - | - | - |
| | | Cloning | 6 | 4 | - | - | - | - |
| | *H. vineae* | Plate | - | - | - | - | - | - |
| | | Cloning | 2 | - | - | - | - | - |
| | *S. cerevisiae* | Plate | 6 | - | 10 | 66 | 92 | 100 |
| | | Cloning | 16 | 28 | 18 | 70 | 84 | 82 |
| | *Smycopsis. vini* | Plate | - | - | - | - | - | - |
| | | Cloning | 4 | - | - | - | - | - |
| | *Z. bailii* | Plate | - | - | - | - | - | - |
| | | Cloning | 2 | - | - | - | - | - |

Yeast diversity detected by direct cloning of the PCR product was higher than by the DGGE technique. Seven different species were identified: *A. niger*, *C. zemplinina*, *H. uvarum*, *Hanseniaspora vineae*, *S. cerevisiae*, *Saccharomycopsis vini* and *Zygosaccharomyces bailii*. However, the total of *C. zemplinina* and *S. cerevisiae* represented 95% of the colonies analysed. Moreover, the remaining species were only isolated in the grape-must samples. Regarding the two major species, as the fermentation progressed the percentage of *S. cerevisiae* increased and *C. zemplinina* decreased. However, this latter species was still present to a significant degree at the end of the fermentation. Again, the fermentation temperature hardly influenced the species distribution. The same species and similar percentages were detected in both

fermentations. It should be noted that the grape-must samples, which showed the highest diversity, were common for both fermentations.

Of particular interest is the comparison of the direct cloning with the random identification of yeast colonies. The culture of the must and wine samples increased the percentages of the major species and decreased the number of species detected (only *H. uvarum* and *H. osmophila* were identified from the minor species). Furthermore, *C. zemplinina* showed an absolute predominance at the beginning of the process while all the colonies analysed at the end of the process belonged to *S. cerevisiae*. The only noteworthy differences between the yeast colonies identified in the fermentations at 13 ºC and 25 ºC are that *C. zemplinina* disappeared more rapidly at low temperature and *H. osmophila* was only isolated in the 25 ºC fermentation.

### 3.3. Enumeration of yeast by real-time quantitative PCR (QPCR)

We used QPCR to enumerate the following main wine yeast groups: total yeasts, *Saccharomyces*, *Hanseniaspora* and *C. zemplinina*. The pair of primers for the quantification of *C. zemplinina* was designed from the D1/D2 region of the 26S rDNA. Due to the high degree of phylogenetic relationship (and the low number of nucleotide substitutions) between the species *C. stellata* and *C. zemplinina*, it was impossible to design completely specific primers for one of these species. However, Sipiczki et al. (2005) showed that most of the wine strains preserved in culture collections or described in recent publications as *C. stellata* were indeed *C. zemplinina*. Likewise we also isolated *C. zemplinina* by DGGE and direct cloning but we never detected the presence of *C. stellata*. Therefore we can assume that these primers are useful to enumerate *C. zemplinina* in wines. The tests of specificity and sensitivity of this pair of primers were satisfactory (correlation coefficient 0.995, slope 3.207 and intercept 39.64) and comparable with the values obtained for the other primers (Hierro, Esteve-Zarzoso, Mas, & Guillamón, 2007; Hierro et al., 2006). A population size of approximately $10^7$ cells/mL was quantified in the grape-must (Table 4). The settling of this must produced a decrease of approximately 50% ($5 \times 10^6$ cells/mL) and the population grew to *ca.* $5 \times 10^7$ cells/mL during the fermentation. The maximum population size remained constant throughout the whole process at 13 ºC whereas it de creased in the latter stages at 25 ºC.

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Table 4. Quantification (cells/mL) of yeast population by QPCR during fermentations at both temperatures (13 °C and 25 °C). Total yeast, *Hanseniaspora, Candida stellata/zemplinina* and *Saccharomyces* yeasts were evaluated.

| | Must | Settled must | Beginning | Middle | Middle-late | End |
|---|---|---|---|---|---|---|
| **13º C** | | | | | | |
| **Total yeast** | 1,28±0,41 x $10^7$ | 4,48±1,91 x $10^6$ | 1,43±0,34 x $10^7$ | 3,41±0,92 x $10^7$ | 4,92±0,19 x $10^7$ | 4,46±2,16 x $10^7$ |
| ***Candida*** | 1,82±0,08 x $10^7$ | 9,05±1,70 x $10^5$ | 2,08±0,41 x $10^7$ | 1,18±0,14 x $10^7$ | 1,75±0,08 x $10^7$ | 6,62±0,58 x $10^6$ |
| ***Hanseniaspora*** | 2,54±0,85 x $10^6$ | 5,51±0,22 x $10^5$ | 1,42±0,13 x $10^6$ | 3,00±1,79 x $10^5$ | 2,69±0,62 x $10^5$ | 1,77±0,22 x $10^5$ |
| ***Saccharomyces*** | 1,10±0,14 x $10^4$ | 8,49±0,43 x $10^3$ | 1,83±0,18 x $10^6$ | 1,63±0,07 x $10^7$ | 1,19±0,11 x $10^7$ | 9,82±1,35 x $10^6$ |
| **C+H+S*** | 2,08 x $10^7$ | 1,46 x $10^6$ | 2,41 x $10^7$ | 2,84 x $10^7$ | 2,97 x $10^7$ | 1,66 x $10^7$ |
| **25 ºC** | | | | | | |
| **Total yeast** | 1,28±0,41 x $10^7$ | 4,48±1,91 x $10^6$ | 3,29±0,35 x $10^7$ | 3,55±0,81 x $10^7$ | 4,35±0,44 x $10^7$ | 1,70±0,14 x $10^7$ |
| ***Candida*** | 1,82±0,08 x $10^7$ | 9,05±1,70 x $10^5$ | 2,79±0,39 x $10^7$ | 1,77±0,66 x $10^7$ | 4,45±0,62 x $10^7$ | 2,92±1,20 x $10^6$ |
| ***Hanseniaspora*** | 2,54±0,85 x $10^6$ | 5,51±0,22 x $10^5$ | 1,48±0,69 x $10^6$ | 1,40±0,27 x $10^5$ | 1,62±0,43 x $10^5$ | 1,29±0,22 x $10^5$ |
| ***Saccharomyces*** | 1,10±0,14 x $10^4$ | 8,49±0,43 x $10^3$ | 6,64±0,25 x $10^6$ | 2,25±0,25 x $10^7$ | 1,93±0,22 x $10^7$ | 6,28±0,27 x $10^6$ |
| **C+H+S*** | 2,08 x $10^7$ | 1,46 x $10^6$ | 3,6 x $10^7$ | 4,03 x $10^7$ | 6,39 x $10^7$ | 9,33 x $10^6$ |

* Represents the addition of the yeast population of the *Candida*, *Hanseniaspora* and *Saccharomyces* yeasts.

*C. zemplinina* and *Hanseniaspora* were the major species in the grape-must, the number of *Saccharomyces* being much lower (~1 × $10^4$ cells/mL). At the beginning of fermentation, all the groups of species grew. The group with the highest counts was *C. zemplinina* (~2 × $10^7$ cells/mL) which represented the majority of the total population. *Saccharomyces* (1.8 × $10^6$ cells/mL at 13 °C and 6.6 × $10^6$ cells/mL at 25 °C) and *Hanseniaspora* (~1.5 × $10^6$ cells/mL) populations were one log unit lower. This latter species decreased its population size during the process to $10^5$ cells/mL. The population of *C. zemplinina* did not increase after the first day of fermentation, but it was constant during the process and only decreased at the end of fermentation. The most important increase in yeast population was registered by the *Saccharomyces* group, which increased more than two orders of magnitude (or 2 log units) the first day and reached a maximum value of 1–2 × $10^7$ cells/mL. However, the percentage of *Saccharomyces* barely reached 50% of the total population in the different days analysed, showing similar percentages to *C. zemplinina*.

Also of interest was the fact that the total of the three groups of species analysed represented more than 80% of the whole population during the process, appearing once again as the major species of the wine-making process. However, these species only represented 33% of the total yeast population in the grape-must, indicating that other species are present, as we have detected with the other techniques used.

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### 3.4. Enumeration of acetic acid bacteria (AAB) and lactic acid bacteria (LAB)

The two main bacterial groups of wine were also counted by QPCR (Fig. 2), using the specific primers described for AAB (González, Hierro, Poblet, Mas, & Guillamón, 2006) and LAB (Neeley, Phister, & Mills, 2005). LAB population showed a very low count in the grape-must ($10^2$ cells/mL). This population ranged from $10^2$ to $10^3$ cells/mL throughout the process, regardless of the temperature of fermentation. The counts of AAB (approximately $5 \times 10^5$ cells/mL) were higher than LAB in the grape-must. The beginning of the fermentation produced a decrease in the AAB population of approximately 2 log units. These populations did not change during the fermentation, with the exception of the last day of fermentation at 13 ℃.

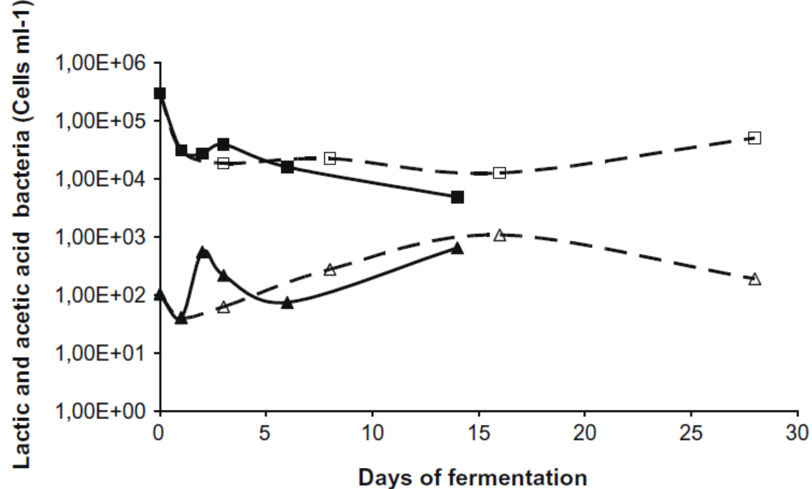


Fig. 2. Evolution of Acetic Acid Bacteria and Lactic Acid Bacteria analysed by QPCR during two fermentations: Lactic Acid Bacteria 25 ℃ (—▲—) and 13 ℃ (- -Δ- -). Acetic Acid Bacteria 25 ℃ (—■—) and 13 ℃ (- -□- -).

## 4. Discussion

Traditional methods of micro-organism quantification and identification rely on culturing the sample, counting and identifying colonies. These studies based on culture-dependent tools are likely to produce biased results based on unrepresentative cultivation conditions (Renouf, Strehaiano, & Lonvaud-Funel, 2007). Minor populations and stressed or weakened cells, which need specific culture conditions, may not be recovered on a plate. These limitations, associated with traditional culture-based methods, have driven microbiologists to develop alternative culture-independent

techniques which are primarily based on the analysis of nucleic acids (Justé, Thomma, & Lievens, 2008).

DGGE has been reported as a powerful technique for the study of the ecology of wine (Cocolin, Bisson, & Mills, 2000; Mills et al., 2002). However, as also reported in previous studies (Andorrà et al., 2008; Renouf et al., 2007), the main drawback of this technique is its low sensitivity. Minor species were barely detected, especially when the best adapted species constituted an overwhelming majority. Consequently, we were only able to detect a high diversity of minor species by DGGE in the grape-must samples in which no species showed a clear predominance. During wine fermentation the predominant species, *C. zemplinina* and *S. cerevisiae*, were the only species detected.

We also used the direct cloning of an amplified ribosomal region of yeasts and the analysis of the clones by restriction analysis or sequencing. This technique detected higher yeast species diversity than DGGE and also permitted the calculation of the percentage or preponderance of the different species. However, it should be taken into account that only a small fraction of the population is being analysed and thus only semi-quantitative or qualitative conclusions should be drawn. Due to the simplicity of current cloning systems, this is an affordable, easy and reliable technique for the study of microbial diversity which avoids the problems associated with the cultivability of micro-organisms. Nevertheless, certain short comings may be attributed to this technique such as the presence of inhibitors in the matrix which interfere with the PCR reactions or differential efficiency in the DNA purification and amplification of the different species. In addition, inter-specific differences such as variation in the copy number of the ITS region may also produce biased results.

Comparing the results of direct cloning with those obtained from yeast isolated colonies, the former also detected more species and the presence of predominant species did not prevent the detection of others present in low quantities. For example, from the middle to the end of the fermentation, few of the colonies analysed were identified as *C. zemplinina* whereas this species was detected in significant percentages in the same samples by direct cloning. The greater detection of this species by cloning may be explained by the inability of these species to grow under the culture conditions used or that with the direct cloning we are amplifying DNA from dead cells. This latter problem may be overcome by cloning cDNA instead of genomic DNA, since RNA is less stable than DNA after cellular death.

The third technique used was the real-time quantitative PCR (QPCR). The DNA extracted from grape-must and wine samples was used with the specific primers and conditions designed in our previous works (Hierro et al., 2006, 2007), with the exception of the primers for the quantification of *C. zemplinina*, which were specifically designed for this study. In our opinion, the main advantage of this technique is that, regardless of the overwhelming presence of a major species, the specificity of the primers designed permits the detection of minor species. Thus, we were able to detect *Saccharomyces* in the grape-must samples and *Hanseniaspora* at the end of the fermentation, even though they each only represented less than 1% of the total population. This sensitivity is inconceivable with the other techniques used. However, there is a possible drawback of the technique such as we used it. The use as template of a very stable molecule such as DNA may inflate the counts by amplifying DNA from dead cells. We are currently assaying the use of a DNA-intercalating dye such as ethidium monoazide bromide, which penetrates only dead cells, avoiding its DNA amplification (Nocker & Camper, 2006). However, the correlation between quantification by plating (data not shown) and quantification by QPCR was satisfactory with some divergences. The total yeast counts of the must and settled-must samples were higher with QPCR than with plating (YPD-agar). As already reported Hierro et al. (2006), the total-yeast primers also amplify other fungal DNA; therefore, the presence of DNA from filamentous fungi, as detected by DGGE and cloning, would increase the values of the population. Our results clearly showed the significant loss of yeast population with the settling of the must (50% reduction). As previously reported (Torija, Rozès, Poblet, Guillamón, & Mas, 2001), *Saccharomyces* strains were the most competitive ones in the process, increasing their population by three orders of magnitude in a few days. However, in this case, the imposition of *Saccharomyces* was not absolute. *C. zemplinina* presented similar values to *Saccharomyces* throughout the fermentation, to the extent that it could be concluded that the grape-must was co-fermented by both groups of species. Regarding the evolution of bacteria population, LAB were practically unaffected by the process, with a constant low population from the beginning. AAB population size was considerably higher than LAB population in the must. The production of carbon dioxide by yeast during alcoholic fermentation substantially reduced the population of AAB.

The idea that temperature may affect the ecology of wine fermentation has been previously reported. Sharf and Margalith (1983) suggested that *H. uvarum* had better

ability than *S. cerevisiae* to grow at lower temperatures and Heard and Fleet (1988) showed that *H. uvarum* and *C. stellata* retained high populations until the end of fermentations at low temperatures. Likewise, Ribéreau-Gayon et al. (2000) reported that the low temperature notably reduced the growth of acetic and lactic acid bacteria. However, in our fermentation conditions, the temperature of fermentation was not a determining factor in the yeast species development. The most notable differences are a rapid disappearance of *Hanseniaspora* at 25 ℃ and a high number of AAB at 13 ℃, possibly as a consequence of the sluggish fermentation rate during the last days of fermentation at low temperature.

In conclusion, DGGE and direct cloning of amplified DNA allowed us to detect higher species diversity compared with plating. Cloning was more sensitive in detecting minor species. The specificity of the primers designed for the QPCR allowed the enumeration of minor microbial groups in spite of the major presence of other species. The results of this study mostly confirmed the importance and distribution during the process of the major yeast species, widely reported in numerous studies. However, the use of these culture-independent techniques evidenced a higher presence and permanence of the non-*Saccharomyces* species and their contribution is not only limited to the first days of fermentation. Also noteworthy is the ubiquitous presence of a significant population of AAB throughout the process. Despite the anaerobic conditions established during fermentation, this population can survive under these circumstances (Bartowsky & Henschke, 2008; Millet & Lonvaud-Funel, 2000). It should be reminded that the fermentations proceeded spontaneously and the lack of inoculation prevented a rapid dominance of *S. cerevisiae*, the most powerful fermentative species. This circumstance might explain these high levels of AAB. Finally, in our working conditions, the temperature of fermentation showed a limited influence on the diversity and distribution of the different wine micro-organisms.

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**Chapter 3**

# Effect of pure and mixed cultures of the main wine yeast species on grape must fermentations

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**Abstract**

Mixed inoculation of non-*Saccharomyces* yeasts and *S. cerevisiae* is of interest for the wine industry for technological and sensory reasons. We have analysed how mixed inocula of the main non-*Saccharomyces* yeasts and *S. cerevisiae* affect fermentation performance, nitrogen consumption and volatile compound production in a natural Macabeo grape must. Sterile must was fermented in triplicates and under the following six conditions: three pure cultures of *S. cerevisiae*, *Hanseniaspora uvarum* and *Candida zemplinina* and the mixtures of *H. uvarum*:*S. cerevisiae* (90:10), *C. zemplinina:S. cerevisiae* (90:10) and *H. uvarum:C. zemplinina:S. cerevisiae* (45:45:10). The presence of non-*Saccharomyces* yeasts slowed down the fermentations and produced higher levels of glycerol and acetic acid. Only the pure *H. uvarum* fermentations were unable to finish. Mixed fermentations consumed more of the available amino acids and were more complex and thus better able to synthesise volatile compounds. However, the amount of acetic acid was well above the admissible levels and compromises the immediate application of mixed cultures.

**Keywords:** *Saccharomyces*, *Candida*, *Hanseniaspora*, *Wine fermentation*, *Volatile compounds*, *Amino acids*.

## 1. Introduction

The fermentation of grape juice into wine is a complex microbial reaction involving the sequential development of various species of yeast. Traditionally, wine has been produced by the natural fermentation of grape juice by yeasts that originate from grapes and winery equipment [1]. Yeasts with low fermentation activity, such as *Candida spp.*, *Hanseniaspora spp.*, *Kluyveromyces spp.*, *Pichia spp.* and *Rhodotorula spp.*, are predominant in grape musts and during the early stages of fermentation. Subsequently, *S. cerevisiae* proliferates, dominating and completing the wine fermentation [2, 3]. Generally, these non-*Saccharomyces* species were considered to be of secondary significance or undesirable to the process. However this trend is changing. In a recent review, Fleet [4] discussed the possibilities of using yeasts other than those from the *Saccharomyces* genus for future wine fermentations and the commercial viability of mixed cultures. These species have great potential to introduce appealing characteristics to wine which may improve its organoleptic quality.

The major non-*Saccharomyces* yeasts present during alcoholic fermentation are *Candida stellata*, currently classified as *Candida zemplinina* [5], and *Hanseniaspora uvarum* (anamorph *Kloeckera apiculata*). Although the population size of these species reduced throughout the wine fermentations, several quantitative ecological studies have indicated that their growth was not completely suppressed, either in spontaneous or in inoculated fermentations [2, 6-8]. Similar studies have shown their capacities to improve wine flavour [9-11] or have evaluated the biotechnological nature of their enzymatic activities [12, 13]. *Candida stellata* is frequently associated with overripe and botrytized grape berries [14-18]. The most interesting oenological characteristic of this species is that it is highly fructophilic [14]. Ciani and Ferraro [19] demonstrated that mixed fermentations containing *C. stellata* and *S. cerevisiae* consumed sugars more completely and postulated that this was due to the preferential use of fructose by *C. stellata*. This yeast may be used in mixed cultures with *S. cerevisiae* for stuck fermentations, where the proportion of fructose is usually higher than glucose. However, more controversial results have been reported about this species' contribution to wine aroma. Some authors have reported the production of high levels of acetic acid [20, 21], glycerol [20, 22] and succinic acid [23] whereas others have found low acetic acid production [24] and low glycerol production [16]. Csoma and Sipiczki [25] asserted that these contradictory results were because *C. stellata* is easily confused with other yeast

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species that colonize the same substrates. This hypothesis is supported by the recent finding that the strain DBVPG 3827, frequently used to investigate the oenological properties of *C. stellata*, belongs to *Starmerella bombicola* [5] and by the description of a new species, *Candida zemplinina* that was previously considered *C. stellata* [5, 26, 27]. Such findings raise doubts about the precise taxonomic position of the oenological *C. stellata* strains described in the literature [25]. *Hanseniaspora* species have been considered great producers of esters, most of them contributing to the flowery and fruity aroma of wines. However, the main ester is ethyl acetate, which in high concentrations produces an unpleasant aroma of glue, solvent, etc. Another characteristic of the excessive growth of *Hanseniaspora* during wine fermentation is the increase in volatile acidity as a result of the synthesis of acetic acid and ethyl acetate. Ciani et al. [28] have recently confirmed the unacceptable increase in ethyl acetate content in a mixed culture of *H. uvarum*/*S. cerevisiae*. *H. uvarum* strains also possess enzymatic characteristics of interest to winemaking because of their technological effects and their contribution to aroma formation. Pectinases, proteases and glycosidases are some of the enzymes secreted by *H. uvarum* which improve the clarification, stabilisation and aroma of wines. Moreira et al. [29] analysed the production of alcohols, esters and heavy sulphur compounds by pure and mixed cultures of apiculate wine yeasts. *H. guilliermondii* produced high levels of 2-phenylethyl acetate, 2-phenylethanol, acetic-acid-3-(methylthio)propyl ester (cooked potatoes aroma) and 3-methylthiopropionic acid. Concentrations of heavy sulphur compounds were also higher in a pure culture of *H. uvarum* than in a pure culture of *S. cerevisiae*.

Consequently, the impact of non-*Saccharomyces* yeasts on wine fermentation cannot be ignored. They introduce an element of ecological diversity to the process that goes beyond *Saccharomyces* species and they require specific research and understanding to prevent any unwanted consequences from their use and to exploit their beneficial contributions [4]. In this study we report the impact of pure and mixed populations of *C. zemplinina*, *H. uvarum* and *S. cerevisiae* on fermentation behaviour, nitrogen consumption and aroma production.

## 2. Material and methods

### 2.1. Experimental fermentations

Fermentations were conducted using several combinations of the commercial strain of *Saccharomyces cerevisiae* QA23 (Lallemand, Inc. Canada) and the strains *Candida zemplinina* CszB4 and *Hanseniaspora uvarum* HuB10 previously isolated from wine fermentations. Both strains were selected on the basis of a preliminary experiment which consisted of a multiple co-inoculation of several strains of the same species in grape must. The strains selected were those with a higher presence at the end of fermentation (data not shown).

Fermentations were conducted on Macabeo must coming from the experimental cellar of the Faculty of Oenology in Tarragona (Spain) during 2007 vintage. This must was sterilised by the addition of 250 mg $l^{-1}$ of dimethyldicarbonate (Sigma-Aldrich, Steinheim, Germany). After settling, 400 ml of must was put in 500 ml bottles. This must contained 180 g $l^{-1}$ of sugar content, which corresponded to 10 % of the probable alcohol degree, a pH of 3.1 and 4.8 g $l^{-1}$ of total acidity of tartaric acid. A total 114.57 mg N $l^{-1}$ of Yeast Assimilable Nitrogen (YAN) was found, 57.16 mg of which was in organic form (amino acids) and 57.41 mg was ammonium. All experiments were done in triplicate fermentations at a controlled temperature of 20 ºC and 150 rpm of stirring on an orbital shaker. Sugar consumption was monitored daily by measuring the density (g $l^{-1}$) of the fermenting must and by enzymatic assay (Roche Applied Science; Germany). Fermentations were considered to be finished when the level of reducing sugars was below 2 g $l^{-1}$.

The musts were inoculated with $10^6$ cells $ml^{-1}$ in all cases. The inocula were *S. cerevisiae* (S), *C. zemplinina* (C), *H. uvarum* (H), *C. zemplinina*/*S. cerevisiae* (CS), *H. uvarum/S. cerevisiae* (HS) and *C. zemplinina*/*H. uvarum*/*S. cerevisiae* (CHS) always at the ratio of 9:1 for non-*Saccharomyces vs*. *Saccharomyces* (4.5:4.5:1 for the triple culture). The total yeast populations were enumerated on plates with YPD medium (2% glucose, 2% Bacto peptone, 1% yeast extract, 2% agar, W/v; Cultimed, Barcelona, Spain). The selective lysine-agar medium (Oxoid, Barcelona, Spain), which is unable to support the growth of *S. cerevisiae* [30], was used to enumerate non-*Saccharomyces* populations.

### 2.2. Nitrogen content analysis

YAN was analysed by the formol index method [31], and the ammonium content was quantified using an enzymatic method (Roche Diagnostics, Germany). The individual amino and imino acids were analysed by OPA and FMOC derivatizations, respectively, using the Agilent 1100 Series HPLC as described by Beltran et al. [32]. Several dilutions of each sample were analysed and averaged using the analysis software. The concentration of each amino acid was calculated using external and internal standards and expressed as mg $l^{-1}$. The software used was Agilent ChemStation Plus (Agilent Technologies, Germany).

### 2.3. Volatile compound analysis

The concentrations of the different volatile compounds were analyzed at the end of each fermentation. The protocol followed by Ortega et al. [33] was modified to determine volatile fatty acids, ethyl esters of fatty acids, higher alcohol acetates and other volatile compounds. The following were added to 15-ml screw-capped tubes: 1.5 ml of wine, 3.5 ml of $(NH_4)_2SO_4$ (45%, w/v), 20 µl of internal standard (4-methyl-2-pentanol (176 µg $ml^{-1}$), 1-nonanol (160 µg $ml^{-1}$), heptanoic acid (150 µg $ml^{-1}$) in ethanol and 200 µl of dichloromethane. The tube was shaken for 30s (3x) and then centrifuged at 4000 rpm for 10 min. Once the phases were separated, the bottom phase (dichloromethane) was transferred to a glass vial insert. The extract (2 µl) was injected in split mode (10:1, 30 ml $min^{-1}$) into an HP-FFAP (Agilent Technologies, Böblingen, Germany) column of 30 m x 0.25 mm, 0.25 µm phase thickness. The temperature program was as follows: 35ºC for 5 min, then raised at 3ºC $min^{-1}$ up to 200ºC and then at 8ºC $min^{-1}$ up to 220ºC. Injector and detector (FID) temperatures were 180ºC and 280ºC, respectively. The carrier gas was helium at 3 ml $min^{-1}$. Volatile compounds were identified and quantified by comparison with standards.

### 2.4. Organic acid analysis

The values of different organic acids were analyzed at the end of the fermentations of the wine samples. Organic acids were determined by HPLC using an Agilent 1100 Series connected to an Agilent multiple wavelength detector (Agilent Technologies, Wilmington, DE). The samples (450 µl) were mixed with 50 µl of formic acid (Internal Standard, 46.84 g $l^{-1}$) and 50 µl were injected into a 300 mm x 7.8 mm

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AMINEX HPX-87H columm (BioRad, Hercules, CA). The solvent used was sulphuric acid 2.5 mM at 0.5 ml min$^{-1}$. The analysis temperature was 70ºC. The concentration of each metabolite was calculated using external and internal standards.

### 2.5. Oenological parameters

The glucose, fructose, glycerol and ethanol content of the wines were analyzed using commercial enzymatic kits (Roche Diagnostics, Germany). Acetic and succinic acids were determined by HPLC as described above. The pH was determined by using a pH-meter Crison MicropH 2000 (Crison, Barcelona, Spain).

### 2.6. Statistical treatment

The data were analyzed with SPSS 15.0 software for Windows (SPSS Inc., Chicago, IL). Analysis of variance was carried out by an ANOVA Tukey test to determine significant differences between the samples. The statistical level of significance was set at $P \leq 0.05$.

## 3. Results

### 3.1. Kinetics and main fermentation products

As expected, the fastest fermentation was with the pure culture of *S. cerevisiae*, considered as control, whereas the slowest fermentations were those inoculated either with a pure culture of *Hanseniaspora uvarum* or *Candida zemplinina* (Figure 1).

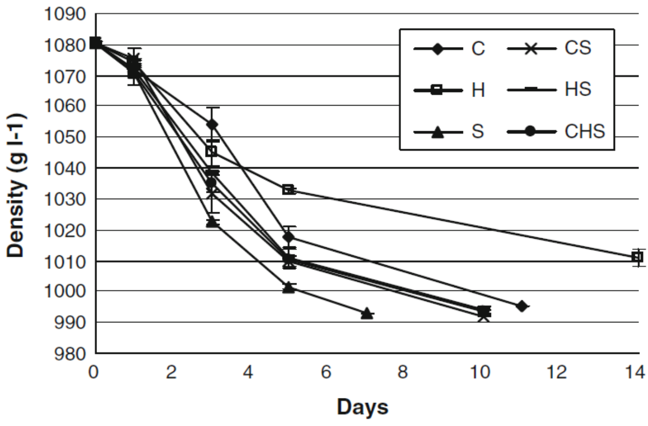


Figure 1. Fermentation kinetics (as density g l$^{-1}$) of the six experimental fermentations: *C. zemplinina* (–♦–), *H. uvarum* (–□–), *S. cerevisiae* (–▲–), *C. zemplinina-S. cerevisiae* (--X--), *H. uvarum –S. cerevisiae* (-----), *C. zemplinina-H. uvarum- S. cerevisiae* (--●--). Each point is expressed as the mean ± standard deviation.

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The pure *H. uvarum* culture was the only condition that did not finish the fermentation (20 g $l^{-1}$ of glucose left in the medium) (Table 1). All the fermentations reached a similar ethanol concentration (around 9.5-10%) with the exception of the *H. uvarum* pure culture, which only reached 4%. Regarding other oenological parameters, the greatest differences among the different cultures were detected in the glycerol and acetic acid concentrations. All the fermentations showed a higher concentration of glycerol and acetic acid than the control *S. cerevisiae* fermentation, with the exception of the unfinished *H. uvarum* fermentation, which produced less glycerol but much more acetic acid.

Table 1. Principal oenological parameters at the end of the different fermentations. Fermentations with Saccharomyces inoculum (S) were taken as the control. All values are expressed as g l-1, with the exception of the pH values, and are the mean ± standard deviation of triplicate fermentations.

| | Glycerol | Succinic acid | Acetic acid | pH | Glucose | Fructose |
|---|---|---|---|---|---|---|
| **S** | 4.56 ± 0.19 | 1.52 ± 0.04 | 0.49 ± 0.03 | 2.94 ± 0.06 | - | 0.87 ±0.04 |
| **C** | 5.91 ± 0.21* | 1.05 ± 0.02* | 0.80 ± 0.05* | 2.97 ± 0.02 | 0.60 ± 0.15 | 1.49 ±0.07 |
| **H** | 3.37 ± 0.23* | 0.50 ± 0.02* | 37.50 ± 0.09* | 2.95 ± 0.02 | 20 ± 0.80 | - |
| **CS** | 5.79 ± 0.49* | 1.82 ± 0.06* | 1.76 ± 0.19* | 2.96 ± 0.05 | 0.40 ± 0.06 | 0.12 ± 0.05 |
| **HS** | 5.31 ± 0.65 | 1.48 ± 0.05 | 1.58 ± 0.06* | 3.05 ± 0.03* | - | 0.55 ± 0.12 |
| **CHS** | 5.41 ± 0.49 | 1.48 ± 0.02 | 1.53 ± 0.11* | 3.01 ± 0.03 | 0.05 ± 0.03 | 0.05 ± 0.02 |

- Not detected

### 3.2. Microbial populations

Total yeast population was very similar in all fermentations and reached a population around $10^8$ cfu $ml^{-1}$ (Fig 2). This population level was reached after 72 hours, except in the case of *H. uvarum* pure culture which reached this maximum population on the fifth day of fermentation. The presence of *S. cerevisiae* in the mixed cultures meant that the maximum total yeast populations were quickly reached. These maximum populations were kept stable during the process, that is, there was no decline phase in last stages of fermentation, and even the population of *C. zemplinina* increased steadily throughout the fermentation. The only exception was the pure *H. uvarum* culture which showed a clear decline during the last stages in accordance with its stuck fermentation.

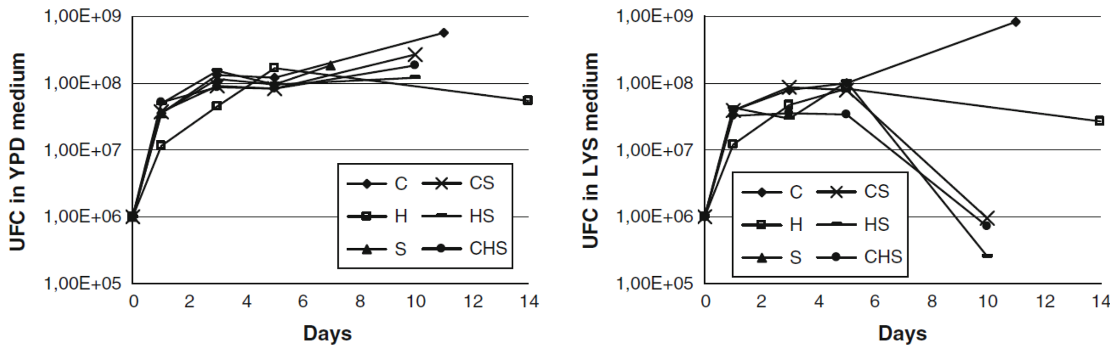


Figure 2. Evolution of yeast population in two different plates, YPD and lysine-agar (LYS) medium, in the different fermentations: *C. zemplinina* (–♦–), *H. uvarum* (–□–), *S. cerevisiae* (–▲–), *C. zemplinina-S. cerevisiae* (--X--), *H. uvarum–S. cerevisiae* (-----), *C. zemplinina-H. uvarum-S. cerevisiae* (--●--). Standard deviation was calculated on each case, and was never higher than 20%.

The non-*Saccharomyces* counts were similar to the total yeast populations (the same order of magnitude) at the beginning of fermentation. However, in the mixed fermentations, these numbers decreased as fermentation proceeded. The comparison between the counts obtained in both culture media (non-selective YPD and selective lysine-agar) clearly proved that most of the yeast population was non-*Saccharomyces* at the beginning of the process but that *Saccharomyces* population took over the process in the middle and at the end of the fermentation. Non-*Saccharomyces* yeasts represented less than 1% of total yeast population at the end of the fermentation.

The counts of the pure non-*Saccharomyces* cultures (C and H) should have been the same in YPD and lysine-agar. This was the case with *C. zemplinina*; however, *H. uvarum* counts were smaller in lysine-agar than in YPD in some samples. This result could be because YPD is a richer medium which supports better growth than lysine-agar, especially when cells are stressed by the presence of ethanol.

### 3.3. Ammonium and amino acid consumption

We analysed the ammonium and amino acid content in the media at different stages of the fermentation. We detected the maximum consumption in the middle of the fermentation because nitrogen release, as consequence of yeast autolysis, was observed in the final phases of the fermentation. This maximum consumption of both individual amino acids and ammonium is shown in the Table 2.

Table 2. Amino acids and ammonium consumed in the middle of the different fermentations. All values are expressed as mg $l^{-1}$ and are the mean ± standard deviation of triplicate fermentations. Fermentations with *Saccharomyces* inoculum (S) were taken as the control.

| Group | AA | Must | S | C | H | HS | CS | CHS |
|---|---|---|---|---|---|---|---|---|
| **Acids & amides** | **Asp** | 51.77 ± 1.06 | 41.98 ± 1.41 | 41.24 ± 1.13 | 44.8 ± 0.85 | 48.76 ± 0.09* | 48.23 ± 0.78* | 47.64 ± 1.95* |
| | **Glu** | 59.39 ± 1.52 | 49.67 ± 1.13 | 55.65 ± 2.12 | 56.53 ± 1.41* | 57.94 ± 0.06* | 55.21 ± 3.01 | 58.37 ± 0.67* |
| | **Asn** | 13.71 ± 0.1 | 13.71 ± 0.0 | 13.71 ± 0.0 | 13.71 ± 0.0 | 13.71 ± 0.0 | 13.71 ± 0.0 | 13.71 ± 0.0 |
| | **Gln** | 166.35 ± 1.06 | 158.49 ± 7.55 | 158.57 ± 9.89 | 131.72 ± 9.20* | 156.47 ± 7.36 | 153.95 ± 7.56 | 154.49 ± 2.72 |
| | *Total acids & amides* | *291.22 ± 3.74* | *263.84 ± 10.09* | *269.177 ± 13.15* | *246.76 ± 11.46* | *276.888 ± 7.35* | *271.1 ± 11.08* | *274.21 ± 5.24* |
| **Aliphatic** | **Gly** | 3.07 ± 0.08 | 2.30 ± 0.10 | 1.53 ± 0.14* | 2.21 ± 0.13 | 2.8 ± 0.11* | 2.73 ± 0.18* | 2.74 ± 0.03* |
| | **β-ala** | 1.75 ± 0.11 | 1.38 ± 0.13 | 1.45 ± 0.14 | 1.07 ± 0.08 | 1.45 ± 0.06 | 1.5 ± 0.12 | 1.22 ± 0.57 |
| | **α-ala** | 40.45 ± 0.51 | 36.94 ± 0.42 | 37.35 ± 0.28 | 36.79 ± 0.71 | 39.11 ± 0.76* | 39.46 ± 0.32* | 39.31 ± 0.19* |
| | **Val** | 10.45 ± 0.53 | 9.26 ± 0.47 | 7.83 ± 0.54 | 9.02 ± 0.62 | 9.78 ± 0.35 | 9.35 ± 0.88 | 9.58 ± 0.37 |
| | **Ile** | 6.07 ± 0.85 | 5 ± 1.13 | 2.06 ± 0.48 | 3.32 ± 0.85 | 5.04 ± 0.74 | 4.22 ± 1.06 | 3.82 ± 0.59 |
| | **Leu** | 9.33 ± 0.54 | 3.77 ± 0.71 | 1.34 ± 0.58* | 6.68 ± 0.48* | 8.09 ± 0.48* | 7.8 ± 0.41* | 7.61 ± 0.67* |
| | *Total aliphatic* | *71.12 ± 2.62* | *58.66 ± 2.95* | *51.56 ± 2.16* | *59.09 ± 2.87* | *66.26 ± 1.97** | *65.03 ± 1.36* | *64.29 ± 1.42* |
| **Aromatic** | **Tyr** | 5.15 ± 0.84 | 1.45 ± 0.57 | 3.99 ± 0.51 | 3.75 ± 0.97 | 4.2 ± 0.61 | 3.65 ± 1.69 | 4.03 ± 0.47 |
| | **Trp** | 9.65 ± 0.95 | 5.13 ± 1.10 | 2.63 ± 0.41* | 4.15 ± 0.78 | 8.32 ± 0.53* | 6.55 ± 0.92 | 6.79 ± 0.32 |
| | **Phe** | 15.93 ± 1.25 | 7.91 ± 1.42 | 4.33 ± 1.41 | 7.4 ± 0.99 | 14.63 ± 0.71* | 13.22 ± 1.84* | 14.1 ± 1.37* |
| | *Total aromatic* | *30.73 ± 3.04* | *14.49 ± 3.72* | *10.96 ± 2.33* | *15.3 ± 2.74* | *27.14 ± 0.91** | *23.42 ± 2.61** | *24.96 ± 1.18** |
| **Hydroxyl** | **Ser** | 21.96 ± 0.52 | 21.08 ± 0.71 | 19.8 ± 0.56 | 21.96 ± 0.84 | 21.78 ± 0.31 | 21.54 ± 0.71 | 21.96 ± 0.0 |
| | **Thr** | 14.97 ± 0.09 | 14.95 ± 0.17 | 14.95 ± 0.11 | 14.89 ± 0.07 | 14.69 ± 0.04 | 14.77 ± 0.19 | 14.94 ± 0.01 |
| | *Total hydroxyl* | *36.93 ± 0.61* | *36.04 ± 0.88* | *34.75 ± 0.68* | *36.85 ± 0.92* | *36.46 ± 0.28* | *36.311 ± 0.90* | *36.9 ± 0.01* |
| **Sulphur** | **Met** | 3.01 ± 0.18 | 1.89 ± 0.25 | 3.01 ± 0.28* | 0.8 ± 0.21* | 1.13 ± 0.15* | 1.97 ± 0.30 | 0.54 ± 0.08* |
| | **Cyst** | 2.15 ± 0.25 | 2.15 ± 0.33 | 2.15 ± 0.26 | 1.05 ± 0.31* | 1.75 ± 0.26 | 1.36 ± 0.13* | 0.91 ± 0.18* |
| | *Total sulphur* | *5.16 ± 0.43* | *4.03 ± 0.59* | *5.16 ± 0.55* | *1.85 ± 0.52** | *2.88 ± 0.09** | *3.33 ± 0.17* | *1.53 ± 0.04** |
| **Basic** | **His** | 6.24 ± 0.26 | 4.53 ± 0.41 | 3.91 ± 0.44 | 3.64 ± 0.37 | 4.69 ± 0.76 | 4.99 ± 0.34 | 5.55 ± 0.19 |
| | **Arg** | 120.06 ± 2.42 | 112.17 ± 2.39 | 111.39 ± 2.80 | 110.75 ± 2.21 | 116.9 ± 2.86 | 116.68 ± 0.54 | 114.61 ± 2.27 |
| | **Lys** | 7.77 ± 0.65 | 6.93 ± 0.85 | 7.08 ± 0.42 | 6.97 ± 0.28 | 7.43 ± 0.85 | 5.88 ± 1.56 | 7.35 ± 0.21 |
| | *Total basic* | *134.07 ± 3.33* | *123.63 ± 3.65* | *122.38 ± 3.66* | *121.36 ± 2.85* | *129.02 ± 2.91* | *127.55 ± 1.92* | *127.51 ± 2.12* |
| | **Pro** | 127.38 ± 0.97 | 113.95 ± 0.74 | 114.96 ± 1.32 | 113.58 ± 0.86 | 105.21 ± 0.98 | 124.83 ± 1.64 | 124.55 ± 0.47 |
| | **NH4+** | 221.47 ± 1.73 | 209.69 ± 1.58 | 209.14 ± 2.03 | 210.03 ± 1.85 | 214.94 ± 2.25 | 217.85 ± 1.84 | 216.87 ± 0.48 |
| | *Total aas* | *696.91* ± 4.07 | *614.64* ± 22.58 | *608.94* ± 23.25 | *594.79* ± 22.04 | *643.85* ± 4.00 | *651.57* ± 18.62 | *653.95* ± 8.23 |
| | ***Total N*** | *154.07* ± 1.49 | *141.58* ± 2.94 | *140.54* ± 3.03 | *136.40* ± 2.87 | *147.04* ± 1.47 | *146.05* ± 2.43 | *145.64* ± 1.43 |
| | ***N org*** | *96.12* ± 1.09 | *86.71* ± 2.36 | *85.81* ± 2.43 | *81.44* ± 2.31 | *90.79* ± 1.07 | *89.05* ± 1.95 | *88.89* ± 1.37 |
| | ***N inorg*** | *57.95* ± 0.48 | *54.87* ± 1.01 | *54.73* ± 1.04 | *54.96* ± 0.99 | *56.25* ± 0.48 | *57.01* ± 0.84 | *56.75* ± 0.12 |

Unfortunately, the low concentration of assimilable nitrogen (YAN) in the grape must meant that the differences in nitrogen consumption were not as remarkable as expected. Ammonium was completely consumed in all the conditions. The mixed cultures consumed more amino acids than the pure cultures. Moreover, these mixed

cultures consumed more of certain groups (aliphatic and aromatic amino acids) than the pure yeasts culture. They also consumed more glutamic acid, aspartic acid, glycine, alanine, leucine and phenylalanine. However, the converse also happened, the mixed cultures consumed fewer sulphur amino acids than the pure *S. cerevisiae* and *C. zemplinina* cultures.

### 3.4. Volatile compounds

The most important aroma forming compounds were analysed in the final wines (Table 3).

Table 3. Volatile compounds at the end of the different fermentations. All values are expressed as mg l-1 and are the mean ± standard deviation of triplicate fermentations. Fermentations with Saccharomyces inoculum (S) were taken as the control.

| Group | Compound | S | C | CS | HS | CHS |
|---|---|---|---|---|---|---|
| **Higher Alcohols** | **1-Propanol** | 9.55 ± 1.26 | 30.58 ± 0.96* | 16.94 ± 4.38 | 11.47 ± 0.30 | 21 ± 1.40* |
| | **2-Methyl-1-propanol** | 24.51 ± 1.53 | 468.86 ± 74.83* | 93.46 ± 0.73 | 55.39 ± 0.75 | 77.4 ± 1.77 |
| | **Isoamyl alcohol** | 167.52 ± 4.33 | 334.63 ± 29.45* | 213.13 ± 17.41 | 202.95 ± 15.50 | 199.86 ± 6.63 |
| | **β-Phenylethanol** | 30.63 ± 4.91 | 227.9 ± 15.26* | 118.15 ± 3.00* | 42.72 ± 2.69 | 61.22 ± 0.02* |
| | *Total higher alcohols* | *232.21 ± 9.51* | *1061.98 ± 29.15** | *441.68 ± 18.06** | *312.53 ± 12.35** | *359.49 ± 9.82** |
| **Fatty acid ethyl esters** | **Ethyl hexanoate** | 0.03 ± 0.01 | 0.12 ± 0.01 | 0.09 ± 0.02 | 0.22 ± 0.11 | 0.1 ± 0.01 |
| | **Ethyl octanoate** | 0.22 ± 0.04 | 3.6 ± 0.06* | 0.61 ± 0.19* | 0.21 ± 0.02 | 0.25 ± 0.03 |
| | **Ethyl lactate** | 0.34 ± 0.04 | 0.89 ± 0.11 | 1.27 ± 0.22* | 2.27 ± 0.05* | 2.54 ± 0.28* |
| | *Total Fatty acid ethyl esters* | *0.6 ± 0.09* | *4.61 ± 0.05** | *1.97 ± 0.39** | *2.71 ± 0.04** | *2.89 ± 0.23** |
| **Higher alcohol Acetates** | **Isoamyl acetate** | 0.25 ± 0.08 | 0.15 ± 0.15 | 0.14 ± 0.01 | 0.70 ± 0.01* | 0.2 ± 0.01 |
| | **Hexyl acetate** | 6.81 ± 0.17 | 13.98 ± 0.68 | 14.34 ± 0.95 | 23.22 ± 4.30* | 9.47 ± 2.09 |
| | **2-Phenylethyl acetate** | 2.99 ± 0.14 | 1.09 ± 0.01* | 2.79 ± 0.16 | 4.23 ± 0.34* | 3.31 ± 0.07 |
| | *Total acetates* | *10.06 ± 0.39* | *15.22 ± 0.55* | *17.27 ± 1.10* | *28.15 ± 3.97** | *12.99 ± 2.01* |
| **SCFA** | **Isobutyric acid** | 1.77 ± 0.01 | 29.08 ± 2.38* | 12.66 ± 1.26* | 6.13 ± 0.47 | 4.35 ± 0.13 |
| | **Isovaleric acid** | 1.97 ± 0.13 | 1.01 ± 0.16* | 2.48 ± 0.38 | 1.52 ± 0.07 | 1.64 ± 0.19 |
| | **Butyric acid** | 0.64 ± 0.02 | 0.34 ± 0.09 | 0.75 ± 0.20 | 0.77 ± 0.04 | 0.64 ± 0.12 |
| | *Total SCFA* | *4.37 ± 0.14* | *30.32 ± 2.44** | *15.89 ± 0.68** | *8.42 ± 0.58* | *6.64 ± 0.06* |
| **MCFA** | **Hexanoic acid** | 3.42 ± 0.46 | 0.31 ± 0.05* | 2.89 ± 0.38 | 1.83 ± 0.19* | 1.74 ± 0.01* |
| | **Octanoic acid** | 2.62 ± 0.45 | 0.23 ± 0.07* | 1.88 ± 0.04 | 0.92 ± 0.11* | 1.62 ± 0.22* |
| | **Decanoic acid** | 1.59 ± 0.32 | 0.12 ± 0.01* | 0.84 ± 0.11* | 0.69 ± 0.17* | 0.56 ± 0.08* |
| | **Dodecanoic acid** | 0.18 ± 0.05 | 1.2 ± 0.18* | 0.56 ± 0.24 | 1.3 ± 0.09* | 0.17 ± 0.01 |
| | *Total MCFA* | *7.82 ± 0.26* | *1.86 ± 0.19** | *6.18 ± 0.55** | *4.74 ± 0.35** | *4.09 ± 0.15** |

Italic values represent the sum of related compounds SCFA short-chain fatty acids, MCFA medium-chain fatty acids. * Means statistically different from the control, P ≤ 0.05

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The pure *H. uvarum* culture fermentation was not analysed because it did not finish fermenting and its high concentration of acetic acid and ethyl acetate made the analysis of other compounds very difficult. *S. cerevisiae* had the lowest production of higher alcohols whereas *C. zemplinina* had the highest. The mixed fermentations produced higher alcohols at levels between those of the pure *S. cerevisiae* and *C. zemplinina*, although levels were closer to those of *S. cerevisiae*. The strong difference between *C. zemplinina* and *S. cerevisiae* was due to a significant increase in each detected compound, whereas the differences between mixed fermentations were mostly due to the increases in 2 phenylethanol and 2 methyl-1-propanol.

The production of ethyl esters is also significantly higher in the presence of non-*Saccharomyces* yeasts and especially in the pure cultures of *C. zemplinina*. In this case, the difference was mostly due to the increase in ethyl octanoate, whereas in the mixed fermentations it was related to the increases in ethyl lactate.

Although all the fermentations produced more acetate esters than the pure *S. cerevisiae* culture, the only significant difference was in the mixed *H. uvarum* and *S. cerevisiae* culture.

The production of short chain fatty acids (SCFA) was also higher in all the fermentations than in the *S. cerevisiae* fermentation. This increase was higher in fermentations which contained *C. zemplinina,* especially when it fermented alone. The main contributor to this difference was isobutyric acid, which was highly synthesised by *C. zemplinina*. On the other hand, medium chain fatty acids (MCFA) concentrations in the *S. cerevisiae* fermentations were always higher than in the other wines, except for dodecanoic acid, which was produced in higher quantities by the non-*Saccharomyces* yeasts.

## 4. Discussion

The aim of this study was to analyze the effect of mixed *Saccharomyces* and non-*Saccharomyces* cultures on amino acid consumption and aroma production in natural grape must, and to determine the interactions among the different microorganisms involved. These fermentations were inoculated with a *Saccharomyces* strain together with a *C. zemplinina* strain and/or a *H. uvarum* strain that was selected according to its fermentation performance. So far, the wine industry has only paid attention to the *S. cerevisiae* strains as fermentative agents, and has ignored the

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possibility of using other yeasts during fermentations. However, interest is growing in the possible contributions of non-*Saccharomyces* yeasts to the fermentation process. Non-*Saccharomyces* species can contribute to the aromatic properties and chemical composition of the resulting wine because they produce more secondary metabolites which contribute to the taste and flavour of the wines [20]. Some authors have even reported that these yeasts produce extracellular enzymes that may provide the wine with properties that are unique to the region where it is produced [13]. In our opinion, however, further research is needed into how individual non-*Saccharomyces* species and strains contribute to wine quality and into the synergy or antagonism between *Saccharomyces* and non-*Saccharomyces* species in the final resulting wines.

It is well-known that non-*Saccharomyces* yeast predominates in the first stages of fermentation before disappearing in favour of *S. cerevisiae*, which has the highest fermentative capacity. This phenomenon is generally ascribed to *Saccharomyces*' higher capacity to withstand increasing concentrations of ethanol and organic acids, decreasing pH and nutritional depletion [34]. However the predominant role of these classic selective pressures is currently being questioned and other, as yet undefined, microbe–microbe interactions are being put forward as potentially significant in influencing yeast successions [10, 35, 36]. Our results clearly proved that *S. cerevisiae* has an antagonistic effect upon *C. zemplinina* and *H. uvarum* strains. The presence of *S. cerevisiae* strongly reduced the other species in the mixed cultures. To date, there have been only a few thorough studies into the causes and the mechanisms underlying this antagonistic phenomenon [36-39]. On one hand, Nissen et al. [37] concluded that the early death of two wine-related yeasts (*Kluyveromyces thermotolerans* and *Torulaspora delbrueckii*) during mixed fermentations with *S. cerevisiae* was not due to the presence of ethanol or any other toxic compound but instead to a cell–cell contact-mediated mechanism. On the other hand, Pérez-Nevado et al. [39] have studied the mechanism involved in the cellular death of two *Hanseniaspora* wine strains (*H. guilliermondii* and *H. uvarum*) during mixed fermentations with *S. cerevisiae* under oenological growth conditions. When *S. cerevisiae* reached cell densities of around $10^7$ CFU $ml^{-1}$, a strong reduction in the *Hanseniaspora* population was observed regardless of the ethanol concentration. The authors hypothesised that one or more toxic compounds produced by *S. cerevisiae* triggers the early death of the *Hanseniaspora* cells, though it has not yet been possible to identify the nature of these compounds.

These yeast interactions had a clear impact on the fermentation kinetics. The presence of *S. cerevisiae* guaranteed a fast fermentation. However, the fermentative behaviour was very different between the pure culture of *C. zemplinina* and *H. uvarum*. Whereas *C. zemplinina* ended the fermentation with a slight delay compared with the *S. cerevisae* fermentations, the *H. uvarum* pure culture was unable to finish it. We did not expect this strain to have such a poor fermentative capacity because in a previous experiment, it was selected on the basis of predominance in synthetic grape must fermentation in competition with other *H. uvarum* strains isolated from wine. This mixed culture of different *H. uvarum* strains was able to consume all the sugars of the synthetic must (data not shown). This controversial result might again be the result of interactions between the yeasts, because a mixture of *H. uvarum* strains was able to end fermentation whereas the predominant strain was unable to finish the fermentation when it was alone.

The dominance of one species over the others may mean that it is better at using the nutrients of the medium. In grape must, nitrogen is considered the main limiting nutrient for optimized growth and good fermentation performance [40]. Several positive and negative interactions have been reported regarding nutrient availability and nutrient limitation [10]. Non-*Saccharomyces* species growing early in the fermentation could strip the medium of amino acids and vitamins, limiting the subsequent growth of *Saccharomyces* [40]. The proteolytic activity of some non-*Saccharomyces* together with the early death and autolysis of these non-*Saccharomyces* could again enrich the medium of nitrogen compounds [41]. In contrast to previous studies [28, 42], we detected a higher consumption of amino acids in the mixed cultures than in the pure cultures. *H. uvarum* pure culture presented the lowest consumption of assimilable nitrogen but it should be taken into account that this yeast was unable to finish the fermentation. However, the most remarkable result was the preferential use of some groups of amino acids in the mixed fermentations compared with the pure cultures. The presence of several yeast species might improve the uptake or consumption of some amino acids by some kind of synergistic mechanism. The metabolism of these three groups of amino acids with differential consumption (aliphatic, aromatic and sulphur amino acids) has a great impact in the synthesis of aroma compounds [43, 44].

Most of the studies with co-inoculation or sequential inoculation of non-*Saccharomyces*/*Saccharomyces* species have highlighted the differences in the

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aromatic profiles obtained in these wines compared with monocultures of *S. cerevisiae*. Thus, *C. stellata* (currently *C. zemplinina*) was associated with a higher production of glycerol, which was confirmed by our data. Moreover, we detected that this strain of *C. zemplinina* produced a huge amount of higher alcohols (approximately 5 times more than the *S. cerevisiae* strain). These compounds can have both a positive and negative impact on the aroma and flavour of a wine depending on the final concentration [44]. It has been reported that concentrations below 300 mg $l^{-1}$ add a desirable level of complexity to wine, whereas concentrations that exceed 400 mg $l^{-1}$ can have a detrimental effect [45]. The monoculture of *C. zemplinina* clearly exceeded this concentration; however, the mixed culture fermentations had a level which can impact positively on the aroma, especially if it is taken into account that the most significant increases were yielded in the β-phenylethanol, which contributes to a desirable floral (rose) aroma [44]. This strain also significantly increased the synthesis of ethyl esters which impart fruity flavours to wine. This increase correlated well with an important increase in short chain fatty acids, the substrate for the synthesis of ethyl esters. To date, this high production of higher alcohols and ethyl esters has not been described for strains of this species, in contrast to *H. uvarum* strains, which have been widely described as great producers of esters [28, 29, 46]. However, this high production of esters goes together with a high volatile acidity production, which makes the wines unacceptable. This was the case with the *H. uvarum* monoculture, which produced such a large amount of acetic acid and ethyl acetate that was impossible to analyze the other minor compounds. All the mixed fermentations with *H. uvarum* presented a desirable increase in esters (especially the acetate esters), however the high production of acetic acid by this strain could jeopardise its use at industrial level. In any case, it should be tested at industrial or semi-industrial volumes because Beltran et al. [47] have already reported a higher production of acetate in small volumes and in less anaerobic fermentations.

## 5. Conclusions

The potential of using mixed cultures in industrial wine production is currently under scrutiny. However, detrimental results such as the production of acetic acid above acceptable levels counteract the benefits of high ester production, as observed in the present study. These benefits could justify the selection of appropriate non-

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*Saccharomyces* yeasts whose production of detrimental products is low and that they interact correctly with *S. cerevisiae*. Furthermore, a better understanding of the nutrient consumption in these mixed fermentations is required for industrial environments as our results suggest that these cultures use amino acids differently.

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UNIVERSITAT ROVIRA I VIRGILI
ADAPTATION AND DEVELOPMENT OF CULTURE-INDEPENDENT TECHNIQUES FOR THE INDENTIFICATION AND ENUMERATION
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Immaculada Andorrà Solsona
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**Chapter 4**

# Effect of mixed culture fermentations on yeast populations and aroma profile

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**Abstract**

The effect of pure and mixed inocula on synthetic grape must fermentation has been determined on the fermentation rate and the main characteristics of the wine produced, the volatile profile, amino acid consumption and yeast population dynamics. Three yeast species were assayed: *Saccharomyces cerevisiae*, *Hanseniaspora uvarum* and *Candida zemplinina*. The proportion of mixed cultures was 90:10 between Non-*Saccharomyces* and *S. cerevisiae*. The population dynamics was followed by plating, microscope counting and QPCR. The aromatic profile was determined by GC, whereas amino acid consumption was determined by HPLC. All the fermentations in which *S. cerevisiae* was present were faster and were conducted by this species. *S. cerevisiae* was also the most effective at converting amino acid consumption into biomass. This efectiveness could be a mechanism for taking over other species during alcoholic fermentation. However, it produced the lowest levels of aromatic compounds.

**Keywords:** Wine flavour, *Saccharomyces*, *Candida*, *Hanseniaspora*, amino acid consumption

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## 1. Introduction

Grape juice fermentation is characterised by the metabolisation of sugars, mostly into ethanol and carbon dioxide. However, sugars are also converted into a wide range of volatile and non-volatile end-products, which influence and determine the aroma and flavour of the wine. The major volatile products of this microbial metabolism, ethanol and carbon dioxide, make a relatively small contribution to wine flavour. Organic acids, higher alcohols and esters, however, are the main group of compounds that form the fermentation bouquet (Romano, Fiore, Paraggio, Caruso & Capece, 2003). During the biochemical transformation, yeasts transform the flavour-inactive compounds present in grape must into flavour-active components in wine. Although these compounds are present in only small quantities, their impact on wine aroma and flavour is detectable and facilitates the expression of grape varietal character (Fleet, 2008).

Of the yeasts that appear at the beginning of wine fermentation, those belonging to Non-*Saccharomyces* genera are predominant. *Candida* and *Hanseniaspora* are the main genera present in the first stages of wine fermentation, while *Saccharomyces cerevisiae* strains are dominant because of their greater sugar consumption and higher ethanol tolerance during the latter stages (Fleet & Heard, 1993; Pretorius, 2000). All the starters on the market belong to the *Saccharomyces* genus. The inoculation of these selected starters is expected to overwhelm the growth of Non-*Saccharomyces* yeasts. However, several quantitative ecological studies have indicated that they were not completely suppressed in either spontaneous or inoculated fermentations (Hierro, Esteve-Zarzoso, González, Mas, & Guillamón, 2006; Hierro, Esteve-Zarzoso, Mas, & Guillamón, 2007; Andorrà, Landi, Mas, Guillamón, & Esteve-Zarzoso, 2008). Therefore, Non-*Saccharomyces* species contribute to the chemical composition and aroma properties of the resulting wine because more secondary metabolites are produced which contribute to the taste and flavour of the wines (Granchi, Ganucci, Messini, & Vicenzini, 2002; Capece, Fiore, Maraz, & Romano, 2005; Garde-Cerdán & Ancín-Azpilicueta, 2006; Moreira, Mendes, Guedes de Pinho, Hogg, & Vasconcelos 2008; Bely, Stoeckle, Masneuf-Pomarède, & Dubourdieu, 2008). In this context, it has been proposed that selected Non-*Saccharomyces* wine yeasts, together with *Saccharomyces* strains as part of mixed and multistarter cultures, should be included to improve the chemical composition and sensory properties of wine, and avoid the unwanted

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compounds that these species can also produce (Ciani, Comitini, Mannazzu, & Dominizio, 2010).

The causes underlying yeast interactions during wine fermentation are not fully understood. The predominance of *S. cerevisiae* during fermentation may be attributed to the fact that viable cells of this yeast at a high density arrest the growth of Non-*Saccharomyces* yeast species in mixed cultures (Nissen & Arneborg; 2003; Nissen, Nielsen, & Arneborg, 2003; Arneborg et al., 2005). These authors postulated that the early death of two wine-related yeasts (*K. thermotolerans* and *T. delbrueckii*) during mixed fermentations with *S. cerevisiae* was due not to the presence of ethanol or other toxic compounds but to a cell–cell contact-mediated mechanism. Pérez-Nevado, Albergaria, Hogg, & Girio (2006) studied the mechanism involved in the cell death of two *Hanseniaspora* wine species (*H. guilliermondii* and *H. uvarum*) during mixed fermentations with *S. cerevisiae* under oenological conditions. When *S. cerevisiae* reached cell densities of around $10^7$ cfu $ml^{-1}$, the *Hanseniaspora* population decreased considerably regardless of the ethanol concentration. The authors hypothesized that one or more toxic compounds produced by *S. cerevisiae* triggers the early death of the *Hanseniaspora* cells. Recently, Albergaria, Francisco, Gori, Arneborg, & Gírio (2010) postulated that these toxic compounds are antifungal peptides secreted by *S. cerevisiae* against Non-*Saccharomyces* species.

The use of culture-independent methods to identify and quantify yeasts is an ideal tool for studying yeast species interaction. Environmental and microbial interactions might produce stressed cells which limit the yeast's ability to grow in a solid culture medium. Most of these methods rely on the direct amplification of yeast DNA from wine by PCR. Phister & Mills (2003) highlighted two main advantages of the direct characterization of microorganisms over yeast enrichment and plating: first, regardless of their capacity to grow in a plate, all the yeast populations are detected; and, second, analysis is fast. Because of its specificity and sensitivity, one of the most promising PCR techniques in food control is the real-time quantitative PCR (QPCR) (Bleve, Rizzotti, Dellaglio, & Torriani, 2003). In the past we developed several protocols to detect microorganisms in wine (González, Hierro, Poblet, Mas, & Guillamón, 2006; Hierro et al., 2006; Andorrà et al., 2008; Andorrà, Landi, Mas, Esteve-Zarzoso, & Guillamón, 2010b). We now aim to study the interactions among the three main yeast species—*S. cerevisiae*, *H. uvarum* and *C. zemplinina*—by monitoring pure and mixed cultures

inoculated in a synthetic grape-must by QPCR. We also analyse the impact of these mixed cultures on nitrogen consumption and aroma production. Due to the presence of aroma precursors in the natural grape must and in order to analyse the effect of yeast metabolism, we perform these fermentations on synthetic must, where no precursors were available.

## 2. Material and methods

### 2.1 Wine fermentations

Fermentations were carried out in synthetic grape must (pH 3.3) as described by Riou, Nicoud, Barre, & Gaillardin (1997), but with some modifications. The final concentration of sugars was 170 g/l (85 g/l glucose and 85 g/l fructose), available nitrogen was 400 mg/l and anaerobic factors were not added. A total of 400 ml of this synthetic must was dispensed in 500 ml fermentors at a controlled temperature of 20ºC and a stirring rate of 150 rpm in an orbital shaker. All the fermentations were inoculated to a final concentration of $10^6$ cells/ml. The yeasts used in this study were: the commercial strain of *S. cerevisiae* QA23 (Lallemand, Inc. Canada) and the strains CszB4 and HuB10 from *Candida zemplinina* and *Hanseniaspora uvarum* isolated in a previous study (Esteve-Zarzoso, Hierro, Mas, & Guillamón, 2010). The inocula were *S. cerevisiae* (*Sc*), *C. zemplinina* (*Cz*), *H. uvarum* (*Hu*), *C. zemplinina*/*S. cerevisiae* (*CS*), *H. uvarum/S. cerevisiae* (*HS*) and *C. zemplinina*/*H. uvarum*/*S. cerevisiae* (*CHS*) always at a Non-*Saccharomyces vs*. *Saccharomyces* ratio of 90:10 (45:45:10 for the triple culture). All the fermentations were performed in triplicate.

The fermentations were monitored by glucose and fructose analysis using an enzymatic kit (Roche Diagnostics). Samples for plating and QPCR were taken in the middle and at the end of fermentation (the middle was regarded as the point at which the total sugar content was about half of the initial content). For total yeast counts, samples were plated on YPD medium and for Non-*Saccharomyces* yeast on Lysine Agar medium, which is unable to support the growth of *S. cerevisiae* (Angelo & Siebert, 1987).

### 2.2. Quantification by real time-PCR

Yeast DNA was extracted from pelleted cells using the method described in Hierro et al. (2006) and 1-50 ng DNA was used for quantification in an Applied

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Bisystems 7300 Fast Real-Time PCR System (Applied Byosistems). Total yeast, *C. zemplinina*, *H. uvarum* and *S. cerevisiae* were quantified and standard curves made according to Andorrà et al. (2010b). All the reagents were from Applied Biosystems and the primers used were from Invitrogen (Germany).

### 2.3. Nitrogen content analysis

The amino acids and ammonium ions were simultaneously analyzed by DEEMM derivatizations using the Agilent 1100 Series HPLC (Agilent Technologies, Germany). Nitrogen compound separation was carried out using a 4.6 x 250 mm, 5 µm ACE C18-HL column (Symta, Madrid, Spain) with a guard column (ACE5 C18-HL) through a binary gradient at a flow of 0.9 ml/min (Gómez-Alonso, Hermosín-Gutiérrez, & García-Romero, 2007).

Several dilutions of each sample were analysed and averaged using the analysis software. The concentration of each amino acid was calculated using external and internal standards and expressed as mg $l^{-1}$. The software used was Agilent ChemStation Plus (Agilent Technologies, Germany). The ratio between the consumption of nitrogen sources (expressed as mg) and the microorganism population was calculated by dividing the consumption of each amino acid group by their population estimated by QPCR and expressed as mg/$10^7$ cells.

### 2.4. Volatile compound analysis

The concentrations of the different volatile compounds were analyzed at the end of each fermentation. The protocol followed by Ortega, López, Cacho, & Ferreira (2001) was modified to determine volatile fatty acids, ethyl esters of fatty acids, higher alcohol acetates and other volatile compounds. The following were placed in 15-ml screw-capped tubes: 1.5 ml of wine, 3.5 ml of $(NH_4)_2SO_4$ (45%, w/v), 20 µl of internal standard (4-methyl-2-pentanol (176 µg $ml^{-1}$), 1-nonanol (160 µg $ml^{-1}$), heptanoic acid (150 µg $ml^{-1}$) in ethanol and 200 µl of dichloromethane. The tube was shaken for 30s (3x) and then centrifuged at 4000 rpm for 10 min. Once the phases had separated, the bottom phase (dichloromethane) was transferred to a glass vial insert. The extract (2 µl) was injected in split mode (10:1, 30 ml $min^{-1}$) into an HP-FFAP (Agilent Technologies, Böblingen, Germany) column of 30 m x 0.25 mm, 0.25 µm phase thickness. The temperature program was: 35ºC for 5 min, which was raised at 3ºC $min^{-1}$ to 200ºC and then at 8ºC

$min^{-1}$ to 220ºC. Injector and detector (FID) temperatures were 180ºC and 280ºC, respectively. The carrier gas was helium at 3 ml $min^{-1}$. Volatile compounds were identified and quantified by comparison with standards.

### 2.5. Organic acid analysis

The values of different organic acids were analyzed at the end of the wine sample fermentations. Organic acids were determined by HPLC using an Agilent 1100 Series connected to an Agilent multiple wavelength detector (Agilent Technologies, Wilmington, DE). The samples (450 µl) were mixed with 50 µl of formic acid (Internal Standard, 46.84 g $l^{-1}$) and 50 µl was injected into a 300 mm x 7.8 mm AMINEX HPX-87H columm (BioRad, Hercules, CA). The solvent used was sulphuric acid (2.5 mM) at 0.5 ml $min^{-1}$. The analysis temperature was 70ºC. The concentration of each metabolite was calculated using external and internal standards.

### 2.6. Oenological parameters

The glucose, fructose, glycerol and ethanol contents of the wines were analyzed using commercial enzymatic kits (Roche Diagnostics, Germany). Acetic and succinic acids were determined by HPLC as described above. The pH was determined by using a Crison MicropH 2000 pH-meter (Crison, Barcelona, Spain).

### 2.7. Statistical treatment

The data were analyzed with SPSS 15.0 software for Windows (SPSS Inc., Chicago, IL). Analysis of variance was carried out by an ANOVA Tukey test to determine significant differences between the samples. The statistical level of significance was set at $P \leq 0.05$. PCA analysis was performed with the same software.

## 3. Results

The *Sc* fermentations needed seven days to finish (residual sugars below 2 g/l), while fermentations conducted by Non-*Saccharomyces* species pure cultures were stuck, as in the case of *Hu,* or needed fourteen days to finish, as in the case of *Cz*. The mixed culture fermentations needed approximately ten days to consume all sugars, except for *CS* fermentation, which was as fast as the *Sc* fermentation. There were no significant differences in the production of ethanol, acetic acid or pH values. The ethanol

concentrations at the end of fermentation ranged between 8.5 and 9.5 %. The acetic acid concentration was high, around 1.5-2 g/l, and the pH was the same in all the fermentations (3.3). There were, however significant differences in the production of glycerol and succinic acid. The glycerol content was 4.7 g/l for *Sc* fermentation and reached a maximum (5.8-6 g/l) in those fermentations in which *C. zemplinina* was present as a pure or mixed culture. *C. zemplinina* also produced more succinic acid than *S. cerevisiae* (0.9 g/l *vs* 0.5 g/l).

### 3.1. Microbial populations

The microbial population was estimated in the middle and at the end of fermentation by microscope counting, plating on YPD and Lysine media, and QPCR (Table 1). Overall, at all the sampling points, yeast population was around $10^8$ cells/ml or CFU/ml. The pure cultures showed good agreement between the enumeration by the three methods (microscope, plating and QPCR). The general trend was to detect slightly higher counts under the microscope than on plates and QPCR. There was also good agreement between the growth in Lysine media and the population of Non-*Saccharomyces* species by QPCR.

**Table 1.** Microbial population analysis. Analyses were made in the middle and at the end of each fermentation. Results were obtained using optical microscopy, plate culture media (YPD and LYS) and QPCR for total yeast, *Saccharomyces*, *Candida* and *Hanseniaspora*.

| | | Microscope | YPD | LYS | Total yeast | *Saccharomyces* | *Candida* | *Hanseniaspora* |
|---|---|---|---|---|---|---|---|---|
| ***Sc*** | *middle* | 3.00±0.23E+08 | 8.52±0.85E+07 | nd | 3.97 ± 0.35E+08 | 2.17 ± 0.21E+08 | nd | nd |
| | *end* | 3.10±0.50E+08 | 1.18±0.38E+08 | nd | 3.02 ± 0.07E+08 | 2.98 ± 0.15E+08 | nd | nd |
| ***Cz*** | *middle* | 3.25±0.19E+08 | 1.31±0.68E+08 | 1.16±0.27E+08 | 1.94 ± 0.27E+08 | nd | 1.14 ± 0.61E+08 | nd |
| | *end* | 5.50±0.42E+08 | 1.69±1.00E+08 | 1.21±0.23E+08 | 1.27 ± 0.09E+08 | nd | 8.16 ± 0.69E+07 | nd |
| ***Hu*** | *middle* | 2.20±0.36E+08 | 6.13±0.54E+07 | 5.77±0.75E+07 | 8.43 ± 0.02E+07 | nd | nd | 1.58 ± 0.38E+08 |
| | *end* | sf | sf | sf | sf | sf | sf | sf |
| ***CS*** | *middle* | 1.58±0.04E+08 | 8.47±0.96E+07 | 6.38±0.48E+07 | 1.86 ± 0.29E+08 | 1.27 ± 0.26E+08 | 1.24 ± 0.19E+08 | nd |
| | *end* | 2.40±0.42E+08 | 1.31±0.05E+08 | 3.09±0.93E+07 | 2.45 ± 0.91E+08 | 1.91 ± 0.38E+08 | 6.92 ± 0.14E+07 | nd |
| ***HS*** | *middle* | 2.00±0.35E+08 | 1.28±0.09E+08 | 7.23±0.45E+07 | 7.06 ± 0.16E+07 | 4.13 ± 0.65E+07 | nd | 1.50 ± 0.33E+08 |
| | *end* | 3.48±0.03E+08 | 1.37±0.43E+08 | 3.47±0.88E+07 | 1.43 ± 0.55E+08 | 1.06 ± 0.26E+08 | nd | 3.84 ± 0.39E+06 |
| ***CHS*** | *middle* | 1.30±0.15E+08 | 1.26±0.54E+08 | 7.22±0.27E+07 | 7.22 ± 0.25E+07 | 4.13 ± 0.23E+07 | 1.70 ± 0.97E+07 | 8.62 ± 0.62E+07 |
| | *end* | 2.95±0.21E+08 | 1.32±1.77E+08 | 3.88±0.85E+07 | 2.57 ± 0.23E+08 | 1.07 ± 0.84E+08 | 8.31 ± 0.87E+07 | 5.18 ± 0.83E+06 |

nd: non detected

sf: stuck fermentation

In the mixed fermentations, the enumeration of total yeast (microscope, growth in YPD and QPCR) again showed very good concordance. The analysis of the growth in Lysine again matched the enumeration by QPCR, with the exception of the mixed *HS* fermentations in which the plate counts were one order of magnitude higher than the QPCR enumeration. In the middle of the mixed culture fermentations, the Non-*Saccharomyces* presented counts that were similar to or higher than *S. cerevisiae*. However, at the end of fermentations, *S. cerevisiae* proved to be the main species, especially in comparison with *H. uvarum*, which seemed to be less competitive at this fermentation stage and presented counts two orders of magnitude lower than *S. cerevisiae*. *H. uvarum* was also unable to finish the fermentation when it was inoculated as a pure culture.

### 3.2. Consumption of nitrogenous compounds

The synthetic must was prepared so that it contained 400 mg N/l of available nitrogen (100 mg N/l as ammonium chloride and 300 mg N /l as amino acids). Although we analysed the samples from the middle and the end of the fermentation, Table 2 shows only the values in the middle because this was the point of maximum consumption. Ammonium was rapidly consumed in all the fermentations, except when *H. uvarum* was present, either as a pure or mixed culture.

*S. cerevisiae* took up almost all the amino acids except proline and the sulfur containing amino acids in which the uptake was between half and two-thirds of the total available. The Non-*Saccharomyces* species are less effective than *S. cerevisiae* in taking up amino acids, leaving considerable amounts of the main amino acids—glutamine, glutamate and threonine (*C. zemplinina*)—and methionine, histidine, aliphatic, and aromatic amino acids (*C. zemplinina* and *H. uvarum*).

Table 2. Ammonium and amino acid analysis in the middle of fermentation. The values are for the consumption (in mg/l) of each amino acid, the percentage of the consumption of each group of amino acids, and the ratio between the total consumption of each group of amino acids and their population estimated by QPCR and expressed as mg/$10^7$ cells.

| | | Must | *Saccharomyces* | *Candida* | *Hanseniaspora* | *Candida-Saccharomyces* | *Hanseniaspora-Saccharomyces* | *Candida-Hanseniaspora-Saccharomyces* |
|---|---|---|---|---|---|---|---|---|
| **Ammonium** | *$NH_4Cl$* | 360.47±18.73 | 358.37±10.45 | 352.16±18.94 | 228.77±11.69* | 357.79±1.38 | 280.53±14.26 | 340.28±26.45 |
| | *Ratio* | - | 9.10±1.11 | 18.55±3.84* | 27.12±1.11* | 19.79±4.41* | 39.71±1.58* | 47.06±2.89* |
| | *% total* | - | 99.42±0.23 | 97.69±1.76 | 63.46±1.48* | 99.25±0.07 | 77.82±2.89* | 94.40±5.19 |
| **Acids & amides** | *Asp* | 65.59±7.35 | 55.28±4.09 | 37.14±6.75 | 49.09±8.05 | 34.85 ± 16.82 | 30.55±1.05 | 29.55±5.37 |
| | *Glu* | 499.06±9.12 | 493.40±6.92 | 452.83±4.05* | 490.44±2.06 | 473.82 ± 1.70 | 471.48±1.64 | 469.32±10.92 |
| | *Gln* | 400.81±5,89 | 388.24±3.05 | 301.86±2.96* | 393.25±3.54 | 337.31 ± 8.18 | 351.11±4.09 | 321.07±21.05* |
| | *Ratio* | - | 23.77±2.70 | 41.59±7.32* | 110.67±1.20* | 46.88±11.21* | 120.89±3.24* | 113.83±10.72* |
| | *% total* | - | 97.05±1.23 | 82.02±3.15* | 96.62±0.78 | 94.31±4.73 | 88.37±0.34 | 84.93±2.73 |
| **Aliphatic** | *Gly* | 19.55±1.56 | 16.29±1.54 | 11.48±1.76 | 13.72±2.03 | 12.47±4.07 | 15.76±0.27 | 14.51±1.50 |
| | *α-ala* | 139.83±0.47 | 138.59±0.22 | 135.44±0.14 | 137.95±0.43 | 138.82±0.78 | 59.02±1.71* | 137.86±0.87 |
| | *Val* | 42.57±0.55 | 40.57±0.59 | 34.18±0.43* | 36.98±0.60* | 40.98±0.57 | 41.52±0.39 | 41.64±0.51 |
| | *Ile* | 29.83±0.99 | 27.38±1.33 | 24.75±2.46 | 25.85±1.78 | 27.06±3.22 | 29.31±0.12 | 29.48±0.02 |
| | *Leu* | 45.68±1.24 | 42.55±1.05 | 40.79±0.27 | 39.93±0.13* | 42.86±1.55 | 44.56±0.08 | 44.67±0.06 |
| | *Ratio* | - | 6.73±0.68 | 12.95±2.22 | 30.18±0.12* | 14.51±3.28* | 26.96±1.09* | 37.20±2.23* |
| | *% total* | - | 95.65±0.65 | 88.90±0.38* | 91.71±0.22* | 94.51±0.13 | 68.54±0.39* | 96.65±0.76 |
| **Aromatic** | *Tyr* | 19.82±1.64 | 19.26±1.58 | 5.28±0.50* | 8.69±1.21* | 16.84±0.50 | 14.54±0.79 | 18.46±1.75 |
| | *Trp* | 130.68±6.78 | 128.75±1.34 | 113.62±0.84 | 104.07±5.98* | 103.59 ± 6.75* | 96.38±2.94* | 111.19±4.74 |
| | *Phe* | 38.7±1.88 | 30.96±0.09 | 23.46±0.15* | 21.62±0.18* | 37.62±0.10* | 36.98±0.01* | 37.15±0.24* |
| | *Ratio* | - | 4.54±0.51 | 7.48±1.39 | 15.93±0.73* | 8.81±2.54* | 20.95±0.14* | 23.14±1.51* |
| | *% total* | - | 94.6±1.74 | 75.24±1.18* | 71.02±0.98* | 83.02±2.86* | 78.17±1.40* | 88.16±1.03 |
| **Hydroxyl** | *Ser* | 73.19±3.79 | 70.99±1.43 | 59.99±2.75 | 66.30±3.23 | 54.74 ± 19.90 | 72.09±0.17 | 62.91±4.91 |
| | *Thr* | 72.85±2.24 | 72.03±0.80 | 43.98±2.97* | 72.31±1.57 | 69.59±1.83 | 68.06±0.64 | 67.69±3.33 |
| | *Ratio* | - | 3.63±0.38 | 5.43±0.67 | 16.44±0.29* | 6.69±0.09* | 19.86±0.57* | 18.14±2.03* |
| | *% total* | - | 97.94±1.45 | 71.20±0.86* | 94.92±2.13 | 96.02±0.32 | 95.06±0.23 | 89.43±3.99* |
| **Sulfur containing** | *Met* | 15.38±0.27 | 12.57±0.41 | 8.94±0.54* | 4.70±0.50* | 8.23±0.01* | 5.90±0.49* | 9.13±0.49* |
| | *Cys* | 16.44±1.35 | 8.98±0.93 | 7.13±0.67 | 7.25±0.86 | 8.39 ± 0.65 | 1.28±0.39* | 4.32±1.04* |
| | *Ratio* | - | 0.54±0.02 | 0.84±0.08* | 1.41±0.18* | 0.91±0.16* | 1.02±0.16* | 1.87±0.17* |
| | *% total* | - | 67.72±2.03 | 50.51±3.49 | 37.54±1.61* | 62.44±8.76 | 22.57±1.96* | 42.29±1.22* |
| **Basic** | *His* | 30.95±1.01 | 29.47±1.41 | 17.82±0.97* | 21.34±0.84* | 29.25 ± 0.74 | 15.22±1.47* | 27.61±0.95 |
| | *Arg* | 339.39±19.27 | 317.71±15.78 | 272.86±23.54 | 214.82±7.94 | 156.82 ± 62.21* | 48.55±9.89* | 110.25±30.65* |
| | *Lys* | 16.9±0.79 | 15.10±1.03 | 13.98±0.76 | 13.96±0.45 | 14.99 ± 0.82 | 15.67±0.03 | 15.64±0.34 |
| | *Ratio* | - | 9.16±0.53 | 15.85±1.33 | 29.66±0.61* | 10.56±2.46 | 11.28±1.97 | 21.39±5.37* |
| | *% total* | - | 93.55±3.01 | 78.67±4.76 | 64.59±2.54 | 81.35±17.50 | 20.51±2.07* | 39.64±5.71* |
| | *Pro* | 433.09±11.38 | 243.74±17.45 | 317.36±9.67 | 227.15±22.96 | 220.75±0.57 | 91.28±27.34* | 163.21±27.55 |
| | *Ratio* | - | 6.15±0.15 | 16.64±2.63 | 26.90±3.00 | 12.21±2.75 | 13.00±4.29 | 22.54±2.72 |
| | *% total* | - | 56.28±4.03 | 73.28±2.23 | 52.45±5.31 | 50.97±0.13 | 21.08±6.31 | 37.68±6.36 |
| **Total amino acids** | | 2863,56±95.15 | 2151.87±74.88 | 1922.83±87.93 | 1949.44±90.99 | 1828.99±102.44* | 1509.29±32.20* | 1715.69±55.56* |

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The uptake of amino acids was also reduced in the mixed fermentations, where much larger amounts of amino acids are left than when *S. cerevisiae* is used. The presence of *C. zemplinina* with *S. cerevisiae* reduces the consumption of aliphatic amino acids (especially tryptophan) and arginine, which is the third most concentrated amino acid in the synthetic must. The presence of *H. uvarum* with *S. cerevisiae* considerably reduced the uptake of alanine which accounted for the significant reduction in the consumption of aliphatic amino acids. The consumption of aromatic amino acids was also reduced mostly due to tryptophan and a large amount of proline, arginine and both sulfur amino acids which were left in the medium in this mixed fermentation. The triple mixed fermentation left higher amounts of glutamine, arginine and both sulfur aminoacids in the medium, confirming that the mixed fermentations generally reduced the uptake of some amino acids quite considerably.

When the ratio of amino acid consumption to yeast cell population is analyzed, it becomes more evident that the yield of the Non-*Saccharomyces* species is much lower than that of *S. cerevisiae*. This lower efficiency is also observed in the mixed fermentations where more nitrogen is needed to reach the same population. In fact, the consumption ratio in these mixed fermentations is similar to that of the species that need more nitrogen. In this term, we can see a clear separation of three consumption groups: *Sc* fermentation; *Cz* and *CS* fermentations; and finally the three fermentations that include *H. uvarum* (*Hu*, *HS* and *HCS*).

### 3.3. Volatile compounds

The most important volatile compounds of the different fermentations are listed in Table 3. *C. zemplinina* proved to be a strong producer of higher alcohols, yet this difference is accounted for the high production of 2-methyl-1-propanol. While the pure cultures showed similar levels of isoamyl alcohol, the mixed cultures showed significantly lower levels.

The acetate esters are more affected by the presence of Non-*Saccharomyces* species. Hexyl acetate is highly produced in *Cz* fermentations and also in the *CHS* fermentation. A higher production of isoamyl acetate is linked to the presence of *H. uvarum* as it is significantly increased in the mixed fermentations where this species is present. 2-phenylethanol acetate is the only acetate ester that is highly produced by *S. cerevisiae* as in the presence of Non-*Saccharomyces* its concentration is significantly

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reduced. However, only *Cz* fermentations increased the overall amount of acetate esters produced. The concentrations of ethyl esters of fatty acids are higher in the mixed culture fermentations, and particularly in the *CHS* fermentation. The main components of these increases are ethyl lactate and ethyl caprylate, and are largely related to the presence of *C. zemplinina* and *H. uvarum*. The concentration of fatty acids (FA) increased considerably in the presence of Non-*Saccharomyces* species. This was accounted for by the fact that butyric acid increases between 7 and 10 times in the presence of Non-*Saccharomyces* species.

**Table 3.** Volatile compounds at the end of fermentations. Volatile compounds were clustered according to their chemical structure. All values are expressed as mg l-1 and are the mean ± SD of triplicate fermentations. Fermentations with *Saccharomyces* inoculum Sc were taken as the control

| | | *Sc* | *Cz* | *CS* | *HS* | *CHS* |
|---|---|---|---|---|---|---|
| | *propanol* | 44.23 ± 2.11 | 32.48 ± 5.45 | 46.02 ± 4.29 | 74.06 ± 7.77 | 67.64 ± 26.94 |
| | *2-methyl-1-propanol* | 14.47 ± 2.05 | 101.64 ± 6.83* | 20.66 ± 4.40 | 19.17 ± 2.61 | 17.05 ± 0.74 |
| | *Isoamyl alcohol* | 41.74 ± 0.52 | 44.69 ± 1.20 | 26.18 ± 2.17* | 30.99 ± 2.31* | 29.11 ± 5.27* |
| | *phe-2-ethanol* | 12.53 ± 3.52 | 15.40 ± 0.15 | 12.24 ± 1.09 | 9.57 ± 0.91 | 10.63 ± 1.18 |
| Higher alcohols | *Total* | 112.97 ± 2.16 | 194.22 ± 0.23* | 105.10 ± 8.45 | 133.79 ± 9.61 | 124.43 ± 24.14 |
| | *Ac isoamyl* | 3.11 ± 0.02 | 3.26 ± 0.08 | 4.14 ± 0.10 | 5.09 ± 0.05* | 5.25 ± 0.35* |
| | *Ac hexyl* | 8.22 ± 0.60 | 25.66 ± 2.98* | 7.68 ± 0.24 | 14.95 ± 1.42 | 17.79 ± 2.84* |
| | *Ac phe-2-ethanol* | 5.06 ± 0.47 | 1.52 ± 0.14* | 3.42 ± 0.27* | 3.42 ± 0.18* | 1.29 ± 0.02* |
| Higher alcohol acetates | *Total* | 16.4 ± 0.73 | 30.43 ± 2.26* | 15.25 ± 0.92 | 23.45 ± 1.10 | 24.33 ± 2.27 |
| | *Ethyl hexanoate* | 0.78 ± 0.04 | 0.82 ± 0.22 | 1.02 ± 0.02 | 1.13 ± 0.04 | 1.21 ± 0.03* |
| | *Ethyl lactate* | 14.94 ± 1.07 | 9.77 ± 2.01 | 16.31 ± 3.03 | 16.27 ± 0.65 | 23.79 ± 0.43* |
| | *Ethyl caproate* | 0.5 ± 0.09 | <dl | 1.45 ± 0.05 | 0.83 ± 0.08 | 0.81 ± 0.08 |
| | *Ethyl caprylate* | 0.78 ± 0.15 | 2.96 ± 0.26* | 1.56 ± 0.12 | 1.36 ± 0.02 | 0.98 ± 0.03 |
| Ethyl ester of fatty acids | *Total* | 17.01 ± 0.74 | 13.55 ± 1.77 | 20.51 ± 2.24 | 19.59 ± 0.42 | 28.80 ± 0.29* |
| | *Caprilic acid* | 1.66 ± 0.39 | <dl | 1.24 ± 0.11 | 1.25 ± 0.44 | <dl |
| | *isobutiric acid* | 11.57 ± 0.17 | 9.42 ± 1.69 | 12.88 ± 2.44 | 17.30 ± 0.36* | 19.68 ± 1.15* |
| | *butiric acid* | 4.13 ± 0.37 | 46.82 ± 5.21* | 33.88 ± 2.11* | 28.29 ± 0.98* | 29.47 ± 9.97* |
| | *isovaleric acid* | 0.91 ± 0.08 | <dl | 2.10 ± 0.14* | 2.56 ± 0.01* | 1.35 ± 0.02* |
| | *valeric acid* | 5.72 ± 1.07 | 1.37 ± 0.06* | 5.05 ± 0.40 | 7.66 ± 1.15 | 8.74 ± 0.04* |
| Fatty acids | *Total* | 23.99 ± 0.32 | 57.62 ± 4.93* | 55.15 ± 2.83* | 57.07 ± 1.75* | 59.25 ± 7.82* |

* Means statistical differently, P≤0.05 <dl: below detection limit

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### 3.4. Principal component analysis (PCA)

A PCA model with two PCs explains 90% of the variability shown in the fermentations studied. PC2, which is mostly formed by the total higher alcohols and acetates (Figure 1), allowed us to differentiate between those fermentations conducted by *S. cerevisiae* and those that were not. PC1 differentiates between the three species, and the Non-*Saccharomyces* species prevail in the mixed cultures. A clear orderly disposition of *S. cerevisiae*, *C. zemplinina* and *H. uvarum* is seen, with the triple mixed culture separated at the end of the scale. PC1 axis represents the ratio of the amides, the aliphatic, aromatic and sulfur-containing amino acids, and ammonium, as well as the production of total ethyl esters of fatty acids. These results, then, seem to confirm considerable differences between pure and mixed cultures in the production of volatile compounds and the consumption of amino acids.

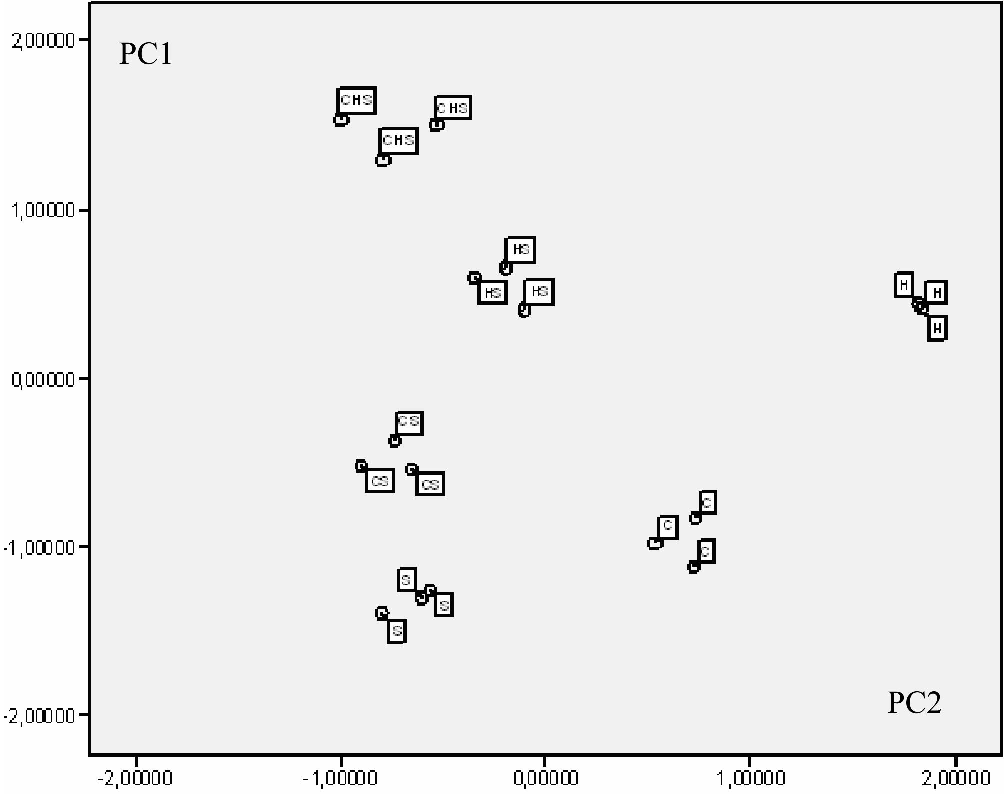


Figure 1. Distribution of the different fermentations according to PCA analysis. In the score plot for the first two principal components, PC1, explains 63.3% of the variation while PC2 explains 26.7%. The main components for PC1 were the ratio of percentage of consumption by their population of amino acids acid and amides (0.941), aliphatic (0.988), aromatic (0.974), sulfur-containing amino acids (0.910), ammonium (0,955) and total fatty acid ethyl esther produced (0.660). Total higher alcohols (0.935) and total acetates (0.880) contributed to PC2.

## 4. Discussion

It is well established that the growth of Non-*Saccharomyces* yeast at the beginning of wine fermentation increases secondary metabolites, thus contributing to the taste and flavour of wines (Romano et al., 2003). But it also produces extracellular enzymes which can interact with grape precursor compounds and enhance the varietal aroma. In our opinion, however, further research is needed into the contribution of individual Non-*Saccharomyces* species and strains to wine quality and the synergy or antagonism between *Saccharomyces* and Non-*Saccharomyces* species in the final resulting wines. In a recent study with a design that was similar to the present one (Andorrà, Berradre, Rozès, Mas, Guillamón, & Esteve-Zarzoso, 2010a), we studied how pure and mixed inocula affected fermentation performance, nitrogen consumption and volatile compound production in a natural Macabeo grape must. In the present study, we aimed to analyse yeast interactions in mixed cultures by using a culture-independent technique such as QPCR. We also analysed nitrogen consumption and aroma production. However, we decided to use a synthetic grape must because natural grape must has some varietal compounds and precursors, which would be transformed by yeasts and would therefore be present in the final wine composition. The synthetic must, then, allowed us to be sure that all the aroma compounds are produced by the yeast metabolism and not the grape precursors.

There are some similarities but also some discrepancies between our previous study (Andorrà et al., 2010a) and the current one. It is confirmed that a low proportion of *S. cerevisiae* in the initial inoculum made the fermentation slower and that when it was absent it was slower still. As expected, the *Hu* fermentation was also unable to finish. It is also confirmed that *C. zemplinina* has a great capacity to produce glycerol (Ciani & Ferraro, 1996), regardless of the type of grape must used (synthetic or natural). A noteworthy difference in the oenological characteristics of the wines obtained by natural or synthetic grape must was the volatile acidity. The amount of acetic acid was clearly higher in all the fermentations with synthetic grape must. This result had already been observed by Beltran, Novo, Guillamón, Mas, & Rozès (2008). The presence of natural grape must components such as unsaturated fatty acids (Thurston, Taylor, & Ahvenaien, 1981) and ß-sitosterol (Luparia, Soubeyrand, Berges, Julien, & Salmon, 2004), and oenological techniques such as the light clarification of must (Delfini &

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Costa, 1993; Moruno, Delfini, Pessione, & Giunta, 1993) and the presence of lees (Guilloux-Benatier & Feuillat, 1993) reduce the volatile acidity.

The yeast population dynamics during wine fermentation has been studied in depth. It is well-known that *S. cerevisiae* has a high capacity to take over the process, even when it is a minor species at the beginning. However, the underlying mechanism that makes *S. cerevisiae* the most competitive species in this environment is far from clear.

We have already mentioned that the greater competitiveness of *S. cerevisiae* has been attributed to cell-to-cell contact (Nissen et al., 2003), the secretion of toxic compounds (Pérez-Nevado et al., 2006; Albergaria et al., 2010) or transient modifications in the medium due to temperature increases (Goddard, 2008). However, all these yeast interaction studies have relied on microbiological methods based on plating, so the presence of viable but non-culturable microorganisms in wine samples (Divol & Lonvaud-Funel, 2005; Millet & Lonvaud-Funel, 2000) may have a special effect on the Non-*Saccharomyces* strains (which are under greater stress or weaker) and give the false idea that they disappear from the fermentation. In the past we used the culture-independent QPCR technique to analyse yeast dynamics during wine fermentations (Hierro et al., 2006; Hierro et al., 2007; Andorrà et al., 2008; Andorrà et al., 2010b). In this study, the counts obtained with this technique were compared with plating in a non-selective YPD medium and in a selective medium for Non-*Saccharomyces*. In general, the correlation between quantification by plating and by QPCR was quite good. In the mixed fermentations, the higher proportion of the Non-*Saccharomyces* in the inocula (90:10) was changed to similar values of both species in the middle of the fermentation and clearly dominated by *S. cerevisiae* at the end. However, this proportion of *S. cerevisiae* at the end of the fermentation depended on the Non-*Saccharomyces* species. In the case of the *CS* fermentation, the *S. cerevisiae* population was one log unit higher, whereas in the *HS* fermentation, the difference was two log units. These results prove once again, then, that *S. cerevisiae* has a competitive advantage over the major Non-*Saccharomyces* species. However, our results also pointed out to a better fitness of *C. zemplinina* than *H. uvarum* in the conditions studied. In any case, with the inocula designed, the Non-*Saccharomyces* were not completely taken over and they definitely contributed to the quality of the final wine.

*S. cerevisiae* may also dominate Non-*Saccharomyces* species because it is better at using the nutrients in the medium. For this reason we analysed the nitrogen consumption in the different fermentations because assimilable nitrogen is the main limiting nutrient for optimised growth and good fermentation performance (Bisson, 1999). We also analysed this consumption in our previous study on natural must (Andorrà et al., 2010a). However, the Macabeo grape must presented a low concentration of nitrogen (114.57 mg N $l^{-1}$), and practically no differences were detected in nitrogen consumption. In this study, the synthetic must was prepared with four times more nitrogen than the natural must in our former study so that possible differences in nitrogen use could be detected between species in pure culture and mixed fermentations. In these conditions, it is clear that the presence of Non-*Saccharomyces* species considerably increases the use of amino acids for biomass production. Of the two species tested, *H. uvarum* is also the least efficient at producing biomass, because it needs to consume more nitrogen to produce the same amount of biomass. In the mixed fermentations, although *S. cerevisiae* takes over the fermentation, the greater need of nitrogen for biomass production in Non-*Saccharomyces* species prevails. The fact that *S. cerevisiae* uses nitrogen more quickly and more efficiently may also account for the better fermentation performance of this species and its capacity to overtake other yeast species.

Ammonium and glutamine are the most interesting nitrogen sources and are primarily consumed by *S. cerevisiae* (Beltran, Novo, Rozès, Mas, & Guillamón, 2004). However, *H. uvarum* and *C. zemplinina* seem to be poor consumers of ammonium and glutamine, respectively. Moreover, their presence in the initial inocula impaired the uptake of these nitrogen sources by *S. cerevisiae*. Also noteworthy is the decrease in arginine and alanine in the *CS* and *HS* fermentations, respectively. The uptake of both amino acids is strongly repressed by the mechanism of Nitrogen Catabolite Repression (NCR) (Magasanik, 1992). A higher concentration of good nitrogen sources in the media, such as glutamine and ammonium, might decrease the assimilation of these NCR amino acids (Beltran et al., 2004). The general reduction in the consumption of aliphatic, aromatic and sulphur amino acids in the mixed cultures may have considerable impact on the synthesis of aroma compounds (Beltran, Esteve-Zarzoso, Rozès, Mas, & Guillamón, 2005; Swiegers, Bartowsky, Henschke, & Pretorius, 2005).

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This study confirms that the *C. zemplinina* strain produces higher alcohols as it did in the Macabeo grape must fermentation. However, production in the synthetic must was five times lower than in the natural grape must. This is clearly related to the differences in nitrogen concentration. The less nitrogen there is available in the fermentation medium, the higher alcohols are produced (Beltran et al., 2005). Moreover, in this study, the 2-phenylethanol, which is the fusel alcohol with the most pleasant aroma (floral, rose), hardly increased. However, there are also clear discrepancies between the two studies. In both cases *C. zemplinina* increased the ester production, but this increase was accounted for by the increase in acetate esters in the synthetic must while only the ethyl esters increased in the fermentation with natural must (Andorrà et al., 2010a). The acetate esters also increased in the fermentations with *Hu* in both the synthetic and natural must. The greater production of acetic acid in the synthetic must fermentations may account for this high production of acetate esters, as it is well known that yeast activates acetyl-transferases as a mechanism for reducing acetic acid toxicity (Plata, Mauricio, Millán, & Ortega, 2005).

In conclusion, the metabolites produced by the yeasts that make up the flavour and aroma of a wine are the result of complex interactions between the different species and strains and the constitution of the fermentation medium (grape must). In this study we have focused on the involvement of the yeast metabolism in the production of flavour active compounds without the participation of the substrate (grape must). The interactions of the main yeasts are clear and, as reflected by PCA, the characteristics due to each species can be detected in the final product. Furthermore, the nutritional requirements of species are very evident: *S. cerevisiae*, for example, is highly efficient at converting nutritional sources into biomass, which could be one of the keys to its success in industrial fermentations, especially those that use grape must in which nitrogen is a clear limiting factor. The mixed fermentations resulted in greater production of most of the volatile compounds and, as other authors have also suggested, may be one way of increasing wine complexity. Thus, the benefits of *Saccharomyces*/Non-*Saccharomyces* mixed cultures should be tested in different grape musts, with different nutritional characteristics and limitations, as they may help modify the expression of the species present in the fermentation.

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## Chapter 5

# Determination of viable wine yeast using DNA binding dyes and quantitative PCR

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**Abstract**

The detection and quantification of wine yeast can be misleading due to under or over estimation of these microorganisms. Underestimation may be caused by variable growing rates of different microorganisms in culture media or the presence of viable but non cultivable microorganisms. Overestimation may be caused by the lack of discrimination between live and dead microorganisms if quantitative PCR is used to quantify with DNA as the template. However, culture-independent methods have been described that use dyes to remove the DNA from dead cells and then quantify the live microorganisms. Two dyes have been studied in this paper: ethidium monoazide bromide (EMA) and propidium monoazide bromide (PMA). The technique was applied to grape must fermentation and ageing wines. Both dyes presented similar results on yeast monitoring. Membrane cell recovery was necessary when yeasts were originated from ethanol-containing media. When applied to grape must fermentation, differences of up to 1 log unit were seen between the QPCR estimation with or without the dye during the stationary phase. In ageing wines, good agreement was found between plating tecniques and QPCR, except for *Z. bailii* and *D. bruxellensis* where much higher counts were occasionally detected by QPCR. The presence of excess dead cells did not interfere with the quantification of live cells with either of the dyes.

**Keywords:** EMA, PMA, spoilage yeast, *Saccharomyces*, *Candida*, *Brettanomyces*, *Hanseniaspora*, *Zygosaccharomyces*

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## 1. Introduction

The detection and quantification of the yeast involved in the wine production process has been widely studied. Most of the methods used focus on the yeasts responsible for the process and the spoilage yeasts, which can alter the taste and aroma of the final wine. Traditional methods based on culturability on solid media are the most commonly used in wineries. They often cause confusion because they are heavily dependent on the microorganisms' physiological status and capacity to grow. Furthermore, the presence of viable but non-culturable microorganisms in wine samples has also been described (Millet and Lonvaud-Funel, 2000; Divol and Lonvaud-Funel, 2005). The traditional methods are also time-consuming and this could be inappropriate for decision taking during alcoholic fermentation. Some spoilage species usually grow slower than other microorganisms in general-purpose culture media and, therefore, they tend to be underestimated in contaminated samples (Loureiro et al., 2004). Attemps have been made to develop and use culture-independent techniques for detecting and quantifying wine yeast species, thus avoiding the problems associated with the traditional culture methods (Cocolin et al., 2000; Mills et al., 2002; Phister and Mills, 2003; Hierro et al., 2006; Andorrà et al., 2008, 2010). Most of these techniques are based on DNA detection and quantification, which avoids the previous step of cultivation. DNA is known to be stable in dead cells, so these DNA techniques may overestimate the population of microorganisms, as the dead population could be included. The use of RNA as an alternative has been proposed, as it is more unstable and easily degraded (Cocolin and Mills, 2003; Hierro et al., 2006). However, as some of the techniques use ribosomal RNA, it has to be considered that ribosomes are also rather stable, although not as much as DNA (Hierro et al., 2006). Furthermore, working with RNA is more demanding and it is prone to contamination with RNA-degrading enzymes, resulting in problems of reproducibility. Moreover, the RNA expression level depends on the physiological status of the cell, which makes it difficult to accurately estimate the size of microorganism population (Nocker and Camper, 2006). Other solutions could be the use of chemicals that can differentiate between live and dead cells (which are generally those that the live cells can either exclude or prevent from entering). Ethidium monoazide bromide (EMA) or propidium monoazide bromide (PMA) are fluorescent photoaffinity labels that bind covalently to nucleic acid after photoactivation. They only enter cells with compromised cell walls and cell membranes.

Once inside the dead cells, the dyes bind to the double stranded DNA, which is covalently bound after photoactivation. The DNA that is covalently bound to these dyes cannot be PCR amplified (Rudi et al., 2004; Nocker et al., 2006). However, Nocker and Camper (2006) proved that the DNA from live cells was selected mainly during DNA extraction and only to a lesser extent during PCR. Signal reduction may be partly due to PCR inhibition but it is also due, more importantly, to a selective loss of genomic DNA from dead cells during the extraction procedure. Thus, only DNA from viable cells-that is to say, cells with membrane integrity-can be detected. Despite being one of the most promising techniques for enumerating microorganisms, QPCR only detects known microorganisms as it requires specific primers. Although both dyes have similar structures and, therefore, similar results might be expected, Pan and Breidt (2007) found differences between them; PMA gave better results than EMA when analysing *Lysteria monocytogenes*. Among the yeasts that have been reported in wine, only *S. cerevisiae* and *Zygosaccharomyces bailii* have been determined by EMA-QPCR (Rawsthorne and Phister, 2009a,b). To the best of our knowledge, no studies have been performed with this method during wine fermentation or using other wine microorganisms.

Although the wine fermentation process is mainly conducted by *Saccharomyces* yeasts, in the initial stages of grape must many Non-*Saccharomyces* yeasts are predominant. *Candida* and *Hanseniaspora* are two of the most common Non-*Saccharomyces* yeasts. Although all these Non-*Saccharomyces* species are normally overgrown by *Saccharomyces*, some of them can survive the alcoholic fermentation and end up spoiling wines by producing unacceptable flavours (Pretorius, 2000; Ribéreau-Gayon et al., 2006). This wine spoilage is especially critical during bulk storage of wines in tanks and barrels prior to packaging. Wine that is exposed to air, as in incompletely filled tanks or barrels, quickly develops a surface flora of weakly fermentative or oxidative yeasts, usually species of *Candida* and *Pichia*. These species oxidise ethanol, glycerol and acids, giving wines unacceptably high levels of acetaldehyde, esters and acetic acid (Ciani et al., 2010). Bulk wines, as well as bottled wines, are also spoiled by fermentative species of *Zygosaccharomyces*, *Dekkera*, *Saccharomyces* and *Saccharomycodes* (Fleet, 2003). The yeast species regarded as the most dangerous to wines are the genus *Dekkera* (Anamorph *Brettanomyces*) and *Zygosaccharomyces bailii* (Loureiro and Malfeito-Ferreira, 2003). Species of *Dekkera* are associated with the production of unpleasant mousy and medicinal taints described as “barnyard-like” or

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"horsey", because they can form tetrahydropyridines and volatile phenolic substances such as 4-ethylguaiacol and 4-ethyl phenol (Grbin and Henschke, 2000; Du Toit and Pretorius, 2000). *Zygosaccharomyces bailii* may cause spoilage by forming gas, sediment and/or cloudiness, and synthesizing other compounds such as succinic, acetic and lactic acid and acetaldehyde and glycerol (Fugelsang and Edwards, 2007). Some of these spoiling yeasts are slow growers so they are difficult to detect on general culture media (Loureiro and Malfeito-Ferreira, 2003).

The aim of this work is to develop a methodology to differentiate live and dead yeasts by using QPCR and apply it to winemaking conditions. We have used both dyes and compared them with the use of non-dye QPCR and plating or microscope counting. As the physiological status of yeast is very different during winemaking processes we have analysed two sets of different populations: during alcoholic fermentations and during ageing. In both cases the yeast populations are very different both in size (around $10^8$ *vs*. $10^4$) and species diversity. As the main limitation is primers availability, the yeast populations analysed by QPCR were total yeast, *Saccharomyces cerevisiae*, *Candida zemplinina, Hanseniaspora sp, Dekkera bruxellensis* and *Zygosaccharomyces bailii*.

## 2. Materials and Methods

### 2.1. Yeast strains and culture conditions

The yeasts used were: *C. zemplinina* CszB4 and *H. uvarum* HuB10 isolated on previous studies (Esteve-Zarzoso et al., 2010), the Active Dry Wine Yeast (ADWY) commercial strain *Saccharomyces cerevisiae* QA23 (Lallemand, Inc. Canada) and two of the main wine spoilage microorganisms, *D. bruxellensis* CECT 1009 and *Z. bailii* CECT 11042, from Spanish Type Culture Collection (CECT). Yeasts were grown in YPD (2% glucose, 2% peptone, 1% yeast extract, W/v; Cultimed, Barcelona, Spain). Plating was done in YPD (supplemented with 2% of agar) and Lysine agar (LYS) (Oxoid, Barcelona, Spain), which is unable to support the growth of *S. cerevisiae* (Angelo and Siebert, 1987).

### 2.2. Optimization of the binding of dyes to DNA of wine yeast

EMA (Molecular Probes Inc. USA) was resuspended in water (5 mg/ml) whereas PMA (Biotium, Inc.USA) was resuspended in 20% DMSO (1 mg/ml). Both solutions were stored in the dark at -20ºC. The light source was a 650-W halogen lamp (Philips). A closed box was constructed with refractory walls and the halogen lamp was placed 20 cm from the sample tubes. Incubation times with the dyes were 5, 10, 15, 20 and 30 minutes and they were tested in the dark. Two different light treatments were studied: in the first the samples were exposed twice to the light for 30 seconds with an interval of 1 minute in ice, and in the second the samples were kept on ice with their lids off, and were then exposed for 5 minutes to light. Different dye concentrations were analysed: for EMA 240, 120, 60, 24 and 12 µM and for PMA 50, 24, 12, 6 and 3 µM. All these parameters were tested against viable and dead cells, with and without dye treatment. Dead cells were obtained from 24h cultures washed with the same volume of distilled water and heated at 65ºC for 20 minutes. The lack of cell viability was confirmed by plating with YPD and LYS media. Cells not treated with EMA/PMA or light exposure were used as controls to evaluate the effect of both dyes.

### 2.3. Use of LIVE/DEAD kits for differentiating live and dead yeast

The effect of ethanol on the reliability of live/dead cell kits was evaluated during the exponential growth of *S. cerevisiae*. After washing the same quantity of cells ($10^6$ cells) was placed in YPD liquid medium with 0, 1 and 5 % ethanol (v/v), and the same quantity of dead cells (killed by heating) was placed in a medium without ethanol. The cells were stained using the LIVE/DEAD® BactLigth™ Bacterial Viability Kit (Molecular Probes Inc, USA) or LIVE/DEAD® Yeast Viability Kit (Invitrogen, USA) and counted using a fluorescence microscope (Leica DM 4000B). To help membrane recovery the same cells were centrifuged, resuspended in fresh YPD and stored for 2 hours at 13ºC. Then, they were stained and counted in the same way. Appropriate controls were used to prevent yeast growth during these 2 hours. Wine samples were analysed either directly or after recovery in YPD medium at 13ºC. Populations were enumerated by plating, microscope counting, QPCR and EMA- or PMA-QPCR.

### 2.4. DNA extraction and QPCR

DNA was extracted from reference strains or wine samples according to Hierro et al. (2006a) and diluted to 1–50 ng/µL. The concentration and purity of DNA was

determined using a Gen-Quant spectrophotometer (Pharmacia, Cambridge, UK). In all cases QPCR was performed in an Applied Biosystems 7300 Fast Real-Time PCR System (Applied Biosystems). Power SyberGreen PCR Master Mix was used according to the manufacturer's instructions (Applied Biosystems, CA). An ABI PRISM 96 well optical plate was used for the reaction. This instrument automatically determined the *Ct*. Yeast quantification was performed by using the primers YEASTF/YEASTR for total yeast (Hierro et al., 2006a), CESPF/SCERR for *Saccharomyces cerevisiaie*, generic CESPF/HUVR for the *Hanseniaspora* genus (Hierro et al., 2007), AF/200R for *C. zemplinina* (Andorrà et al., 2010), DBRUXF/DBRUXR for *D. bruxellensis* (Phister and Mills, 2003) and ZBF1/ZBR1 for *Z. bailii* (Rawsthorne and Phister, 2006). All the primers anneal the ribosomal gene region. Standard curves were calculated for each type of microorganism in triplicate samples using both cell and purified DNA dilution with or without dye. Furthermore, the interference of dead cells was tested by adding a constant population of $10^6$ dead cells/ml to concentrations of viable cells that ranged from $10^3$ to $10^7$ and recalculating the standard curves.

### 2.5. Natural wine fermentation and sampling

Macabeo grapes from the experimental cellar of the Faculty of Oenology in Tarragona (Spain) were used. After destemming and crushing, $SO_2$ was added (60 mg/l) and the must was settled at 10ºC to separate the particles by density. The clear grape must was transferred to 80 l tank, and the fermentation was conducted spontaneously without yeast inoculation at a controlled temperature of 13 ºC. The alcoholic fermentation was monitored by daily density analysis. Samples were taken from the fermenting must until the end of fermentation, which was considered to be over when the residual sugar concentration was below 2 g/l. Samples were kept in ice and analysed a few hours after sampling.

### 2.6. Analysis of ageing wines

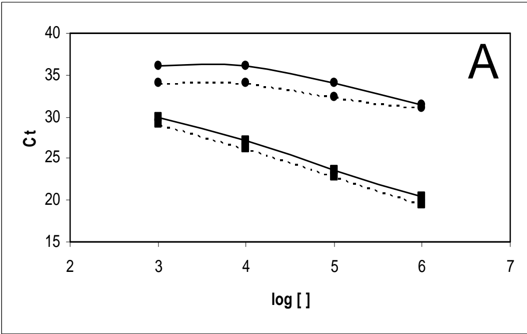
Five different wines were taken from the experimental cellar of the Oenology Faculty in Tarragona (Spain). They had all been aged for different times. Sample A was in the first year of ageing in an oak barrel. Samples B and C were in their second year of ageing in oak barrels. Samples D and E were taken from spoiled wines. In these samples, to increase the detection limit, the wine was analysed either directly or

concentrated 10- or 50-fold. The same sample was analysed at the three different concentrations to test for the possible presence of inhibitors of the PCR reactions.

## 3. Results

### 3.1. Optimization of the dye treatments

The optimal conditions for QPCR after both dyes had been used were determined using live, dead and a mix of live/dead cells. In all cases the yeast population ranged from $10^3$-$10^7$ cells/ml. The optimum EMA and PMA concentrations were 24µM and 6µM, respectively. Higher concentrations had inhibitory effects on viable cells and lower concentrations could not reliably distinguish the dead cells (data not shown). With these parameters the differences between the enumeration of viable cells in the samples treated with or without dyes were not significant as only dead or live cells were in each sample. Dye treatment was optimal after 10 minutes incubation in the dark, followed by two 30 second exposures to light with an interval of 1 minute in ice to prevent the samples from overheating. Using these parameters, standard curves were calculated using viable or dead cells (Fig. 1A). EMA- and PMA-QPCR with dead cells produced Ct close to 35, similar to the values obtained with the Non-Template Control (NTC). This value decreased only slightly when the population of dead cells was high. In all cases, the differences between the Ct of dead and viable cells were between 5 and 10 Ct. Viable cells, on the other hand, showed similar curves with both dyes, with slopes ranging from 3.1 to 3.7. The presence of $10^6$ dead cells did not interfere with the quantification of viable cells yielding curves with similar slopes (Fig 1B).



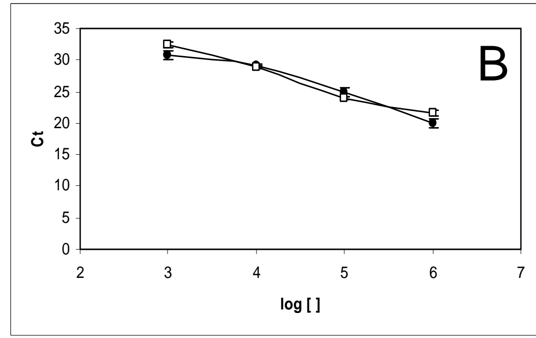




Figure 1. A) Differentiation between live and dead cells after EMA- or PMA-PCR treatments. (■ live cells, ● dead cells, continuous lines use EMA and broken line use PMA). B) Effect of the presence of $10^6$ dead cells/ml on the enumeration of live cells by PMA -QPCR (-□- Presence of $10^6$ dead cells/ml; -●-without dead cells).

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### 3.2. Analysis of ethanol interference

Because fermentation and wine samples contain varying amounts of ethanol, we decided to analyse the effect ethanol has on the dyes. When wine samples are directly treated with EMA or PMA and enumerated with QPCR, recovery is poor in contrast to high colony population on plates. To test the extent to which ethanol affects membrane permeabilizaty to dyes, two different LIVE/DEAD kits for fluorescence microscopy counting were used. It was confirmed that all direct wine samples contained "dead cells", that is to say cells with compromised membrane permeability (data not shown). YPD medium containing ethanol was used to test membrane permeabilitiy to dyes on a population of $10^6$ cells/ml. Yeast populations in 1 or 5% ethanol yielded different amounts of dead cells. However, after recovery for 2 hours at 13 ºC in YPD, most of them were accounted for as alive (Table 1). The YPD recovery was analysed in natural wine samples by enumerating the population with EMA- or PMA-QPCR for total yeast. An unknown sample was plated on YPD: 1.7 x $10^6$ CFU/ml was recovered and a population of 2.2 $10^6$ cell/ml was counted under the microscope. Enumeration with EMA-QPCR estimated a population of 5 $10^4$ cells/ml. However, the value was $10^6$ cells/ml after two hours of YPD recovery at 13ºC. This, then, was the general protocol used to enumerate wine-related samples. Once the appropriate conditions had been set, the standard curves were calculated and used to estimate viability (Table 2). Good correlations and curve values (slope and intersection) were obtained and the linearity was set for populations between $10^3$ and $10^7$ cells/ml.

Table 1. Analysis of different microscope viability kits for analysing cell membrane permeability in the absence or presence of ethanol. Green is associated with live yeast, while red is considered with dead yeast. Cilindrycal Intravacuolar Structures are considered to be indicators of metabolic activity of the yeast.

| | Directly | | | | After 2 hours at 13ºC in a YPD medium | | | |
|---|---|---|---|---|---|---|---|---|
| | Bacterial Viability kit N cel/ml | | Yeast Viability kit N cel/ml | | Bacterial Viability kit N cel/ml | | Yeast Viability kit N cel/ml | |
| YPD | green | 7,00E+05 | + | 1,80E+05 | green | 5,00E+05 | + | 8,70E+05 |
| | red | 2,00E+05 | - | 3,10E+04 | red | 0,00E+00 | - | 0,00E+00 |
| YPD + 1% Ethanol | green | 1,50E+05 | + | 1,50E+05 | green | 6,50E+05 | + | 1,50E+06 |
| | red | 1,15E+06 | - | 0,00E+00 | red | 1,00E+05 | - | 0,00E+00 |
| YPD + 5% Ethanol | green | 3,00E+05 | + | 0,00E+00 | green | 8,50E+05 | + | 9,30E+05 |
| | red | 8,00E+05 | - | 2,80E+05 | red | 0,00E+00 | - | 0,00E+00 |
| YPD * | green | 0,00E+00 | + | 0,00E+00 | green | 0,00E+00 | + | 0,00E+00 |
| | red | 5,50E+05 | - | 3,10E+05 | red | 8,00E+05 | - | 2,50E+05 |

* Dead cells

Table 2. The Slope, intersection and correlation coefficient (R2) of standard curves from serial dilution of yeasts *S. cerevisiae*, *H. uvarum*, *C. zemplinina*, *Z. bailii*, and *D. bruxellensis* cells calculated by QPCR, and with or without the dyes.

| | QPCR | | | EMA-QPCR | | | PMA-QPCR | | |
|---|---|---|---|---|---|---|---|---|---|
| | slope | intercept | $R^2$ | slope | intercept | $R^2$ | slope | intercept | $R^2$ |
| Total yeast | -3,69 ± 0,07 | 41,47 ± 1,65 | 0,986 ± 0,01 | -3,75 ± 0,65 | 42,31 ± 1,21 | 0,994 ± 0,00 | -4,01 ± 0,31 | 44,69 ± 0,57 | 0,968 ± 0,02 |
| *S. cerevisiae* | -3,12 ± | 38,18 ± | 0,989 ± | -3,19 ± 0,22 | 38,17 ± 1,35 | 0,997 ± 0,00 | -3,76 ± 0,05 | 43,65 ± 0,25 | 0,982 ± 0,01 |
| *H. uvarum* | -3,48 ± 0,14 | 39,05 ± 0,76 | 0,999 ± 0,00 | -2,95 ± 0,04 | 38,75 ± 0,39 | 0,992 ± 0,00 | -3,37 ± 0,11 | 40,60 ± 0,92 | 0,992 ± 0,00 |
| *C. zemplinina* | -3,21 ± 0,02 | 39,64 ± 0,18 | 0,995 ± 0,00 | -3,36 ± 0,03 | 40,84 ± 0,39 | 0,988 ± 0,00 | -3,40 ± 0,11 | 40,81 ± 0,91 | 0,992 ± 0,00 |
| *D. bruxellensis* | -3,39 ± 0,22 | 37,40 ± 0,98 | 0,999 ± 0,00 | -3,47 ± 0,06 | 38,56 ± 0,38 | 0,999 ± 0,00 | -3,50 ± 0,08 | 39,02 ± 0,05 | 0,998 ± 0,00 |
| *Z. bailii* | -3,64 ± 0,05 | 39,09 ± 0,22 | 0,999 ± 0,00 | -4,04 ± 0,06 | 42,62 ± 0,51 | 0,974 ± 0,00 | -3,58 ± 0,01 | 39,00 ± 0,13 | 0,993 ± 0,00 |

### 3.3. Analysis of an alcoholic fermentation

During an alcoholic fermentation of Macabeo grape must at 13ºC in semi-industrial conditions (80 l), samples were taken and analysed by plating, microscope counting and QPCR with or without the dyes (Figure 2). The fermentation took 21 days to finish (residual sugars below 2g/l). The QPCR analysis was used to evaluate the main yeast during the alcoholic fermentation: total yeast, *S. cerevisiae, Hanseniaspora* and *C. zemplinina*. The first fermentation sample was taken before settling and the second after settling. Between the must and the settled must the number of cells decreased from $10^6$ to $10^5$ cells/ml. The values for total recovered yeast in plates and the estimation by QPCR were similar throughout the fermentation except at the last point (20 days), at which values obtained by plating, and EMA- and PMA-QPCR were one log unit lower than those obtained by QPCR without dye treatment (Fig 2A). This observation was extended to the values obtained with the *S. cerevisiae* primers and compared with plating in YPD (Fig 2B). The difference between viable counting (both plating and dye-QPCR) and QPCR started only at mid fermentation (8 days) and it was already one log unit lower after 12 days. These differences were higher in some cases with Non-*Saccahromyces* species. The culturable population of Non-Saccharomyces was recovered in LYS medium. The QPCR values for *Hanseniaspora* were slightly higher than those obtained by LYS plating, starting at $10^6$ cells/ml and declining to about $10^5$ cells/ml, staying at a similar level throughout the fermentation (Fig 2C). However, the viability values with both EMA- and PMA-QPCR were very similar to one another but much lower than those recovered by plating during the second phase of fermentation. The values obtained for *C. zemplinina* were very similar throughout the fermentation with values between $10^5$ and $10^4$ at the end of fermentation without a clear decline at the end. After the initial fermentation samples when the difference between all the QPCR for *C. zemplinina* and LYS plating were very different, during the rest of the

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fermentation similar *C. zemplinina* QPCR enumeration and LYS plating were obtained. Thus, *Hanseniaspora* seemed to make a considerable contribution to the colonies recovered with LYS plating at the beginning of fermentation whereas *C. zemplinina* seem to be the main contributor during most of the fermentation and at the end. However, it should be emphasized that other species not included in this study may also be present in the colonies recovered in LYS medium.

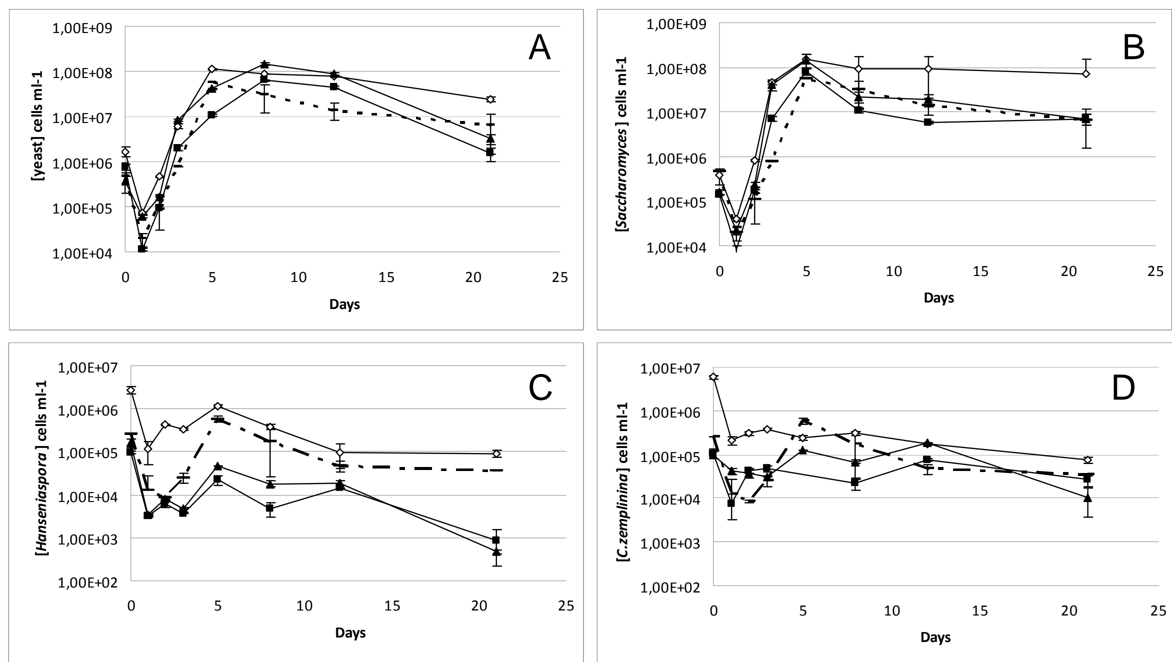


Figure 2. Yeast monitoring during spontaneous Macabeo fermentation. The techniques used were plating (YPD and Lysine Agar) and qPCR analysis, with or without the dyes. Total yeast (A), *Saccharomyces* (B), *Hanseniaspora* (C), *Candida zemplinina* (D) yeasts were evaluated. (••• YPD plates; -•- LYS plates; -◊- QPCR; -■- EMA-QPCR; -▲- PMA-QPCR).

### 3.4. Detection of viable yeast in ageing wine

Matrix interference was tested by direct wine analysis (1 ml), or 10- and 50-fold concentrated samples. The results of enumeration were similar to those of the initial samples (data not shown). However, as the direct and 10-fold concentrated samples were very close to the detection limit, we used the 50-fold concentration for the ageing wine analysis. In general, the populations detected by the three QPCR methods were very similar and always within the same range (Table 3). *S. cerevisiae* and *D. bruxellensis* were present in all the wines, although in very low quantities (except for *D. bruxellensis* in spoiled wines, all the others were $10^2$ cells/ml or lower) and only *H. uvarum* was not detected in any samples (data not shown). *C. zemplinina* was only

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found in wine D in very low counts whereas *Z. bailii* was present in all the ageing wines but not in the spoiled ones. As expected, spoiled wines had a notable presence of *D. bruxellensis* because they presented the typical "Brett" character. Only in wine D was the plate recovery much lower than the counts obtained by QPCR enumeration (by two orders of magnitude).

Table 3. Microorganism enumeration in different ageing and storage wines analysed by QPCR with or without the dyes, and compared to CFU recovered in YPD and LYS plating.

| | | Wine A | Wine B | Wine C | Wine D | Wine E |
|---|---|---|---|---|---|---|
| YPD plates | Culturable yeast | $2{,}07 \pm 1{,}68 \times 10^{2}$ | $9{,}15 \pm 2{,}62 \times 10^{2}$ | $2{,}00 \pm 0{,}0 \times 10^{2}$ | $2{,}23 \pm 0{,}41 \times 10^{3}$ | $2{,}65 \pm 0{,}92 \times 10^{2}$ |
| LYS plates | Culturable non-*Sacch* | $4{,}00 \pm 5{,}66 \times 10^{1}$ | - | $8{,}30 \pm 5{,}23 \times 10^{1}$ | $2{,}39 \pm 0{,}55 \times 10^{3}$ | - |
| QPCR | Total yeast | $4{,}91 \pm 0{,}19 \times 10^{2}$ | $4{,}77 \pm 1{,}71 \times 10^{2}$ | $1{,}68 \pm 0{,}17 \times 10^{2}$ | $5{,}28 \pm 0{,}08 \times 10^{3}$ | $4{,}65 \pm 0{,}25 \times 10^{4}$ |
| | *S. cerevisiae* | $1{,}08 \pm 0{,}24 \times 10^{1}$ | $9{,}57 \pm 3{,}83 \times 10^{0}$ | $1{,}34 \pm 0{,}43 \times 10^{1}$ | $9{,}23 \pm 2{,}09 \times 10^{3}$ | $2{,}06 \pm 0{,}67 \times 10^{2}$ |
| | *C. zemplinina* | - | - | - | $4{,}46 \pm 0{,}84 \times 10^{1}$ | - |
| | *D. bruxellensis* | $2{,}17 \pm 0{,}15 \times 10^{1}$ | $2{,}09 \pm 0{,}06 \times 10^{2}$ | $2{,}07 \pm 0{,}12 \times 10^{1}$ | $2{,}02 \pm 0{,}24 \times 10^{2}$ | $2{,}18 \pm 0{,}12 \times 10^{4}$ |
| | *Z. bailii* | $1{,}23 \pm 0{,}12 \times 10^{3}$ | $3{,}44 \pm 0{,}01 \times 10^{0}$ | $1{,}78 \pm 0{,}19 \times 10^{2}$ | - | - |
| EMA-QPCR | Total yeast | $1{,}96 \pm 0{,}32 \times 10^{2}$ | $4{,}63 \pm 0{,}54 \times 10^{2}$ | $6{,}98 \pm 0{,}70 \times 10^{1}$ | $2{,}01 \pm 0{,}28 \times 10^{3}$ | $5{,}95 \pm 0{,}32 \times 10^{4}$ |
| | *S. cerevisiae* | $1{,}05 \pm 0{,}28 \times 10^{1}$ | $8{,}68 \pm 1{,}18 \times 10^{0}$ | $8{,}53 \pm 0{,}82 \times 10^{0}$ | $3{,}05 \pm 0{,}70 \times 10^{3}$ | $7{,}38 \pm 0{,}91 \times 10^{2}$ |
| | *C. zemplinina* | - | - | - | $3{,}86 \pm 3{,}45 \times 10^{1}$ | - |
| | *D. bruxellensis* | $1{,}99 \pm 0{,}01 \times 10^{1}$ | $2{,}03 \pm 0{,}16 \times 10^{2}$ | $7{,}96 \pm 1{,}05 \times 10^{0}$ | $2{,}01 \pm 0{,}35 \times 10^{2}$ | $2{,}98 \pm 0{,}17 \times 10^{4}$ |
| | *Z. bailii* | $7{,}56 \pm 0{,}13 \times 10^{2}$ | $5{,}48 \pm 1{,}03 \times 10^{0}$ | $9{,}95 \pm 1{,}74 \times 10^{1}$ | - | - |
| PMA-QPCR | Total yeast | $3{,}21 \pm 0{,}87 \times 10^{2}$ | $2{,}38 \pm 0{,}40 \times 10^{2}$ | $7{,}26 \pm 1{,}46 \times 10^{1}$ | $2{,}15 \pm 0{,}26 \times 10^{3}$ | $5{,}88 \pm 0{,}23 \times 10^{4}$ |
| | *S. cerevisiae* | $1{,}37 \pm 0{,}32 \times 10^{2}$ | $1{,}38 \pm 0{,}17 \times 10^{1}$ | $4{,}40 \pm 1{,}20 \times 10^{1}$ | $4{,}71 \pm 2{,}96 \times 10^{3}$ | $1{,}08 \pm 0{,}03 \times 10^{3}$ |
| | *C. zemplinina* | - | - | - | $1{,}23 \pm 0{,}85 \times 10^{1}$ | - |
| | *D. bruxellensis* | $2{,}42 \pm 0{,}15 \times 10^{1}$ | $1{,}24 \pm 0{,}04 \times 10^{2}$ | $1{,}64 \pm 0{,}22 \times 10^{1}$ | $6{,}10 \pm 0{,}22 \times 10^{2}$ | $2{,}35 \pm 0{,}20 \times 10^{4}$ |
| | *Z. bailii* | $5{,}93 \pm 0{,}01 \times 10^{2}$ | $1{,}61 \pm 0{,}17 \times 10^{0}$ | $9{,}27 \pm 0{,}44 \times 10^{1}$ | - | - |

- means non-detectable

## 4. Discussion

The use of different monoazide dyes (EMA and PMA) that bind to the DNA to differentiate live from dead cells was described 7 years ago (Nogva et al., 2003). However, they were mostly used on bacterial cells in which PMA showed better results than EMA (Pan and Breidt, 2007; Nocker et al., 2006). Because of their limited application in yeast (Rawsthorne and Phister, 2009a,b) and to differences found in the optimal conditions of use (Nocker and Camper, 2006; Rudi et al., 2004; Lee and Levin, 2006), we focused on determining how these dies could be used for optimal effect in yeast and alcoholic fermentation. It is well known that they can enter cells which have compromised membrane/cell wall system (Rudi et al., 2004), yet little is known about the possible effects of ethanol on their entering cells. Ethanol has clear effects on membrane permeability (Mishra and Prasad, 1988; Alexandre et al., 1994; Ding et al., 2009) and the increase in ethanol is the main characteristic of alcoholic fermentation.

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Thus, the effect of ethanol had to be controlled first as concentrations increased during alcoholic fermentation and second during the analysis of the final wines when ethanol levels are high. An initial estimation by EMA- and PMA-QPCR showed that the direct analysis of wine samples yielded fewer cells than those that were culturable and, thus, we considered that the membrane was permeable to both dyes. Cells were recovered by removing them from ethanol and allowing them to recover in YPD medium in conditions in which no growth is possible (2 h at 13º C). We had previously observed cell membrane recovery in compromised cells by contact in optimal yeast medium such as YPD (Redón et al., 2008).

Once we had set the best conditions for using EMA and PMA, we analysed the differences between them and compared with the results obtained with the non-dye QPCR. We observed no differences between the two dyes either with live and viable cells or in the presence of dead cells. The presence of dead cells did not interfere with the total estimation of viable cells because high counts ($10^6$) did not affect the method linearity between $10^3$ and $10^7$ cells/ml. These results agree with those of Rawsthorne and Phister (2009a) who detected population of 13 cells/ml in matrixes of $10^5$ dead cells/ml. The non-dye QPCR could not differentiate between dead and live cells and, accounted for all of them. When it was used in controlled conditions (different combinations of cells killed by heating and live cells), agreement was always good between the enumeration of the colonies recovered on plates, counts under the microscope with LIVE/DEAD kits and the enumeration with EMA- or PMA-QPCR. The standard curves obtained also showed good slopes with all the primers tested.

We tested the use of both dyes to alcoholic fermentation in two different sets of samples. On the one hand, we analysed an alcoholic fermentation and, on the other, some ageing wines. During alcoholic fermentation, alcohol increases and the yeast population changes. To enforce the yeast variability and to have samples for a longer period of time with more variables, we performed a low fermentation temperature without selected yeast inoculation. The fermentation took 20 days and was led by *S. cerevisiae*, as usual, also at low temperature (Hierro et al., 2007; Andorrà et al., 2010). With the exception of the first samples taken (before and after settling) for which the total yeast counts made by the three QPCR methods and plate recovery were one log unit higher than *S. cerevisiae*, the values were always very similar and showed that *S. cerevisiae* was the main yeast recovered and fully viable. Only at the end of

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fermentation did the non-dye QPCR show very high counts, whereas plate and dye-QPCR showed a decline in viability. At the first points, the two major Non-*Saccharomyces* yeast, *C. zemplinina* and *Hanseniaspora sp* accounted for the difference between the total yeast counts and *S. cerevisiae* as in previous studies in the same winery (Constantí et al., 1997; Beltran et al., 2002; Hierro et al., 2007). Whereas the primers for *C. zemplinina* and *S. cerevisiae* are species-specific, the ones for *Hanseniaspora* are genus specific (Hierro et al., 2007). Although *H. uvarum* was the most frequently isolated species in this cellar, *H. osmophila* and *H. guilliermondii* have also been found (Hierro et al., 2006b; Andorrà et al., 2010), so this methodology does not discriminate among them. Later, during the fermentation, these species survive: *Hanseniaspora sp* is the main species at the beginning of the fermentation and *C. zemplinina* survives in higher alcohol concentrations at low temperature, as observed by Llauradó et al. (2002). In all the samples taken from this alcoholic fermentation we found very good agreement between the QPCR dyes and plate recovery, both with YPD for total yeast and with LYS medium for Non-*Saccharomyces* yeasts.

During wine ageing the yeast population is several orders of magnitude lower than during alcoholic fermentation, which is a new challenge for low population estimation. In this situation, and even after sample concentration, counting under the microscope, gave a poor estimation as not enough yeasts were seen to be considered reliable. However, we recovered a significant population in both YPD and LYS media and also detected reliable amounts in the three QPCRs. It was disappointing to find that all the methods used recovered low populations in all the ageing wines. Most of the viable cells were also culturable and no differences were observed with the methods. Only in one of the spoiled wines (Wine E) did we find a discrepancy between the culturable yeast and the recoveries with QPCR. The high levels of *D. bruxellensis* were undetected by plates yet identified by all three QPCRs, which gave very similar values. In this case, we detected viable but not culturable cells. In fact, the slow growth of these species may account for this lack of culturability (Loureiro and Malfeito-Ferreira, 2003).

In conclusion, we believe that we now have a new culture-independent methodology for determining microbial diversity in winemaking. Most of the present knowledge on food microbiology has been acquired from the microorganisms that have been recovered on culture media. For many years the limitations of the protocols used were evident and, to overcome them, culture-independent methods have been proposed. We had already worked with some of these culture-independent

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methodologies, despite being aware of the limitations of working with DNA due to its high stability and the impossibility of distinguishing between live and dead cells. We now believe that we have circumvented this problem by using two dyes coupled with QPCR to differentiate between live and dead cells in several situations with high alcohol concentrations and even low yeast populations. However, the main handicap of this technology is the availability of species-specific primers for detecting the different species. Our laboratory is now working on increasing the availability of primers addressed to more yeast species of interest in oenology.

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**Chapter 6**

# Analysis and direct quantification of *Saccharomyces cerevisiae* and *Hanseniaspora guilliermondii* populations during alcoholic fermentation by fluorescence *in situ* hybridisation, flow cytometry and quantitative PCR

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**Abstract**

Traditionally, it was assumed that non-*Saccharomyces* (NS) yeasts could only survive in the early stages of alcoholic fermentations. However, recent studies have shown the persistence of NS populations throughout the fermentation process by applying culture-independent methods. The aim of the present work was to analyse and quantify *Saccharomyces cerevisiae* (Sc) and *Hanseniaspora guilliermondii* (Hg) populations during alcoholic fermentations by using classical and culture-independent methods, such as fluorescence *in situ* hybridisation (FISH), quantitative PCR (QPCR) and flow cytometry. Species-specific FISH probes labeled with fluorescein (FITC) were used to directly hybridise Sc and Hg cells from single and mixed cultures. The results obtained by both culture-independent methods (FISH and QPCR) showed in general good agreement, revealing the presence of high populations (ranging $10^7$-$10^8$ cells/ml) of both yeasts throughout fermentations. When fermentations were performed in pure cultures there was also a good agreement with plating for both species. However, during mixed fermentations Hg lost its ability to grow on plates after 4 to 6 days, while enumeration by FISH and QPCR showed the persistence of high Hg populations (about $10^8$ cells/ml) that remained constant along the complete fermentation (as long as 10 days). The rRNA levels of cells during the course of mixed fermentations was also analysed by using flow cytometry in combination with FISH probes. The fluorescence intensity conferred by the species-specific FISH probes was considerably lower for Hg than for Sc. Moreover, when the effect of boiling the cells was analysed by flow cytometry the stability of the rRNA content of cells showed species-dependence, being more sensitive for Sc than for Hg species.

**Keywords:** wine, culture-independent methods, RNA stability.

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## 1. Introduction

The transformation of grape must into wine involves the coexistence and succession of different yeast species. The microorganisms present in the berry surfaces are composed mainly by non-*Saccharomyces* (NS) yeasts which predominate during the early stages of the alcoholic fermentation. These are soon overtaken by the growth of *Saccharomyces cerevisiae* (Sc) that dominates the mid to final stages of the fermentation (Fleet & Heard, 1993; Fleet, 2003). Despite this typical growth pattern has long been accepted, it was established mainly by plating methods. Indeed, more recent studies have questioned this pattern by using molecular methods that revealed the persistence of NS populations throughout the fermentation process (Fernandez et al., 1999; Cocolin et al., 2000; Andorrà et al., 2010; Zott et al., 2010). The early displacement of NS wine yeasts is quite controversial. Previously, it was thought that this was mainly due to the lower tolerance of NS species towards the increasingly adverse conditions established in the medium (low pH values, high levels of ethanol and organic acids, nutrient depletion, etc.) as the fermentation progresses (Fleet & Heard, 1993). More recently, the dominance of Sc has been attributed to other factors, such as growth arrest mediated by cell-to-cell contact mechanisms (Nissen et al., 2003), and the secretion of toxic compounds (Pérez-Nevado et al., 2006; Albergaria et al., 2010). However, most of these studies has been carried out by using classical plating methods which are laborious, time-consuming and somewhat unreliable (Giraffa, 2004) and only detect culturable populations.

Molecular techniques have been developed and applied to control microbial growth and to characterise the microflora of different processes and environments. These methods are generally faster, more specific, more sensitive and more accurate, allowing the precise study of the microbial populations and their diversity (Justé et al., 2008). Molecular techniques can be used to identify or genotype microorganisms previously grown on a culture media (commonly known as a culture-dependent techniques), or applying them directly to a sample (known as a culture-independent techniques) (Rantsiou et al., 2005). The application of culture-independent methods gives a better knowledge of the true microbial diversity, avoiding the biases that growth and isolation by enrichment platting might introduce. Furthermore, culture-dependent techniques can underestimate the size and diversity of a given population since they do not account for non-culturable populations, such as sublethally injured and/or viable but

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non-culturable (VBNC) cells, which may fail to grow on plates and are common in wine (Millet & Lonvaud-Funel, 2000). Underestimating VBNC and/or injured populations can be important since these are still metabolically active (Oliver, 2005; Mills et al., 2008). Several culture-independent techniques have been develop and used for detecting and quantifying wine yeast species (Cocolin et al., 2000; Mills et al., 2002; Phister & Mills, 2003; Hierro et al., 2006, 2007; Andorrà et al., 2008, 2010).

One of the most promising methods, due to its simplicity and rapidity, is the fluorescence *in situ* hybridisation technique (FISH). This technique combines the simplicity of microscopic observation with the specificity of DNA/RNA analysis. Furthermore, most of the molecular techniques do not provide information regarding the morphology of cells, their number and spatial distribution within a given environment. In theory, FISH technique can detect single cells but in practice, however, the detection limit is often $10^4$ cells/ml, since enumeration is usually carried out by hemocytometry. This limitation can be overcome by concentrating the samples prior to hybridisation and counting (Blasco et al., 2003). Another limitation of using FISH by hemocytometry is the insufficient automation which is required for high throughput sample analysis (Amann et al., 2001). This can be solved by using flow cytometry (FC) in combination with FISH for a selective enumeration of mixed microbial populations, which allows a high resolution and highly automated analysis (Amann et al., 1990). The main advantage of this technique is its sensitivity, which can detect a cell in a million. FC in combination with FISH probes has been used to identify and analyze mixed microbial populations (Amann et al., 1990; Wallner et al., 1993; Rigottier-Gois et al., 2003).

Xufre et al. (2006) developed 26S rRNA gene probes for identification of numerous wine-related yeast species, including Sc, *Candida stellata*, *H. uvarum*, *H. guilliermondii* (Hg), *Kluyveromyces thermotolerans*, *K. marxianus*, *Torulaspora delbrueckii*, *Pichia membranifaciens* and *P. anomala*. Stender et al. (2001) detected the slow growing yeast *Dekkera bruxellensis.* These authors, however, used cultivation and isolation steps prior to application of the FISH technique and thus the identification of those samples did not account the overall cell population but just the culturable cells.

The aim of this work was to analyse Sc and Hg populations by using plating and culture-independent methods based on RNA (FISH) and DNA (QPCR). These methodologies were used for direct quantification and identification of Sc and Hg populations in single cultures and mixed fermentations performed on synthetic media and on a simulated wine. FC in combination with FISH probes was used to quantify

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fluorescence intensity of hybridised cells of Sc and Hg during mixed fermentation and also to determine the stability of the rRNA content of the cells.

## 2. Materials and Methods

### 2.1. Yeasts strains, inoculum cultures and growth media

The yeasts strains used were Sc CCMI 885 (Culture Collection of Industrial Microorganisms, LNEG, Lisbon) and Hg NCYC 2380 (National Collection of Yeast Cultures, Norwich, UK). Both strains were first isolated from Portuguese wines, Sc from Alentejo and Hg from Douro wine regions. Yeasts were maintained on YMPD-agar slants (1% dextrose, 0.5% peptone, 0.3% malt extract, 0.3% yeast extract, 2% agar, w/v) and stored at 4ºC.

Inocula of Hg and Sc were prepared by transferring biomass of one YMPD-agar slant (pre-grown for 48 h at 30 ºC) into 50 ml of YMPD medium in 100 ml flasks that were incubated for 16 h at 30 ºC and 150 rpm.

Single cultures of Sc and Hg were performed on YMPD media and mixed fermentations on synthetic grape juice (SGJ), prepared as described by Pérez-Nevado et al. (2006).

### 2.2 Single cultures on YMPD medium

Single cultures of Sc and Hg were carried out in 500 ml flasks filled with 250 ml of YMPD medium. Each flask was inoculated with $1\times10^4$ cells/ml of the respective yeast strain and incubated without agitation at a constant temperature of 20ºC. Both fermentations were performed in duplicate and monitored by plate counting and by direct application of the FISH technique, using species-specific FITC-labelled probes and DAPI staining. Hybridised cells were enumerated in a Neubauer chamber using an epifluorescence microscope (Olympus BX-60, Tokyo, Japan).

### 2.3 Alcoholic fermentations

Two mixed fermentations (shaken and static) were performed in duplicate in 500 ml flasks filled with 250 ml of SGJ and inoculated with $1\times10^5$ cells/ml of each yeast strain (Hg and Sc). Shaken fermentations were conducted under constant agitation of 100 rpm in an orbital shaker (Unitron, Infors, Switzerland). All the experiments were conducted at controlled temperature of 20ºC. The monitoring of fermentations was

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made by daily samplings for cellular density quantification and determination of sugar consumption and production of ethanol. The yeast population was determined by the classical plating method and also by FISH and QPCR.

To simulate the second part of the alcoholic fermentations, a commercial red wine was diluted with sterile distilled water till ethanol concentration reached 30 g/l. The medium was supplemented with glucose and fructose to attain a final concentration of 55 g/l for each sugar and 3 g/l of yeast extract in order to simulate the average sugar concentration of a middle wine fermentation. This medium was then inoculated with $10^6$ cells/ml of each strain (Sc and Hg) with inocula previously grown (for 16 h at 30ºC) on YMPD broth. The fermentations were conducted in duplicates with constant agitation (100 rpm) at a controlled temperature of 20ºC. Sugar consumption was monitored daily by both density measures and by HPLC. Samples were taken each 24 hours until the end of fermentation (sugars below 2 g/l). Yeasts were monitored by using plating counts, and by direct application of the FISH (with species-specific probes and DAPI) and QPCR techniques.

### 2.4 Fluorescence *in situ* hybridization (FISH)

#### 2.4.1 Oligonucleotide FISH probes

Oligonucleotides ranging from 15 to 20 nucleotides and targeted to the D1/D2 domain of 26S rRNA of the yeasts species Sc and Hg were synthesized and labelled with the fluorochrome Fluorescein IsoThioCyanate (FITC) at the 5′-end. The species-specific FISH probes used in this work were those previously designed and validated by Xufre et al. (2006) with the following sequences for Hg and Sc, respectively: 5’-CAATCCCAGCTAGCAGTAT-3’ and 5’-TGACTTACGTCGCAGTCC-3’. Furthermore, a universal eukaryote FISH probe (EUK 516 5’-ACCAGACTTGCCCTCC-3’) was also used as a positive permeabilisation control.

#### 2.4.2 FISH procedure

The FISH procedure used the protocol described in Xufre et al. (2006) and adapting it to the direct use from fermenting samples. The adaptation involved the permeabilisation and the hybridization steps. Briefly, several permeabilization treatments were tested as different concentrations of lysozyme, zymoliase, ultrasounds, triton and ethanol. And two different concentrations of hybridization solution at different

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reaction time were also studied. Thus, the detailed protocol for direct analysis resulted as follows:

One ml of fermentation samples were centrifuged for 5 min at 5,000 g, the cells were washed once with 1x phosphate-buffered saline (PBS - 130 mM sodium chloride, 10 mM sodium phosphate buffer, pH 7.2) and incubated with 4% (v/v) of paraformaldehyde for at least 3 h at 4 ºC and strong agitation. Fixed cells were thereafter centrifuged for 2 min at 10,000 g and resuspended in 1volume *per* volume of 1x PBS buffer and ethanol (98%) and kept at -20ºC until required. Approximately $10^6$ fixed cell samples were prior to hybridisation centrifuged discard the supernatant and then hybridised in 40 µl hybridisation buffer (0.9 M sodium chloride, 0.01 % w/v sodium dodecyl sulphate, 20 mM Tris-HCl and 0.025 % v/v formamide) and 10 µl of FITC-labelled probe (50 ng/µl) and incubated at 46 ºC for at least 3 h. After centrifugation, cells were resuspended in 100 µl of washing buffer (25 mM Tris/HCl and 0.5 M NaCl) and incubated for 30 min at 48 ºC. Before enumeration, the previous suspensions were centrifuged, resuspended in 100 µl of 1x PBS and doubled stained with DAPI (1 µg/ml) (4,6-diamidino-2-phenylindole), a fluorescent dye that stains the double stranded-DNA, by incubation for 1 min at room temperature. Approximately 5 µl of cell suspension was mixed with 5 µl of Vecta Shield (an anti-fading agent, Vector Laboratories, USA), spotted onto a Neubauer chamber and enumerated by epifluorescence microscopy (Olympus BX-60, Tokyo, Japan). Total cells were enumerated using a NU-MWB filter (wavelength for DAPI) and Hg and Sc hybridised cells using a U-MWB filter (wavelength for FITC).

**2.5 DNA extraction and QPCR**

DNA was extracted according to Hierro et al. (2006). QPCR was performed in an Applied Biosystems 7300 Fast Real-Time PCR System (Applied Biosystems). Power SyberGreen master mix was used according to the manufacturer's instructions (Applied Biosystems, CA). An ABI Prism 96 well optical plate (Applied Biosystems) was used for the reaction. The instrument automatically determined the Ct. Yeast quantification was performed using the primers, YESTF/YESTR (Hierro et al., 2006) for total yeasts, CESPF/SCERR for *Saccharomyces* and CESPF/HUVR for *Hanseniaspora* (Hierro et al., 2007). Standard curves were done for each type of microorganisms in triplicate.

### 2.6. Flow Cytometry

Cells from single and shaken mixed fermentation of Hg and Sc were hybridised with the respective FISH-probes and analysed by FC. Fluorescence of the hybridised cells was quantified with a FAC-Scan FC (Biosciences). The argon ion laser was tuned to an output of 15 mW at 488 nm. Forward-angle light scatter (FSC) and side-angle light scatter were detected with a 530 (±30) nm band pass filter and fluorescence (FL1) was detected with a 530 (±30 nm) nm band pass filter. FITC-probes were measured with deionised filtered (0.2 μm Millipore membranes) water as sheath fluid. The FC automatically stabilised the optical alignment and standardised the fluorescence intensities of the hybridised cells.

Data acquisition and processing: The parameters FSC, SSC and FL1 were recorded and for each measure 20,000 events were stored in list modes. The WinMDI software (v.2.8) was used for subsequent analysis. Probe-conferred fluorescence was determined as the mean (geometric) of the fluorescence values emitted by single hybridised-cells (of Sc and Hg) measured in the FL1 detector and recorded on a gate that was first defined in a FSC-versus-SSC density plot. This mean value was then divided by the mean value of the fluorescence emitted by non-hybridised cells. Samples from mixed fermentation were first analysed without hybridisation in a FSC-versus-SSC density plot. Due to the considerably different size of Sc and Hg cells it was possible to obtain separated gates containing the entire cell population of each yeast species. Mixed samples were hybridised in separate with each species-specific probe and analysed with and without probe. FISH experiments were performed in triplicate for each sample (6 replicates for mixed samples) in independent hybridisation assays and fluorescence values measured on the FC at various time intervals (30 min, 1h, 2 h and 4 h) after application of the FISH procedure. No significant loss of fluorescence was detected for at least 4 h. The stability of the cells rRNA was analysed by FC, measuring the fluorescence intensity emitted by hybridised cells after boiling (10 min at 100ºC) and also after RNase treatment (10 μl of RNase solution (0.2 mg/ml) added to 100 μl of sample for 10 min at 65ºC).

### 2.7 Analytical methods

Glucose and fructose consumption and ethanol production were determined by HPLC (Merck, Darmstadt, Germany) using a Sugar-Pak™ column (Waters, Milford, USA) and a refractive index detector. The samples were eluted at 90ºC using a

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degassed aqueous mobile phase containing 50 mg/l Ca-EDTA, at a flow rate of 0.5 ml/min. All samples were analysed in triplicate.

### 2.8 Plating counts

Quantification of Hg and Sc populations during mixed fermentations was performed by the classical plating method. Samples, taken aseptically throughout fermentations, were inoculated onto YMPD-agar plates after appropriate dilution in sterile water. In the mixed fermentations, CFU counting of Hg was determined on YMPD-agar plates containing 0.01% of cycloheximide, a selective medium where Sc is unable to grow. CFU counting of Sc was calculated as the difference of the total CFU counts obtained on YMPD-agar plates (without cycloheximide) and the CFU counts of Hg. All plates were incubated at 30ºC for 2-4 days and CFUs enumerated after no increase of colonies was observed on plates.

## 3. Results

### 3.1. Validation of direct application of FISH

In order to validate the direct application of FISH, thus avoiding previous cultivation on enriched media, single cultures of Sc and Hg were performed on YMPD medium. Samples from those cultures were directly hybridised with the respective FISH probes, stained with DAPI and enumerated on a Neubauer chamber by epifluorescence microscopy. The cell population of those fermentations was also followed by plating. FISH technique correlated well with plating counts for both yeasts along cultivation (Fig.1). Besides, results showed that under single cultivation both strains were able to grow on YMPD-agar plates long after sugar exhaustion, which occurred within 24 h, although a slight decrease was observed in the cellular density of Hg after 72 h of cultivation (by both methods). This means that cells kept their culturability even after long periods of starvation.

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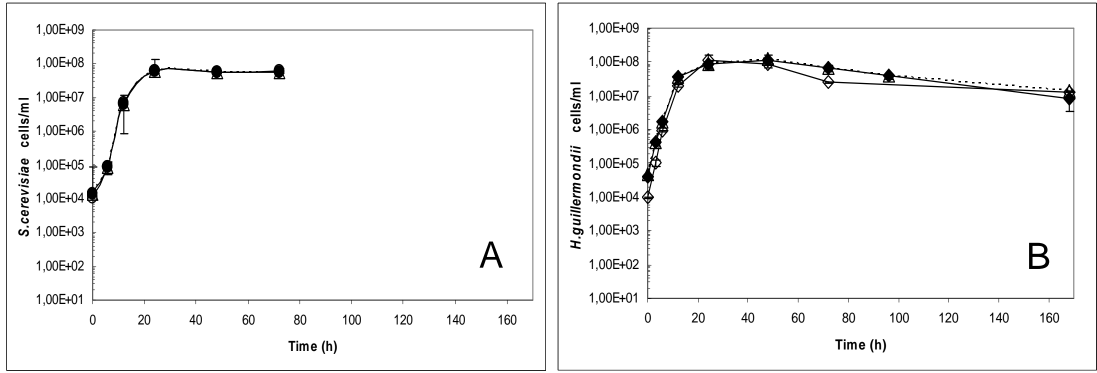


Figure 1: Yeast population evolution during single Sc (A) and Hg (B) fermentations carried out on YMPD medium, as determined by FISH and plating (-●-, Sc-FISH; -○-, Sc-CFU; -♦-, Hg-FISH; -◊-, Hg-CFU;-Δ-, total cells-DAPI). (mean values ± SD).

The FISH procedure was applied directly to samples from mixed fermentations and microscopic observation of the hybridised cells (Fig.2) confirmed the species-specificity of the FISH probes. In addition, hybridised cells of Hg (Fig.2-A) and Sc (Fig.2-C) compared well with total cells counts (Fig.2-B,D) measured by DAPI staining, thus confirming and validating the effectiveness of the FISH procedure and the reproducibility of enumeration by hemocytometry.

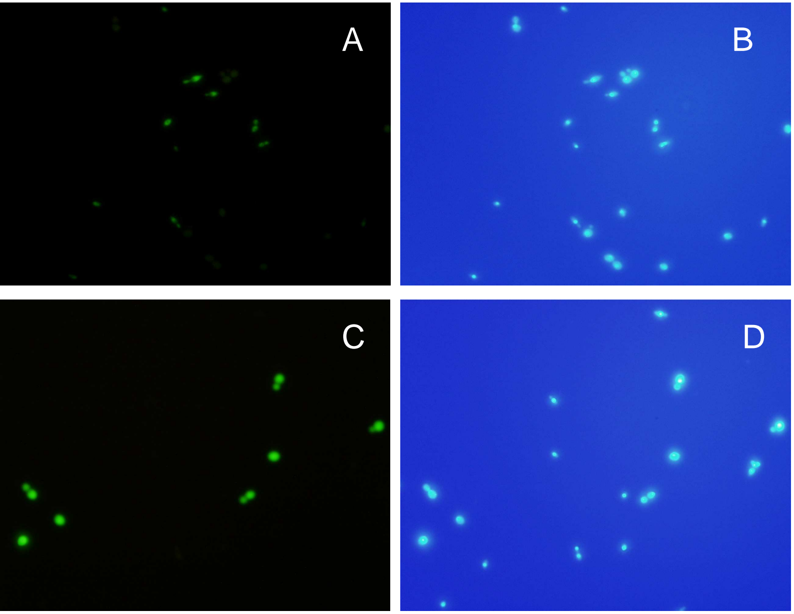


Figure 2. Microscope visualisation of fluorescence signals emitted by Sc and Hg cells of mixed fermentation samples hybridised with FITC-labelled FISH-probes and stained with DAPI. Images of cells hybridised with Hg-FISH probe captured on the filter for FITC (A) and on the filter for DAPI (B). Images of cells hybridised with Sc-FISH probe captured on the filter for FITC (C) and on the filter for DAPI (D).

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### 3.2. Monitoring of mixed fermentations on SGJ

Sugar consumption and ethanol production during shaken and static fermentations are represented in Fig.3-A and A', respectively. The time required to consume sugars present on the SGJ was 6 and 10 days for shaken and static fermentation, respectively. In both cases, the glucose consumption rates were higher than those of fructose and residual sugars present on final wine was less than 2 g/l of fructose. Both fermentations produced similar amounts of ethanol with final concentrations of about 100 g/l.

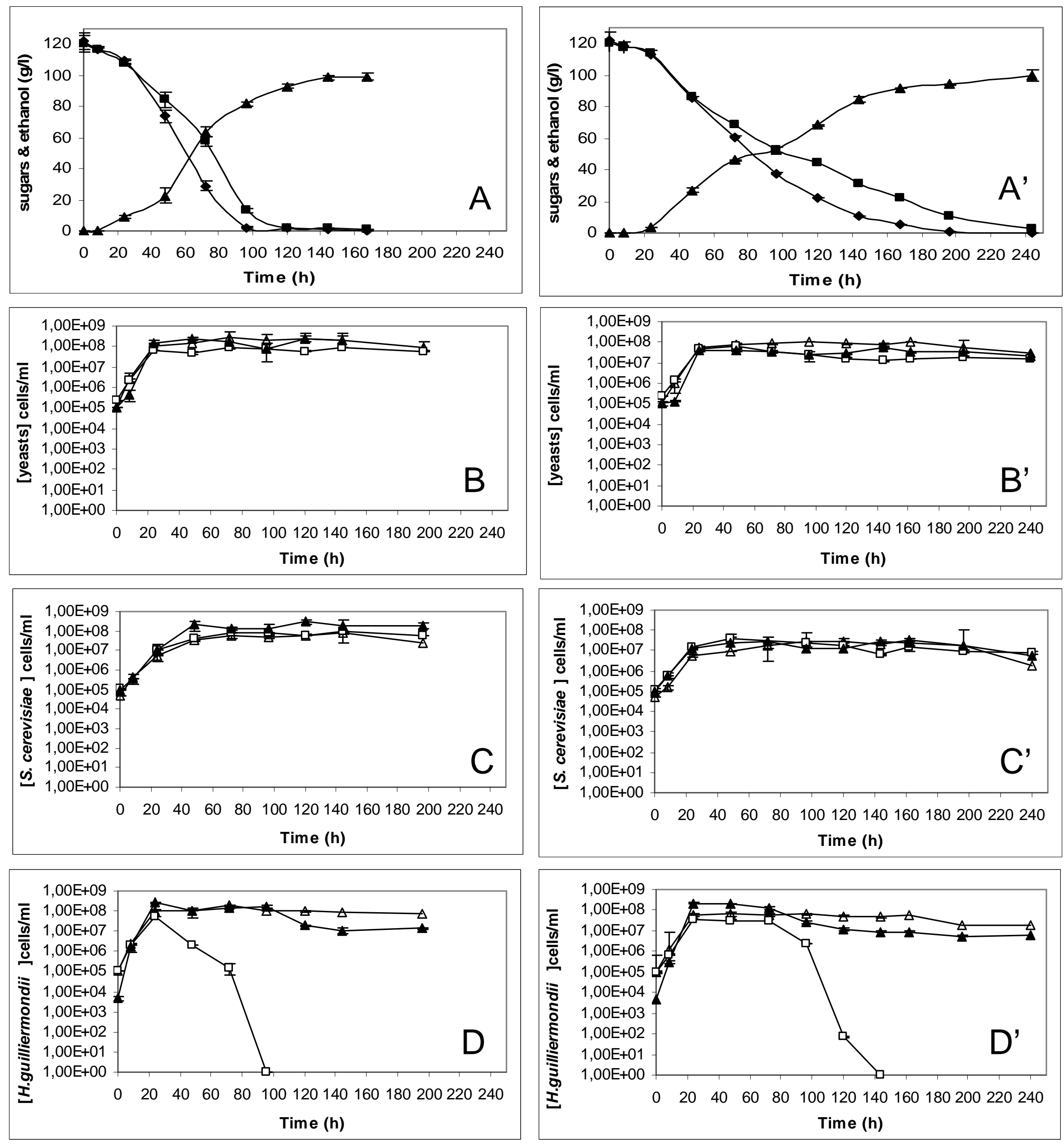


Figure 3. Sugar consumption and ethanol production in shaken (A) and static fermentation (A') (-▲-, ethanol; -■-, fructose; -♦-, glucose). Evolution of total yeasts (B, B'), *S. cerevisiae* (C, C') and *H. guilliermondii* (D, D') species in shaken and static mixed fermentation analysed by FISH (-Δ-), QPCR (-▲-) and plating (-□-).

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The cell density evolution during shaken and static fermentations is represented in Fig. 3 for total yeast (B, B'), Sc (C, C') and Hg (D, D') populations, as determined by plating, FISH and QPCR methods. Results showed that in both fermentations yeasts raised their initial cell populations from $10^5$ cells/ml to $10^7$-$10^8$ cells/ml (Fig.3-B, B') within 24 to 48 h. During the stationary phase growth the cell density profiles of Hg (Fig.3-D, D') and Sc (Fig.3-C, C') significantly diverged by plating but were quite similar when determined by FISH and QPCR. Using both culture-independent techniques (FISH and QPCR), Hg maintained its population around $1\times10^8$ cells/ml for 100 h in the shaken fermentation, although a 10-fold decrease was observed after that time by QPCR, whereas Sc kept a constant cellular density of about $6\times10^7$ cells/ml. However, after 200 h of cultivation in the static fermentation the cell population of both yeast species slightly decreased. During all fermentation the results showed that the yeast populations in the shaken fermentations were slightly higher than the static ones. Independently of method used (culture-dependent or not), no major decline was observed on the cell population of Sc in all fermentations for at least 200 h, while Hg totally lost its ability to form colonies when plated after 4 days and 6 days in shaken and static fermentations, respectively.

### 3.3. Monitoring of mixed fermentation on simulated wine

In the simulated wine fermentation yeasts showed similar fermentation pattern than that of SGJ consuming glucose faster than fructose (Fig. 4A). The initial ethanol of the media was 30 g/l and after 96 h of fermentation the residual sugars were lower than 2 g/l and ethanol achieved a final concentration of 80 g/l. Total yeast and Sc population increased up to $10^8$ cells/ml in the first 24h, maintaining these cell density throughout the process, as measured by both culture-independent methods (FISH and QPCR) (Fig. 4 B, C). Meanwhile, Hg began to loose its cultivability on YMPD-agar plates after 24 h and did not grow at all on plates after 96 h. When analyzed by culture-independent methods (FISH and QPCR), Hg population did not increase its population and was constant during all the fermentation slightly over $10^6$ cells/ml. In this case, no change was observed by QPCR, which enumerated always higher population than FISH.

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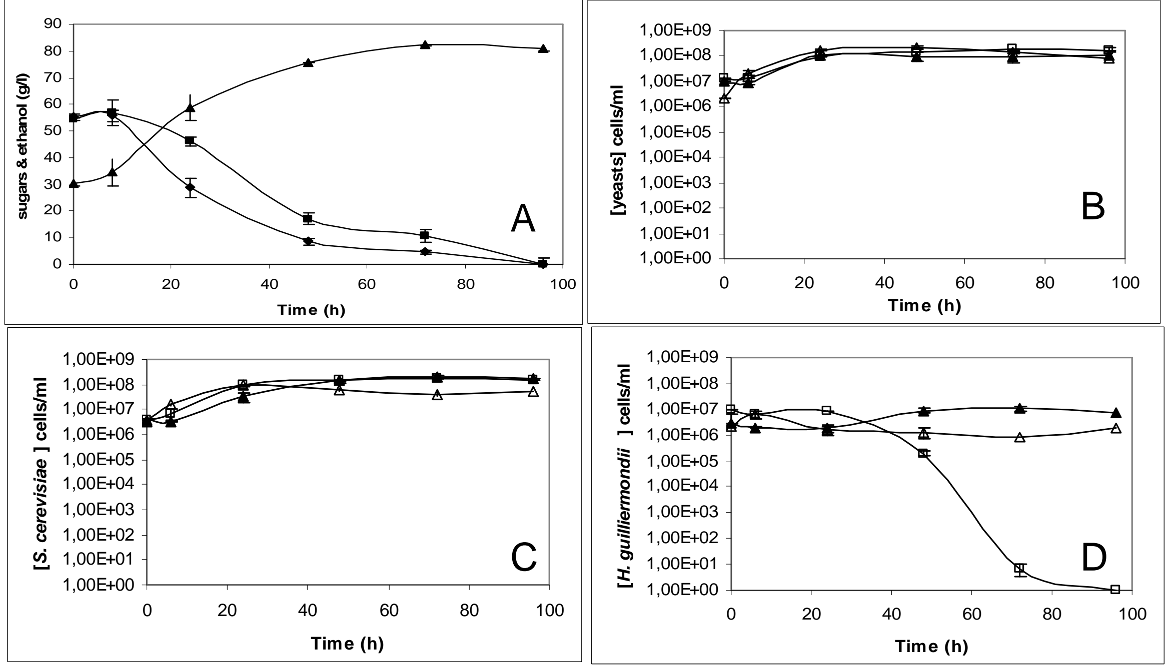


Figure 4. Sugar consumption and ethanol production in simulated wine fermentation (A) (-▲-, ethanol; -■-, fructose; -♦-, glucose). Evolution of total yeasts (B), *S. cerevisiae* (C) and *H. guilliermondii* (D) species in simulated wine fermentation analysed by FISH (-∆-), QPCR (-▲-) and plating (-□-).

### 3.4. FC in combination with FISH probes

In order to evaluate changes in the rRNA content of cells during the fermentations and to avoid the subjectivity of operator microscope counting, FISH procedure was used in combination with FC. Furthermore, the RNA stability towards boiling or/and RNase cell treatments was also analysed. Cells from pure and mixed cultures hybridised with the respective FISH probes were analysed by FC and data for cell size (FSC), internal heterogeneity (SSC) and fluorescence (FL1) acquired. Initially, pure cells suspensions of Hg and Sc were analysed by FC for non-hybridised cells and cells hybridised with the respective species-specific FISH probes (data not shown). Results showed that due to the higher cell size of Sc by comparison to Hg cells it was possible to design, in a SSC-versus-FSC density plot, different gates for each yeast species. Autofluorescence of Hg and Sc yeasts was 1.21 for Hg and of 3.5 for Sc, as determined by the relative fluorescence intensity (FL1 signals in a LOG scale) emitted by non-hybridised cells of each species. The specific fluorescence intensity conferred by each FISH probe to the respective yeasts cells (Hg and Sc) was determined dividing the relative fluorescence intensity of hybridised cells by the respective autofluorescence value.

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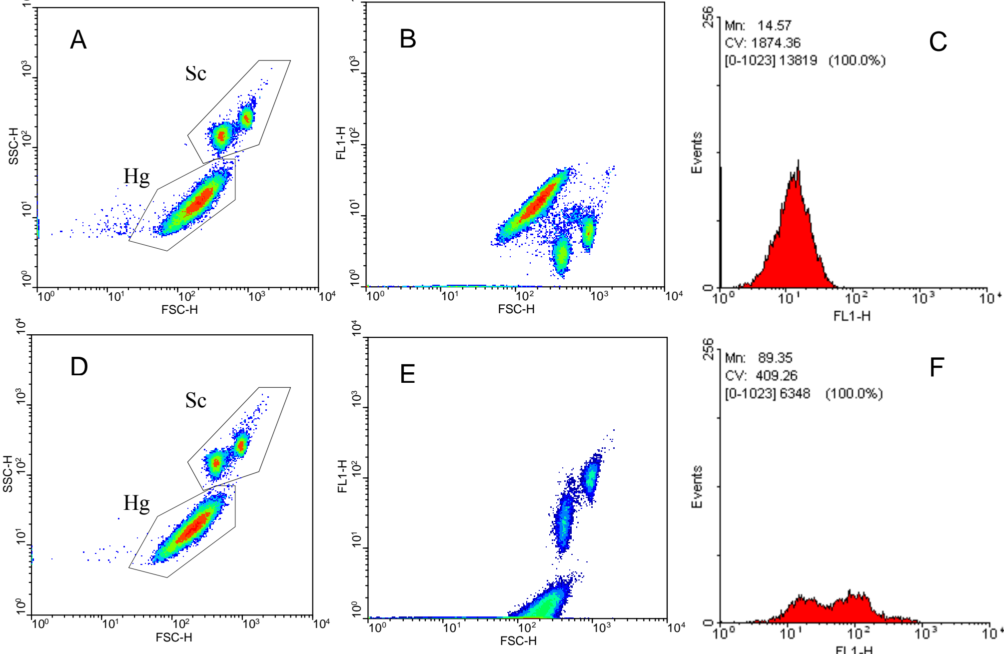
Similar quantification and analysis was done for samples of the mixed fermentation (at times 24, 48, 96, 144 and 192 h) with cells hybridised with either Hg or Sc species-specific FISH probes (in independent essays for each probe) and analysed by FC (Fig.5). Results showed that Sc population exhibited much higher heterogeneity along the fermentation than Hg population, as exemplified in Fig.5-A and D, originating two subpopulations within the same sample, which probably depends on whether cells are budding or not (Fig.5-A). The number of Hg and Sc cells within a given sample and the respective fluorescence intensity conferred by each FISH probe was determined along the mixed fermentation (Table 1).

Figure 5. Density plots (A,B,D,E) and histograms (C,F) obtained by FC analysis (20,000 events recorded) of a 144 h mixed fermentation sample with cells hybridised with the species-specific FISH probes for Hg (A,B,C) and for Sc (D,E, F). Histograms represent the distribution among the entire population analysed of the relative fluorescence intensity emitted by hybridised cells of Hg (C) and Sc (F) within the respective gates, as defined in the SSC-versus-FSC density plots. Mn, is the mean value of the relative fluorescence intensity of the entire cell population within the respective gate.




Results showed that the relative fluorescence intensity emitted by Hg-hybridised cells was always lower than that of Sc cells. Moreover, Sc cells exhibited 10 times higher fluorescence during the exponential phase than the stationary phase, conversely to what happened to Hg cells that only slightly decreased their relative fluorescence intensity (Table 1).

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Table 1. Flow cytometry analysis of hybridised cells of Hg and Sc during the shaken mixed fermentation and also of cells that were hybridised after boiling or RNase treatment at the end of fermentation. The values represent the mean value (20,000 events recorded per analysis) of at least 3 independent experiments performed for each FISH-probe.

| Time-sampling (h) | number of Hg cells mean±SD | number of Sc cells mean±SD | fluorescence of Hg cells mean±SD | fluorescence of Sc cells mean±SD |
|---|---|---|---|---|
| 24 | 17658 ± 774 | 774 ± 72 | 14.66 ± 0.97 | 132.41 ± 8.20 |
| 48 | 15460 ± 620 | 2914 ± 495 | 15.94 ± 1.75 | 121.78 ± 8.71 |
| 96 | 12824 ± 228 | 6690 ± 258 | 14.49 ± 1.09 | 20.74 ± 1.88 |
| 144 | 13826 ± 338 | 5848 ± 459 | 12.61 ± 0.54 | 27.55 ± 2.33 |
| 196 | 11703 ± 456 | 7725 ± 458 | 9.49 ± 0.52 | 17.05 ± 1.16 |
| Sample 196 h | Boiled cells for 10 min at 100 ºC | 0 h | 10.23 ± 0.26 | 13.08 ± 0.05 |
| | | 24 h | 8.33 ± 0.25 | 1.25 ± 0.01 |
| | | 96 h | 10.02 ± 0.01 | nd |
| | Rnase treated cells | 0 h | 1.14 ± 0.10 | 1.91 ± 0.43 |

nd: not determined

The stability of the rRNA of these yeasts was analysed by measuring the fluorescence intensity emitted by dead cells that were boiled (10 min at 100 ºC) and/or treated with RNase and thereafter hybridised with the respective FISH-probes. Results showed the disappearance of RNA immediately after RNase treatment for both yeast species, but not for death by boiling. In the later case, 24 hours were needed to reduce the levels of the RNA content of Sc cells, while in Hg the levels of RNA were kept rather constant even after 96 hours following boiling (Table 1).

## 4. Discussion

During spontaneous wine fermentations there is a typical growth pattern where the NS yeasts, belonging to the natural microflora of grape musts, grow during the early stages of alcoholic fermentation (4-5% v/v ethanol) and then begin to die off, leaving way to Sc strains to dominate and complete the fermentation process (Fleet & Heard, 1993; Fleet, 2003). However, recent studies have challenged these yeast population dynamics and revealed the presence of NS populations throughout the fermentation process (Andorrà et al., 2010; Zott et al., 2010). These results have been obtained mainly due to the use of culture-independent techniques that allow detecting non-culturable yeast cells during alcoholic fermentations (Millet & Lonvaud-Funel, 2000). In

the present study, the evolution of Sc and Hg populations during single and mixed fermentations was analysed by using FISH in combination with epifluorescence microscopy and FC and also QPCR. The cell density profiles obtained for both yeast species with these molecular methods were compared with those obtained by classical plating on YMPD-agar medium.

We used QPCR which was previously developed and used during alcoholic fermentations (Hierro et al. 2007; Andorrà et al., 2008, 2010) and it targets DNA, which due to its stability it is presumed to detect total non-autolysed yeast population (Hierro et al., 2006). Indeed, a good correlation was found for both yeasts between QPCR and FISH results, which seem to confirm that both techniques measure cells in similar physiological states. Application of FISH technique to identify different yeast colonies obtained at various stages of wine fermentations was first applied by Xufre et al. (2006) and the species-specific probes designed and validated on that work to identify Sc and Hg were used in the present work. The permeabilization and hybridization procedure of FISH was optimized to be applied directly on samples during alcoholic fermentations, avoiding previous plate cultivation. Results obtained confirmed the species-specificity of both probes, since no cross-species hybridization was detected. In addition, hybridised cells of Hg and Sc compared well with total cells counts obtained with DAPI staining, revealing the effectiveness of the FISH procedure and microscopic enumeration. Thus, FISH proved to be a consistent and reproducible method for direct quantification of Sc and Hg populations during mixed fermentations and single cultures. Furthermore, FISH technique only detects intact cells and since the target was rRNA, it is generally assumed that quantification yields viable, active populations (Giraffa & Carminati, 2008).

The cell densities profiles of Sc and Hg as determined by platting were totally consistent with the ones previously found by Pérez-Nevado et al. (2006) and Albergaria et al. (2010) for the same strains in mixed fermentations performed on SGJ. Indeed, Sc maintained its cultivability for at least 10 days in both mixed fermentations, while Hg lost the ability to form colonies after 4 and 6 days in shaken and static fermentations, respectively. However, as measured by both culture independent methods, QPCR (DNA) and FISH (rRNA), the Hg population remained at high levels (up to $10^7$ cells/ml) during the entire fermentation process. However, this species population declined one order of magnitude when enumerated by QPCR during the second half of the fermentation, which did not occur by FISH analysis. The QPCR was targeted to the DNA and was previously developed and applied to monitor the yeast population during

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alcoholic fermentations (Hierro et al. 2007; Andorrà et al., 2008, 2010). In the case of Sc the enumeration by the different methods yielded always similar results, even in the simulated wine fermentation that started with a certain concentration of ethanol (Fig. 4). In the later case, Hg cell population did not increase either by FISH or by the agar-plating method, even in the first days of fermentation.

Our results are similar to those found by Cocolin & Mills (2003) which studied the changes in the yeast population within wine fermentations as a response to sulphur dioxide additions by using PCR-DGGE target to DNA and rRNA. Furthermore, they also found that NS population could maintain high levels of DNA or RNA almost after 20 days of being uncultivable like our results in which Hg lost its ability to form colonies although maintain their DNA/RNA for almost 240 hours. Those authors concluded that NS yeasts may persist in a non-culturable state within standard wine fermentations and that platting alone is insufficient to monitor the various yeast populations. Indeed, the use of direct molecular methods for characterising microbial environments often reveals different populations than observed by plating analysis. This can be due to several factors, including the existence of VBNC populations, injured populations and/or dead populations in which the cellular DNA or RNA is protected from degradation. For this reason, Cocolin & Mills (2003) stated that future work is required in order to discriminate between VBNC, injured and dead populations within wine.

No growth of Hg could be observed on agar plates after 4-6 days in both mixed fermentations performed on SGJ; while FISH counts revealed the existence of up to $10^8$ cells/ml of Hg during the fermentation containing enough RNA to hybridise with the FISH probes and emit a fluorescence signal. The lack of growth of Hg when added to wine at mid fermentation occurred after 48 h. The levels of ethanol when Hg stopped growing in plates were completely different, and thus, this factor can be ruled out as the main cause for the loss of culturability by this species. However, under single cultivation Hg was able to grow on YMPD-agar plates long after sugar exhaustion, which occurred within 24 h. This means that Hg cells kept their culturability even after long periods of starvation, at least during 136 hours. Moreover, previous work carried out with the same yeast strain also showed that under single fermentation Hg was able to keep its culturability for long time (up to 15-20 days) while under mixed fermentations Hg cells began to loose its ability to grow on YMPD-agar plates after 1-3 days of fermentation depending on the initial Sc/Hg ratio (Pérez-Nevado et al., 2006, Albergaria, 2007). In

the present work, by applying molecular methods (either FISH or QPCR), high Hg populations (up to $10^8$ cells/ml) were present throughout the mixed fermentations. Put together, all these findings raise the question to know both how many of these cells are metabolically active and how these metabolically active Hg populations would influence the final wines. In addition, it remains to be determined if these populations correspond to VBNC, injured and/or dead cells.

The yeast population during mixed fermentations was analyzed by FC using cells hybridized with the respective species-specific FITC-labeled probes. The use of FISH combined with FC can be used for the analysis of the different physiological states, by determining the levels of rRNA during the different stages of winemaking. Quantification of the number of Sc and Hg cells along the mixed fermentation by FC compared well with results from FISH microscopic enumeration. The fluorescence intensity of Sc cells was much higher (up to 10 times) during the exponential phase than in stationary phase, whereas for Hg cells only a slight decrease of fluorescence was observed.

The observed results have to be put in relation with the kinetics and main physiological changes of the microbial population during alcoholic fermentation. Yeasts during winemaking are found in different growth stages (lag, exponential and stationary phases) and variable rRNA copy number was found with differences up 4-fold lower (corresponding to 2 Ct when estimated by QPCR) in the stationary phase samples than in the lag and exponential phase (Hierro et al., 2006). Thus, the decrease in fluorescence by Sc hybridised cells after the exponential phase indicated higher protein synthesis during this phase. Since the number of intact ribosomes approximately reflects the rates of protein synthesis, rRNA has been proposed as a more representative target for assessing cell viability and as a general good marker of metabolic activity (Ward et al., 1992; Vardervliet et al., 1994; Felske et al., 2000). However, RNA is easily degraded by RNase enzymes, which is avoided in FISH technique since there is no need for previous extraction and manipulation of RNA thus the degradation of RNA is prevented. The stability of the rRNA was evaluated for both yeasts by either boiling cells (10 min at 100 ºC) or treating cells with RNases (10 min at 65ºC) revealing that Hg boiled cells maintained similar amounts of rRNA for at least 96 h, while Sc boiled cells lost all rRNA within 24 h after boiling. RNase treatments completely destroyed the rRNA content of cells in both yeast species. Moreover, boiled cells of Hg hybridised after RNase treatment didn't show any fluorescence, this confirmed that there wasn't any non-specific hybridisation between FISH-probe and

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other cell structures. Thus, the fluorescence emitted by the hybridised cells should be attributed entirely to hybridisation of FISH probes with the respective rRNA target-sequence for yeast species. These results allow concluding that the fluorescence intensity conferred by these FISH-probes can be taken as an indirect measure of the cellular rRNA content. However, it should be emphasized that there is a significant presence of Hg rRNA after boiling, which could be due to two main reasons, survival to boiling by Hg or maintenance of ribosomal structure for long after cell death.

The effect of the viable but non culturable microorganisms during the alcoholic fermentation is not clear enough. Microorganisms can be present in changing physiological states during the different stages of winemaking. These physiological states could change from fully alive to almost dead, with different metabolic activities that could alter easily the fragile status of a wine by a release of metabolites. There is a strong need for doing research in these “late-live” statuses that could be really relevant in the last phases of the wine maturation, ageing, bottling and storing prior the consumption. Although in the present work we have used different culture-independent methodologies for estimating the viability of the cells. However, this viability has to be put in terms of metabolic activity and, still, this concept has to be further defined and oriented according the aims of the determination. Thus, in winemaking, for instance, the concept of metabolic activity related to non-*Saccharomyces* yeasts should be measured according to the production or release of unwanted metabolites (or in other words, spoilage potential) and thus the methods used should incorporate this potential for an appropriate estimation of “metabolic activity” of non culturable cells.

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UNIVERSITAT ROVIRA I VIRGILI
ADAPTATION AND DEVELOPMENT OF CULTURE-INDEPENDENT TECHNIQUES FOR THE INDENTIFICATION AND ENUMERATION
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Immaculada Andorrà Solsona
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# GENERAL DISCUSSION

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# GENERAL DISCUSSION

*- Culture-independent methods*

The study of microbial population dynamics during wine fermentation has traditionally been conducted using culture-dependent methods. Nevertheless, these methods fail to characterize the microorganisms that grow slowly or do not grow well on a plate, or whose population size is too small to be detected by regular sampling. Several factors influence the ability of cells to reproduce, and the detection of cells in samples by culturing (Lloyd & Hayes, 1995). It is well known that starvation and other multiple stresses underlying bioprocesses and natural environments induce cells to adopt non-culturable states to withstand stress and survive (Tonon & Lonvaud-Funel, 2000; Ganesan et al., 2007). In such states, microorganisms lose their ability to grow on nutrient non-selective media that normally support their growth, but exhibit metabolic activity to various extents. Responses to stress vary according to the species and the type of stress, and there are numerous specific stress-resistant forms (Oliver, 2005). Resistance to different stress forms, like chemical or heat shock, osmotic stress and dehydration, etc. has been attributed to entry into VBNC.

Nowadays, then, there is a clear interest in developing more efficient and rapid methods for assessing the presence of microorganisms. Molecular culture-independent techniques identify and quantify microorganisms regardless of their ability to grow or form colonies, because they do not require microorganisms to be previously cultured. The presence of these states has led to the introduction of several culture-independent techniques so that these different states of viability can be characterized and quantified as well as the diversity of microorganisms (Justé et al., 2008). In this study a variety of culture-independent techniques have been adapted and developed: QPCR, DGGE and direct cloning of amplified DNA. These techniques are also quicker than culture-dependent techniques.

DGGE is a good tool for studying the diversity of an unknown sample; it is both reliable and rapid. The main advantage is that an exhaustive knowledge of the microbial genomes is not required because universally conserved regions are used for the amplification. The main drawback is that it has difficulty in detecting minor species, especially when the best adapted species are an overwhelming majority (Renouf et al., 2007). Its detection limit in a pure culture is $10^3$ cells/ml. However, in a mixed culture, it is higher. In fact, populations lower than 1% of the major species are not detected

(Prakitchaiwattana et al., 2004). In the work reported here the detection limit was similar to those reported by other authors (Cocolin et al., 2001; Prakitchaiwattana et al., 2004). In wine fermentation, when the *S. cerevisiae* population rose to $10^8$ cells/ml, none of the yeasts with population sizes lower than $10^6$ cells/ml were detected. However, the detection limit for bacteria species was around $10^3$ cells/ml because no competitive DNA from other bacterial groups was present in these wine samples. Thus, DGGE is ideal for detecting species diversity in a mixed population with similar relative proportions, although the massive presence of a given species decreased the chances of detecting the minor species. One possible solution could be to design specific primers for some groups of microorganisms of interest. These primers would not amplify the main groups, and the technique would still be used as a tool for studying microorganism diversity.

Direct cloning of the amplified ribosomal region of yeasts is another culture-independent technique that has been developed for studying wine yeast diversity. This technique can detect a greater diversity of species than DGGE and their relative quantification. The main problem of this technique is that it analyzes only a small fraction of the population, as the number of colonies analyzed is limited, and the results are therefore only semi-quantitative or qualitative. It is a straightforward and reliable technique, although it is time-consuming and tedious.

The QPCR technique accurately quantifies minor microbial groups, regardless of an overwhelming presence of other microbial groups. Its detection limit is very low, around $10^2$ cells/ml, and can even be lowered further by concentrating the sample. Reproducibility is good and it is rapid. The only limitation is that the DNA sequence of the microorganisms studied needs to be known. It is therefore more useful for studying the changes of known microorganisms during a process such as alcoholic fermentation, than for studying microbial diversity. The development of new primers or sequences of important microorganisms could be one of the goals of the studies carried out with this technique, so that the approach to the population dynamics of a particular process would be more accurate. In this thesis, a new pair of primers has been designed for *C. zemplinina* from the D1/D2 domain of the 26S rRNA gene. The specificity and sensitivity of these primers are satisfactory, and comparable to those of the other primers used in the study.

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One possible drawback of the culture-independent techniques is the use of the very stable molecule DNA as template in the PCR reaction. This may overestimate the counts by amplifying the DNA from dead cells. One way round this problem would be to use RNA or dyes to eliminate the amplification of DNA from dead cells (Nocker & Camper, 2006). Taking these problems into account, two culture-independent methods were developed to differentiate between live and dead cells; FISH and the EMA-, PMA-QPCR technique. The former uses the rRNA as a target and the latter uses a dye treatment to differentiate between live and dead cells after DNA extraction.

FISH proved to be a good tool to be applied directly for identifying and quantifying a variety of viable yeast species. The target used in this study is rRNA which has long been reported to be a good representative target for viability (Vandervliet et al., 1994). Felske et al. (2000) used rRNA as a marker for general metabolic activity, because the number of ribosomes reflects the rates of protein synthesis. Hierro et al. (2006) also used rRNA to quantify yeast, but the fact that the gene expression depends on the physiological state of the cell might be a drawback. Yeasts in wines are found in different growth stages (lag, exponential and stationary phases) and the rRNA copy number was up to two cycle thresholds (Ct) lower in the stationary phase samples than in the lag and exponential phases (Hierro et al., 2006). An alternative to rRNA could be messenger RNA, which has also been chosen for viability studies; however, variable half-lives and expression patterns can make mRNA less attractive (Sheridan et al., 1998). FISH technique does not require the RNA to be previously extracted and manipulated, which prevents the RNA from degrading.

Some studies have compared the results obtained with RNA or DNA. Cocolin & Mills (2003) studied the changes in yeast population as a response to sulphur dioxide additions using DGGE with DNA and rRNA. These authors reported that cells could maintain high levels of DNA or RNA almost after 20 days of being unculturable. In agreement with our results, the changes of different yeast species using DNA or RNA did not present significant differences. Furthermore, in this study, dead cells were analysed after boiling and/or RNase treatment. The results showed that RNA disappeared when RNase was used, but that for *S. cerevisiae* it took 24 hours to reduce RNA when the cells were killed by boiling. The same results were obtained by Hierro et al. (2006). In the present thesis, we detected that RNA levels were the same 96 hours after a culture of *H. guilliermondii* had been boiled. This may be due to the fact that some cell structures were maintained after boiling in *Hanseniaspora* species, which lose

their ability to form colonies and keep the same levels of rRNA. However, the ribosome can be a rather stable organelle and depending on the species, the structure and the associated rRNA can survive for different lengths of time before they show any significant reduction in number.

FISH proved to be a good technique for analysing populations in which various microorganisms coexist. In fact, several microorganisms can be detected simultaneously using different probes stained with specific fluorochromes. Finally, the use of FISH combined with flow cytometry enables the method to be automated and different microorganisms to be quantified in the same analysis in a short time. This technique has the added value that it reduces the subjectivity of microscope counting. Flow cytometry can quantify fluorescence intensity, which was much higher during the exponential phase of *S. cerevisiae* than the rest of the stationary phase and all the growth phases of *H. guilliermondii*. FISH targeting rRNA seemed to be useful for detecting and quantifying microorganisms, and it can also be a good tool for studying the viability of yeast species. Furthermore, FISH combined with flow cytometry can be used to analyse the different physiological states, by determining the rRNA levels during the different stages of winemaking.

The second technique used for differentiating dead and live cells was QPCR with a previous dye treatment with EMA or PMA, which enter the dead cells and prevent their DNA from being detected (Rudi et al., 2005; Nocker & Camper, 2006; Nocker et al., 2006). The QPCR technique with the previous dye treatment allows a rapid quantification of viable cells.This technique showed a good correlation between EMA-, PMA-QPCR and cell plating in an exponential phase. The direct analysis of wine samples had to be modified to correct the effect of ethanol on yeast membrane permeability. When the dyes were applied directly to wine yeasts, the enumeration was much lower than on agar plates. To confirm that this underestimation in the dye-treated samples was the result of the higher permeability of the plasma membrane of cells exposed to ethanol, two different commercial kits were used to test yeast viability (Van Zandycke, 2003; Zhang & Fang, 2004). The use of these kits also showed that the viability of cells directly from wine samples with high ethanol content was not as high as the culturable population. Only a cell recovery step for two hours at 13ºC provided a good correlation between plates and “live cells”. In further studies, this procedure of removing DNA from dead cells should also be applied to DGGE and direct cloning of

the amplified ribosomal region to prevent the amplification of the DNA of dead cells. In this quantification, the detection limits of EMA- and PMA-QPCR decreased to 1 cell/ml by sample concentration. Interference of the matrix was tested, but it did not have any effect. Both techniques should also be applied to the detection and quantification of different wine bacteria species so as to provide more in-depth knowledge of the microbial aspects of the process.

Finally, it is important to underline that all the culture-independent techniques which use amplification may have associated biases. Firstly, wine contains such inhibitors as phenols, polysaccharides and pigments and their presence has been reported as interfering the PCR process. This could reduce the effectiveness of the amplification (Tessonniere et al., 2009). Although Hierro et al. (2006) showed small differences in the standard curves obtained by using culture media or wine, our results showed that these compounds did not interfere at all, even when samples concentrated 50-fold were used. Secondly, the efficiency in the DNA purification and amplification of the different species may not be the same. And finally, inter-specific genomic differences such as variations in the copy number of the ribosomal region may produce errors. To prevent them, it is recommendable to have electrophoretic patterns (for the DGGE technique) or standard curves (for the QPCR) with the same media in which the analysed species are likely to be found.

*- Oenological applications*

One of the main objectives of this thesis was to use these methods to analyse the population dynamics of microbiota during winemaking. DGGE and QPCR techniques were used to monitor a variety of wine fermentations, and the effect of various oenological practices, such as the addition of sulphur dioxide and yeast inoculation, was studied. In the same winery, other members of our group, Constantí et al. (1998) and González et al. (2005), studied the effect of these practices upon yeast and AAB population dynamics, respectively, although they used culture-dependent techniques. Their results showed that these practices restricted the growth of indigenous yeasts and bacterial populations. Our results, however, were obtained with culture-independent techniques and did not show the same population dynamics: non-*Saccharomyces* species and AAB were not suppressed by these practices, their growth was merely restricted. Sulphur dioxide, then, modified the physiology and/or the state of the microorganisms but it did not kill them. This was also observed by Cocolin & Mills

(2003) when they studied the effect of sulphur dioxide using DGGE with DNA and RNA as a target for yeast species. Both yeast inoculation and sulphur dioxide kept the lactic acid bacteria populations at very low levels. Meanwhile, acetic acid bacteria were only slightly affected by these two practices, as indicated by QPCR and DGGE. The former showed that the population was between $10^4$-$10^5$ cells/ml, and the latter that two species of AAB were present throughout the process. A good correlation between both techniques was obtained for AAB detection. The same cannot be said of yeasts; whereas *S. cerevisiae* was present throughout the process with both techniques, *Hanseniaspora* was only detected with QPCR, and DGGE only detected *Hanseniaspora* the first day of fermentation, when *S. cerevisiae* did not reach the maximum population, according to QPCR.

Direct cloning of the amplified ribosomal region of yeasts was applied together with DGGE and QPCR to study the population dynamics of different microorganisms during fermentations at different temperatures (low and standard temperatures). The results were also compared with those of culture-dependent techniques. The fermentation temperature has been reported to affect the ecology of wine fermentation. Sharf & Margalith (1983) suggested that *H. uvarum* was more capable than *S. cerevisiae* of growing at lower temperatures, and Heard & Fleet (1988) showed that *H. uvarum* and *C. stellata* retained high populations until the end of fermentations conducted at low temperatures. Finally, Ribéreau-Gayon et al. (2006) reported that LAB and AAB populations were reduced by low-temperature fermentations. However, our results did not show the same population dynamics when we used culture-independent techniques. In this case, the population of *Hanseniaspora* was as expected: it decreased throughout the process and at lower temperatures numbers were higher. At the end of both fermentations, *Hanseniaspora* reached population sizes around $10^5$ cells/ml. Meanwhile, the population of *C. zemplinina* stayed constant and only decreased at the end of fermentation, achieving populations higher than $10^6$ cells/ml. *C. zemplinina* presented the highest populations in the fermentation conducted at 25ºC throughout the process, while at low fermentation temperatures the population was highest at the end. However, *C. zemplinina* seems to be well adapted to the must fermentation medium, as is *S. cerevisiae*.

The use of culture-independent techniques showed a high presence and permanence of non-*Saccharomyces* yeasts and bacteria species throughout the wine

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fermentation, not only in the first days of fermentation. We found a permanent population of AAB species, despite the anaerobic conditions of the alcoholic fermentation. Our results agree with those obtained by Millet & Lonvaud-Funel (2000) and Bartowsky & Henschke (2008).

These techniques have also been applied to analyse yeast dynamics in media that had been inoculated with a mix of different yeasts. Mixed yeast inoculation has been proposed as an oenological practice to increase the complexity of wines (Ciani et al., 2010), although questions about the survival of the different species and the control of the population dynamics still need to be answered (Mills et al., 2008). It has always been assumed that *S. cerevisiae* takes over the fermentation soon after it starts, so the survival of the non-*Saccharomyces* yeasts is restricted in the very early phases of the fermentation (Fleet, 2003). However, our previous results contradicted these expectations and several fermentations were set up to analyse the population dynamics of different yeast species used as inocula in both pure and mixed fermentations. One fermentation was conducted with natural must and the other with synthetic must. The former contained the grape precursors and low nitrogen sources; the latter was a standard medium. The fermentation capacity of the different species studied was very different. *C. zemplinina* ended the fermentation with a slight delay compared with *S. cerevisiae*, but the *H. uvarum* pure culture was unable to finish it. The mixed fermentations also had different characteristics. In all cases, the non-*Saccharomcyes* were not completely taken over and it must be assumed that they were present throughout the fermentations and definitely contributed to the quality of the final wine.

When EMA- or PMA-QPCR techniques were applied to monitor wine fermentations, the results were different from those obtained without these dyes treatment, showing that not all the cells present during the alcoholic fermentation were alive. Thus, the results mentioned above should be revised. Meanwhile, the evolution of *H. guilliermondii* and *S. cerevisiae* in a synthetic medium followed by FISH, QPCR and plating showed that *H. guilliermondii* lose their ability to form colonies despite the continuing high levels of RNA and DNA. Quantification by QPCR and FISH showed no significant differences. Finally, EMA- and PMA-QPCR techniques were also applied to quantify different wine yeast species in ageing and stored wines; in this case the results showed the same population of yeasts quantified by QPCR with or without the dye treatment, indicating that all the cells were alive, although not all of them could form a

colony. As far as we know this was the first time that this technique had been applied to monitor different wine microorganisms during alcoholic fermentation.

*- Impact of wine microorganisms conducting the fermentation*

Wine is the result of the interaction of different yeast species, in particular the interaction of non-*Saccharomyces* yeasts and *Saccharomyces* spp. Romano et al. (2003) postulated that it is advantageous to formulate and use mixed starter cultures. In our study, we concluded that *C. zemplinina* produce higher glycerol content than *S. cerevisiae*, as previously described (Ciani & Ferraro, 1996, 1998; Ferraro et al., 2000). That was not the only change, because also the production of 2-methyl-1-propanol was higher and of phenylethyl acetate lower. Instead, *H. uvarum* produced higher quantities of isoamyl acetate (Moreira et al., 2008). In the present study we found that, in general, non-*Saccharomyces* species increased the production of higher alcohols, their acetates and fatty acids. Pure non-*Saccharomyces* either do not ferment well or produce an excess of some volatile compounds, but mixed cultures presented good fermentation rates and lower concentrations of volatile compounds. Although more experiments are needed, particularly on an industrial scale, the present study shows that mixed fermentations are an alternative for conducting alcoholic fermentations. Furthermore, the initial must seems to be very important in the production of volatile compounds. Amino acids in the must are the precursors of higher alcohols. Hernandez-Orte et al. (2002) demonstrated that there is a close relationship between the amino acid composition of must and the final content of important volatile compounds in wine.

Finally, the consumption of amino acids depends on their availability in the medium and the yeast species conducting the fermentation. *S. cerevisiae* are known to consume a variety of nitrogen sources (Magasanik & Kaiser, 2002; Beltran et al., 2004). A particularly important observation of the present study was the dramatic difference between *S. cerevisiae* and the other non-*Saccharomyces* species in the use of nitrogen sources. *S. cerevisiae* was very efficient at using nitrogen, whereas the non-*Saccharomyces* species needed larger amounts of nitrogen to build the same biomass. In mixed cultures, and because of the greater presence of non-*Saccharomyces* species, the nitrogen/biomass efficiency was also lower than that of *S. cerevisiae*. Because nitrogen is limited in natural grape musts, the competition of the different species for the nitrogen available could be an additional factor that explains the prevalence of *S. cerevisiae* in natural alcoholic fermentations.

*- Impact of viable but non-culturable microorganisms in wine*

It is not clear what effect these viable but non-culturable microorganisms have on alcoholic fermentation. Microorganisms can be present in different physiological states during the different stages of winemaking. These physiological states can vary from fully alive to almost dead, and their different metabolic activities could easily alter the fragile status of a wine by releasing uncontrolled metabolites. There is a strong need to carry out research in these "late-live" statuses, which can play an important role in the last phases of wine maturation, ageing, bottling and storing prior the consumption. This research could focus on cell state and how to determine the state in different phases, in order to understand the impact the cells might have on the final product. In the last part of this study, in which a mixed fermentation of *H. guilliermondii* and *S. cerevisiae* was analysed, FISH showed that the population of *H. guilliermondii* was a little higher than *S. cerevisiae* throughout the fermentation.

To sum up, culture-independent techniques need to be developed to control the microbiota associated to wine production. If the microorganisms are not previously cultured, results are obtained faster and the microbial population can be better understood. EMA- or PMA-QPCR applied to wines and wine fermentations may prevent the DNA from dead cells from being included as live cells. A combination of EMA- or PMA-QPCR, FISH and flow cytometry, then, may be the best options for simultaneously analysing and quantifying a given population. Nevertheless, culture-independent techniques, and especially those that can determine the cell physiological status, are useful for diversity studies. One possible solution could be to combine some of the modifications mentioned above (EMA, PMA) with DGGE. This technique can be improved also by designing primers to prevent the exclusive amplification of the dominant species, like *S. cerevisiae*.

New techniques to differentiate between live and dead microorganisms have been developed or adapted so that they can be used to better control the wine production process. Further research is under way, not only to understand the identity of the species responsible for wine spoilage or wine character, but also to quantify their spoiling or production capacity. Identifying the species or strains present in a wine fermentation, then, and also the presence or absence of a particular enzyme can be good tools for controlling the quality of wine. This could be done at different levels: enzyme activity, gene presence or gene expression. Perhaps the best level is gene

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expression which can easily be quantified by QPCR. Other new technologies such as pyrosequencing, metagenomics, etc. might be applied in winemaking to have better microbiological control of the overall process.

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# GENERAL CONCLUSIONS

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# GENERAL CONCLUSIONS

## BIOTECHNOLOGICAL CONCLUSIONS

1. Culture-independent techniques enable different wine yeasts and bacteria species to be rapidly detected and quantified without the selectivity of growing cells in a culture medium.

2. The main advantage of QPCR is the identification and quantification with a high specificity and sensibility. Its main limitation is the availability of specific primers. If it is combined with the DNA binding dyes EMA or PMA viable yeasts species can be rapidly identified and quantified.

3. DGGE is a good tool for carrying out diversity studies for yeasts, and acetic and lactic acid bacteria. The main problem is the detection limit of minor species in mixed populations. This could be overcome by designing specific primers that exclude the majority species.

4. The direct cloning of amplified ribosomal genes of yeasts shows a greater diversity of species than DGGE with a semi-quantitative estimation. The main drawback is that it is time consuming.

5. FISH was successfully applied for *S. cerevisiae* and *H. guilliermondii*. In combination with Flow Cytometry different microorganisms can be quantified in the same analysis in a short time and the different physiological states of wine yeasts can be determined.

UNIVERSITAT ROVIRA I VIRGILI
ADAPTATION AND DEVELOPMENT OF CULTURE-INDEPENDENT TECHNIQUES FOR THE INDENTIFICATION AND ENUMERATION
OF MICROORGANISMS IN WINE FERMENTATIONS
Immaculada Andorrà Solsona
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**OENOLOGICAL CONCLUSIONS**

1. Culture-independent techniques showed a higher presence and permanence of non-*Saccharomyces* species and acetic acid bacteria during the fermentation.

2. Inoculation and the addition of sulphur dioxide did not suppress the growth of non-*Saccharomyces* yeasts species or acetic acid bacteria. Only lactic acid bacteria were affected by these practices.

3. The consumption of different nitrogen sources is affected by the presence of different species. *S. cerevisiae* is extremely efficient at using nitrogen to develop high populations and this could be critical in taking over the alcoholic fermentation.

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# ANNEXES

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# ANNEX 1

## 1. Culture media

### 1.1. YPD

Yeast Extract Peptone Dextrose. General medium to grown yeast

| | | |
|---|---|---|
| Glucose | 20 g/L | distilled water |
| Peptone | 20 g/L | |
| Yeast Extract | 10 g/L | |

This medium could be liquid or solid adding 20 g/L of agar

Autoclave at 121ºC for 15 min.

### 1.2. LYS (Angelo & Siebert, 1987)

This medium supports the growing of non-*Saccharomyces* yeast. *Saccharomyces* yeast could not grow in a medium which unique source of Nitrogen is Lysine. Then, this is fairly used to distinguish between *Saccharomyces* and non-*Saccharomyces* yeast.

| | | |
|---|---|---|
| Lysine medium | 66 g/L | distilled water |
| Lactate potassium | 4 ml/L | |

Bring to boil this medium with constant agitation, to avoid any overheating.

When the medium is around 50ºC add 1 ml lactic acid 10% to adjust the pH at 5.

Proceed to distribute in plates, around 20 ml per plate.

| | |
|---|---|
| Lactate potassium | 18 ml lactic acid 85% |
| | 14 g KOH |

### 1.3. MRS

This is the general medium to grow lactic acid bacteria.

| | | |
|---|---|---|
| MRS medium | 55 g/L | distilled water |
| DL-Malic | 6 g/L | |
| Fructose | 5 g/L | |
| Cysteine | 0,5 g/L | |

This medium could be liquid or solid (adding 20 g/L of agar).

Autoclave at 121ºC for 15 min.

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### 1.4. GY

Glucose media. This is general to grow acetic acid bacteria.

| | | |
|---|---|---|
| Glucose | 10 g/L | distilled water |
| Yeast Extract | 10 g/L | |

3% Calcium carbonate can be added to be able to diferentiate the acid production for AAB forming a halo surrounding the AAB colony.

This medium could be liquid or solid (adding 20 g/L of agar).

Autoclave at 121ºC for 15 min.

## 2. Synthetic must

| | | |
|---|---|---|
| Glucose | 100 g/L | distilled water |
| Fructose | 100 g/L | |
| DL-malic acid | 5 g/L | |
| Citric acid | 0.5 g/L | |
| Tartaric acid | 3 g/L | |
| $KH_2PO_4$ | 0.75 g/L | |
| $K_2SO_4$ | 0.5 g/L | |
| $MgSO_4\ 7H_2O$ | 0.25 g/L | |
| $CaCl_2\ 2H_2O$ | 0.16 g/L | |
| NaCl | 0.2 g/L | |
| $NH_4Cl$ | 0.47 g/L | |

Autoclaved at 121ºC for 15 min, once autoclaved add the next solution previous sterilized;

| | |
|---|---|
| Amino acid solution | 13.09 ml/L |
| Oligoelements solution | 1 ml/L |
| Vitamines solution | 10 ml/L |
| Anaerobiosi factors | 1 ml/L |

Adjust pH 3.3 (with NaOH 10 M)

- Amino acid solution

| | | |
|---|---|---|
| Tyrosine (Tyr) | 1.5 g/L | (heat at 100ºC) |
| Tryptophan (Trp) | 13.4 g/L | (70ºC) |
| Isoleucine (Ile) | 2.5 g/L | (70ºC) |

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| | | |
|---|---|---|
| Aspartic acid (Asp) | 3.4 g/L | (degas $CO_2$) |
| Glutamic acid (Glu) | 9.2 g/L | (degas $CO_2$) |
| Arginine (Arg) | 28.3 g/L | |
| Leucine (Leu) | 3.7 g/L | |
| Threonine (Thr) | 5.8 g/L | |
| Glycine (Gly) | 1.4 g/L | |
| Glutamine (Gln) | 38.4 g/L | |
| Alanine (Ala) | 11.2 g/L | |
| Valine (Val) | 3.4 g/L | |
| Methionine (Met) | 2.4 g/L | |
| Phenylalanine (Phe) | 2.9 g/L | |
| Serine (Ser) | 6.0 g/L | |
| Histidine (His) | 2.6 g/L | |
| Lysine (Lys) | 1.3 g/L | |
| Cysteine (Cys) | 1.5 g/L | |
| Proline (Pro) | 46.1 g/L | |

Sterilization by filtration, divided into aliquota and keep at -20ºC.

- Oligoelements solution

| | |
|---|---|
| $MnSO_4$ $H_2O$ | 4 g/L |
| $ZnSO_4$ $7H_2O$ | 4 g/L |
| $CuSO_4$ $5H_2O$ | 1 g/L |
| KI | 1 g/L |
| $CoCl_2$ $6H_2O$ | 0.4 g/L |
| $H_3BO_3$ | 1 g/L |
| $(NH_4)_6Mo_7O_{24}$ | 1 g/L |

Sterilization by filtration, divided into aliquota and keep at 4ºC.

- Vitamines solution

| | |
|---|---|
| Myo-inositol | 2 g/L |
| Pantothenate calcium | 0.15 g/L |
| Thiamine hydrochloride | 0.025 g/L |
| Nicotinic acid | 0.2 g/L |
| Pyridoxine | 0.025 g/L |

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Biotine 3 ml/L from 100 mg/ml

Sterilization by filtration, divided into aliquota and keep at -20ºC.

- Anaerobiosis factors

| | |
|---|---|
| Ergosterol | 1.5 g |
| Oleic acid | 0.5 ml |
| Tween 80 | 50 ml |
| Ethanol | up to 100 ml |

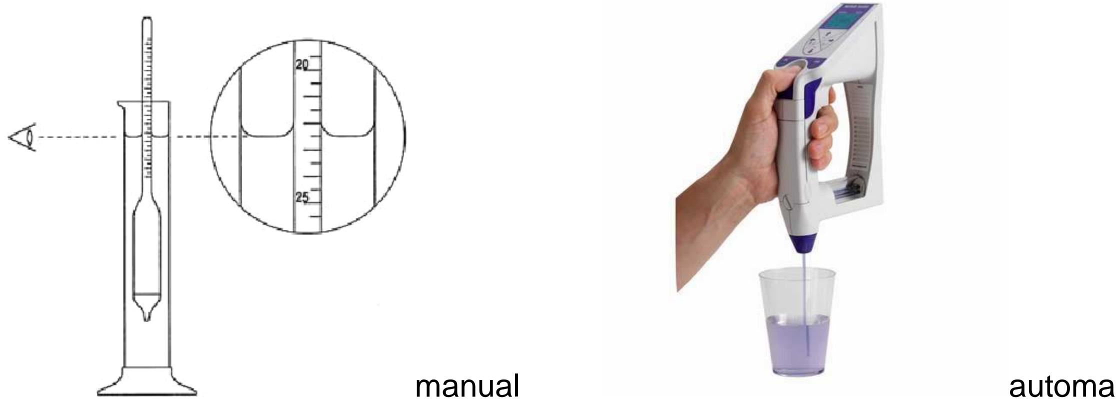
Heat the solution at 70ºC to dissolve

Maintain this solution at 4ºC in aliquots.

**3. Monitoring wine fermentations**

**3.1. Density**

During wine fermentation yeasts metabolize sugars; the decrease of sugars is directly proportional to the decrease of density. This is the method most used to follow the alcoholic fermentation, because of the simplicity of this technique. There are different methods to measure the density, using manual or automatized densimeters.




manual automatized

**3.2. Optical density**

The spectophotometer measure the absorbance at 600 nm. This absorbance is directly proportional to the biomass formed by microorganisms. The cells in a medium have the capability to disperse the light, this make to the medium change their turbidity, being more turbid. The absorbance can be directly related to the biomass although standard curves have to be created for each microorganism.

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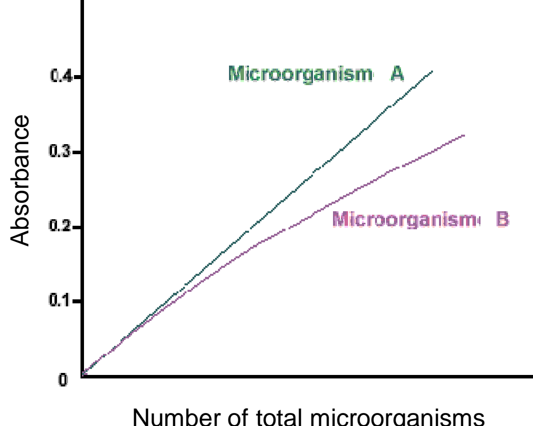
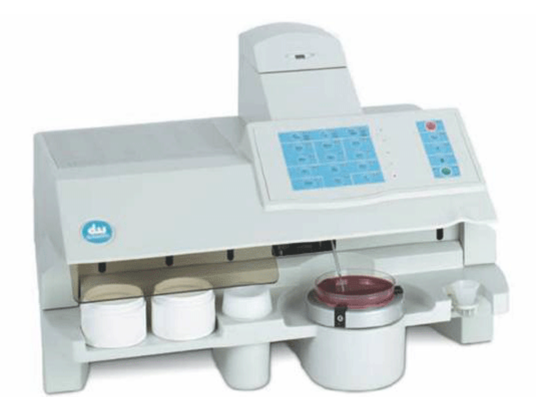



### 3.3. Plate counting

Different microorganisms will plated in a medium and incubated during specific time and conditions, after this incubation time each cell will form a colony and counting the colonies will be obtained the number of colony form units (UFC/ml).

For plating, in this thesis, was used a Whitley Automatic Spiral Plating (AES Laboratoire, France), and for counting the colonies was used and automatic colony counter ProtoCol HR (Microbiology international, USA).



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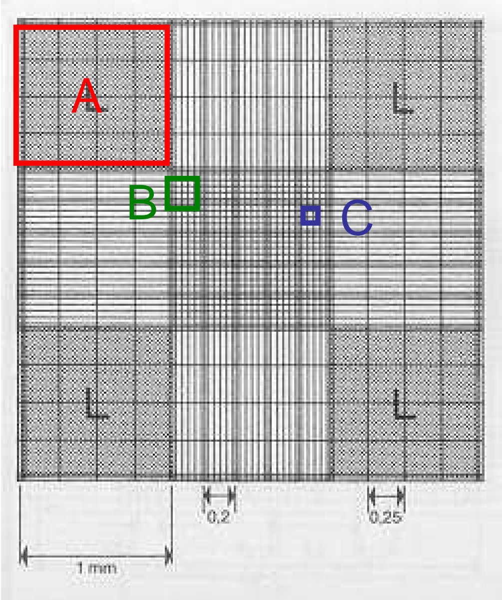
### 3.4. Microscope counting

The number of total microorganisms can be determined counting the cells with a microscope and a Neubauer chamber. Following there is a representation of a Neubauer chamber, with the different squares which can be used to count the cells.




Relation among different squares,
A = 16 B
B = 25 C

Profundity = 0.1 mm

Volume A = $1 \times 1 \times 0.1 = 0.1$ mm$^3$ = $10^{-4}$ ml
Volume B = $0.25 \times 0.25 \times 0.1 \times 10^{-3} = 6.25\ 10^{-6}$ ml
Volume C = $0.05 \times 0.05 \times 0.1 \times 10^{-3} = 2.5\ 10^{-7}$ ml

Equation

(Cells number) / (Square number x Square volume x dilution) = number of cells/ml

**4. DNA Extraction of wine microorganism by mini plant kit (Qiagen)** (Hierro et al., 2006)

1. Collect 1ml of a homogeneous cell solution. Centrifuge 10 minuts at 12000 rpm.
2. Wash the pellet with 1000 ml of distilled water. Centrifuge 10 minuts at 12000 rpm.
3. Cells are resuspended in lysis Buffer AP1. The solution is transferred into a microcentrifuge tube of 2 ml with 1 g of glass bits with 0.5 mm of diameter.
4. The wall of yeasts is broken with a high agitation in the mini bead-beater (Biospec Products Inc., Bartlesville, Okla.), this agitation is conducted during 1 minut a max. velocity then 1 minut in ice, and this is repeated 3 times, to avoid an overheating of the sample.
5. Centrifugate for 1 min at 10000 rpm.
6. Transfer the solution (cells disrupted + Buffer AP1) to an eppendorf (aprox. 400 µl) and 4 µl RNasa A. Vortex and incubate for 10 min at 65ºC. Invert tube 2-3 times during incubation.
7. Add 130 µl Buffer AP2. Mix and incubate for 5 min on ice.
8. Centrifugate the lysate for 5 min at 14000 rpm at 4ºC.

9. Pipet the lysate into a QIAshredder Mini spin column in a 2 ml collection tube. Centrifugate for 2 min at 14000 rpm.
10. Transfer the flow-through fraction (450 µl aprox.) into a new tube without disturbing the pellet. Add 675 µl of Buffer AP3/E and mix by pipetting.
11. Transfer 650 µl of the mixture into a DNeasy Mini spin column in a 2 ml collection tube. Centrifuge for 1 min at 10000 rpm. Discard the flow-through. Repeat this step with the remaining sample.
12. Place the spin column into a new 2 ml collection tube. Add 500 µl Buffer AW, and centrifuge for 1 min at 10000 rpm. Discard flow-through.
13. Add another 500 µl Buffer AW. Centrifuge for 2 min at 14000 rpm.
14. Transfer the spin column to a new 1.5 ml microcentrifuge tube, and add 100 µl Buffer AE for elution. Incubate for 5 min at room temperature. Centrifuge for 1 min at 10000 rpm.

## 5. Electrophoresis

### 5.1. Solutions

| | | |
|---|---|---|
| Buffer TBE 10 X | Tris base | 108 g/L |
| | Boric acid | 55 g/L |
| | EDTA | 7.5 g/L |

Adjust the pH at 8 with NaOH

Loading buffer
- 0.10 % (p/v) bromophenol blue
- 50 % (v/v) glycerol
- 10% (v/v) TBE 10 X
- 40 % (v/v) distilled water

Agarosa MP (Boehringer Mannheim)

Etidium Bromide (Fluka, Sigma)

Molecular ladder normaly used 100 pb DNA (Invitrogen)

### 5.2. Procedure

Measure the agarose to create the gel, higher the agarose concentration higher the retention of the fragments. Normally we use 1% p/v and to increase the separation between different bands we use 2-3 % p/v.

Dissolve the agarose in buffer TBE 1 X by heating. Carefully bring the solution just to the boil to dissolve the agarose. Let the solution cool down to about 60 °C. Add etidium bromide to a final concentration of 0.4 µg/ml, and pour the solution it into the gel rack. Insert the comb at one side of the gel, about 5-10 mm from the end of the gel. When the gel has cooled down and become solid, carefully remove the comb. The holes that remain in the gel are the wells or slots. Put the gel, together with the rack, into a tank with TBE 1 X. The gel must be completely covered with TBE 1 X, with the slots at the end electrode that will have the negative current. Then the sample and the DNA ladder have to be injected, first the sample has to be mixed with 2-4 µl of charge buffer. Then the voltage is adjusted, normally between 60-90 V and finally the gel is observed through a transiluminator UV system. The image was captured using MiniBis Pro (DNR Bio-Imaging Systems Ltd., Israel).

**6. Molecular techniques**

**6.1. RFLP rDNA** (Guillamón et al., 1998)

This technique was applied to identify yeasts species. Consist first in an amplification of the region comprised between the 18S and 26S rDNA gene. And latter a digestion with several restriction enzymes.

The first stage of this technique is the amplification, the primers used are,

ITS1 5'- TCCGTACGTGAACCTGCGG - 3'

ITS4 5'- TCCTCCGCTTATTGATATGC - 3'

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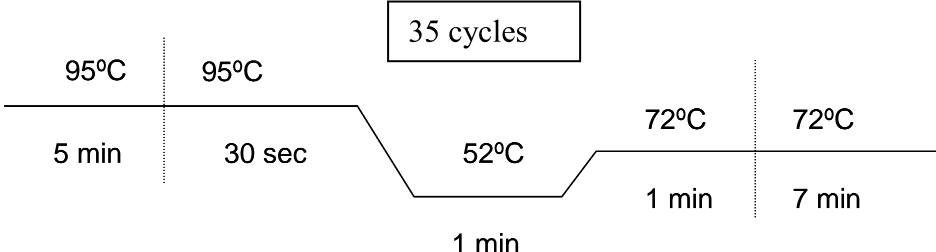
The mix for the amplification is done in 50 µl of total volume containing,

| | |
|---|---|
| Primer ITS1 (10 µM) | 1 µl |
| Primer ITS4 (10 µM) | 1 µl |
| dNTPs (40 mM) | 4 µl |
| $MgCl_2$ (50 mM) (Ecogen) | 3 µl |
| Buffer Taq 10x without Mg. (Ecogen) | 5 µl |
| *Taq* DNA polymerasa (Ecotaq, Ecogen) (5 U/ µl) | 0.5 µl |
| $H_2O$ milli-Q | 33 µl |
| DNA | 2.5 µl |

The PCR conditions are,




Once obtained the amplified fragment, the fragment can be tested using a gel electrophoresis (1% p/v), after it is done the digestion. This can use different restriction enzymes. In this work was used, *Cfo*I, *Hae*III, *Hin*fI, *Dra*I (Roche Diagnostics). The mix used to conduct the digestion is in 20 µl of total volume.

| | |
|---|---|
| Enzyme | 1 µl |
| Specific buffer for each enzyme | 2 µl |
| $H_2O$ milli-Q | 9 µl |
| Amplified DNA | 8 µl |

This reaction was conducted overnight at 37ºC. Finally the product is carried in an eletrophoresis gel (3% p/v) to conduct the separation of the digestion bands. To characterize each species every gel need a molecular weight marker (MWM), normally we used 100 pb. Each species is characterized by the digestion bands and the amplified bands comparing with a known amplified and restriction bands belonging to specific species. The restriction patterns to conduct the identification were listed in Guillamón et al. (1998) and Esteve-Zarzoso et al. (1999).

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### 6.2. Sequencing of the region D1/D2 from rDNA (Kurtzman & Robnett, 1998)

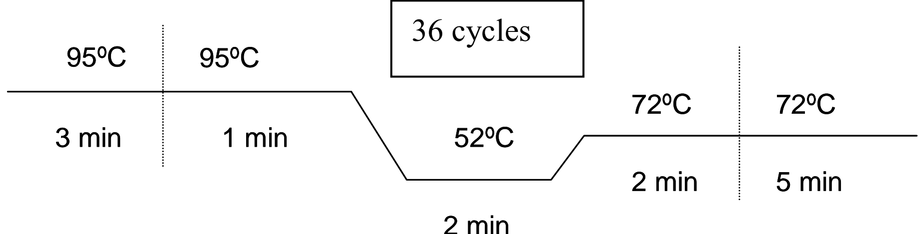
This technique was used to identify species.This technique consists in an amplification and sequencing. The primer used for the amplification was,

| | |
|---|---|
| NL-1 | 5'- GCATATCAATAAGCGGAGGAAAAG - 3' |
| NL-4 | 5'- GGTCCGTGTTTCAAGACGG – 3' |

The mix of the amplification contains in 50 µl of total volume,

| | |
|---|---|
| Primer NL-1 (10 µM) | 1 µl |
| Primer NL-4 (10 µM) | 1 µl |
| dNTPs (40 mM) | 1 µl |
| $MgCl_2$ (50 mM) (Ecogen) | 2.5 µl |
| Buffer Taq 10x, without Mg. (Ecogen) | 5 µl |
| *Taq* DNA polymerasa (Ecotag, Ecogen) (5 U/µl) | 0.5 µl |
| $H_2O$ milli-Q | 37 µl |
| DNA | 2 µl |

The PCR programmed was,




Finally this amplified is sent to purified and sequencing by Macrogen Inc. facilities (Seoul, Korea) using an ABI3730 XL automatic DNA sequencer. The result sequence is compared with the data base sequences European Molecular Biology Laboratory (EMBL) by BLAST (Basic Local Aligment Search Tool).

### 6.3. Q-PCR

This technique is used to quantify exactly the population of different yeasts species and lactic and acetic acid bacteria. The primers designed for this technique were,

-The primers for general yeasts were designed in the region D1/D2 26S gene (Hierro et al., 2006)

YEAST-F 5'-GAGTCGAGTTGTTTGGGAATGC-3'
YEAST-R 5'-TCTCTTTCCAAAGTTCTTTTCATCTTT-3'

The amplicon obtained is 124 pb

- The primers for *S. cerevisiae* were designed in the region ITS2 and 5,8S gene (Hierro et al., 2007)

CESP-F 5'-ATCGAATTTTTGAACGCACATTG-3'
SCER-R 5'-CGCAGAGAAACCTCTCTTTGGA-3'

The amplicon obtained is 175 pb

- The primers for *Hanseniaspora* were designed in the region ITS2 and 5,8S gene (Hierro et al., 2007)

CESP-F 5'-ATCGAATTTTTGAACGCACATTG-3'
HUV-R 5'-AACCCTGAGTATCGCCCACA -3'

The amplicon obtained is 121 pb

- The primers for *C. zemplinina* were designed in the region D1/D2 of the 26S gene (Andorrà et al., 2010)

A-F 5'-CTAGCATTGACCTCATATAGG-3'
200-R 5'-GCATTCCCAAACAACTCGACTC-3'

The amplicon obtained is 60 pb

- The primers for *D. bruxellensis* were designed in the region D1/D2 of the 26S gene (Phister & Mills, 2003)

DBRUX-F 5'-GGATGGGTGCACCTGGTTTACAC-3'
DBRUX-R 5'-GAAGGGCCACA TTCACGAACCCCG-3'

The amplicon obtained is 79 pb

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- The primers for *Z. bailii* were designed in the region D1/D2 of the 26S gene (Rawsthorne & Phister, 2006)

ZBF1 5′-CATGGTGTTTTGCGCC-3′
ZBR1 5′-CGTCCGCCACGAAGTGGTAGA-3′

The amplicon obtained is 122 pb

- The primers for lactic acid bacteria were designed in the region 16S V4 and V5 (López et al.,2003)

WLAB1 5'-TTCGGATTTATTGGGTATTCACCGCG-3'
WLAB2 5'-TCGAATTAAACCACATGCTCCA-3'

The amplicon obtained is 400 pb

- The primers for acetic acid bacteria were designed in the region 16S rDNA (Gonzalez et al., 2006)

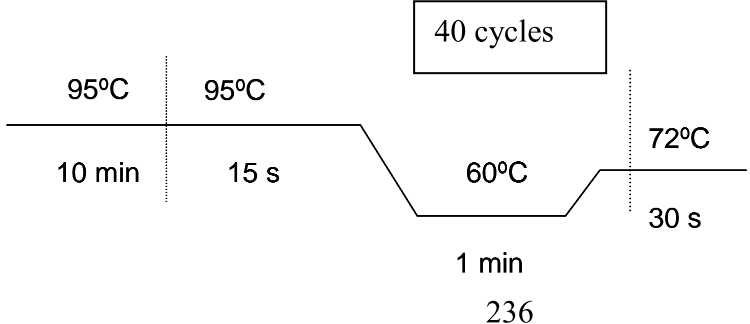
QA1-F 5'-TCAAGTCCTCATGGCCCTTATG-3'
QA2-R 5'-CGCCATTGTAGCACGTGTGTA-3'

The amplicon obtained is 55 pb

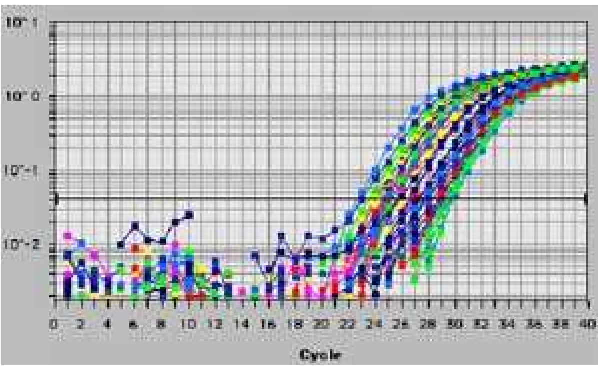
The PCR reaction is done in a total volume of 25 µl with,

| | |
|---|---|
| Power SybrGreen Master Mix | 12.5 µl |
| Forward primer (7 µM) | 0.75 µl |
| Reverse primer (7 µM) | 0.75 µl |
| DNA | 5 µl |
| Distilled water | 6 µl |

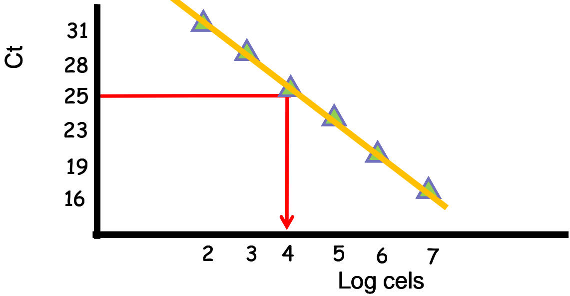
The QPCR conditions are,



In this thesis Power SyberGreen (Applied Biosystems, USA) was used as a binding agent, this binds to the double-chained DNA. The QPCR measure the fluorescence signal emited by Power SyberGreen at the end of each cycle. The information obtained is represented as an amplification curve that provides the cycle number for which the intensity of donor emission increases compared with the background noise. This cycle number is called the cycle threshold (Ct) and is inversely proportional to the number of copies of the sample, thus it can be used to evaluate the initial quantity of sample numerically (DNA or cells) with great precision.




To determine the number of cells or DNA is necessary to create standard curves by plotting the Ct versus the concentration of the cells or DNA, this is made with some dilutions of the cells with a known DNA concentration or number of cells. For each concentration of DNA or number of cells the QPCR gives a Ct, this value will be used to create the standard curves for each pair of primers.




### 6.3.1. EMA/PMA QPCR

Before the DNA extraction was done the binding of dyes to the DNA from dead cells to avoid the quantification of this DNA using the QPCR. The procedure was,

1. Collect 1 ml of a homogeneous cell solution

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2. Centrifuge 10 min at 12000 rpm. Discard the supernatant and add 1 ml of sterile distilled water.
3. Add EMA (final concentration 24 μM) or PMA (6 μM) and maintain the solution in dark during 10 min.
4. Two exposures for 30 seconds on the light (650 W halogen lamp at 20 cm from the samples), with an interval of 1 minut in ice were used for photolysis.
5. Centrifuge 10 min at 12000 rpm. Discard the supernatant to carcinogenic compounds. Add 1 ml of sterile distilled water to eliminate the excess of the dyes unbound.
6. Centrifuge 10 min at 12000 rpm. Discard the supernatant to carcinogenic compounds.
7. Extract the DNA directly by mini plant kit Qiagen and procede with the QPCR.

### 6.4. DGGE

In DGGE fragments of the same length but with different sequences can be separated. Separation of the DNA amplicons is based on the decreased electrophoretic mobility of a partially melted double-stranded DNA molecule in polyacrilamide gels containing a linear gradient of DNA denaturants.

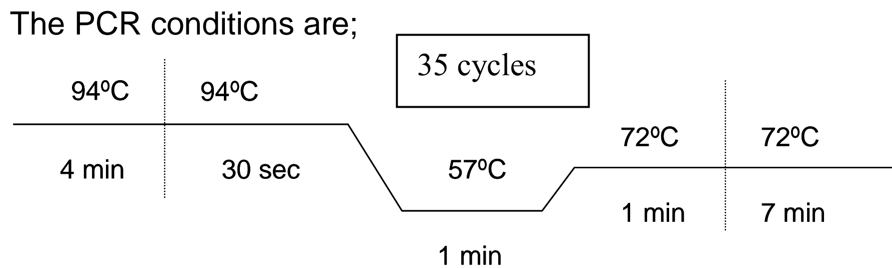
During the first step was made an amplification with a suitable primers, one of them must have a GC clamp (underlined sequence on the primers). The primers used in this thesis were:

- The primers for general yeasts were designed in the 28S rRNA gene. (Meroth et al., 2003a).

U1$^{GC}$ 5'-GCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCC CCGCCCCGTGAAATTGTTGAAAGGGAA-3'

U2 5'-GACTCCTTGGTCCGTGTT-3'

The PCR conditions are;




The amplicon obtained is around 300 pb.

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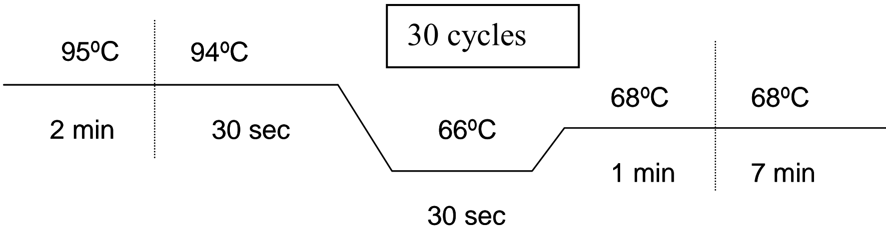
- The primers for lactic acid bacteria were designed in the 16S rRNA gene (V3 region) (Meroth et al., 2003b)

| | |
|---|---|
| $L1^{GC}$ | 5'-GCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCC CCGCCCCCAGCAGTAGGGAATCTTCC -3' |
| HDA2 | 5'- GTATTACCGCGGCTGCTGGCAC -3' |

The PCR conditions are;

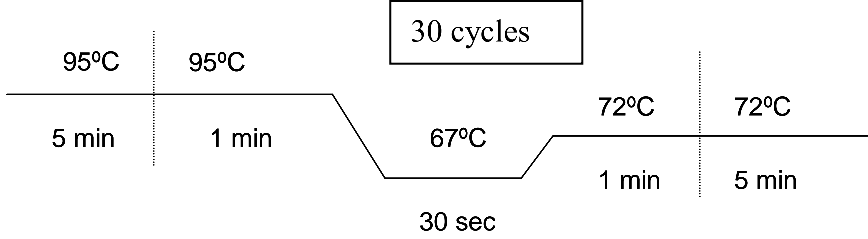



The amplicon obtained is around 185 pb.

- The primers for acetic and lactic acid bacteria were designed in the 16S rRNA gene (V7 and V8 region) (Lopéz et al., 2003).

| | |
|---|---|
| $WBAC1^{GC}$ | 5'- GCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCC CCGCCCCGTCGTCAGCTCGTGTCGTGAGA -3' |
| WBAC2 | 5'- CCCGGGAACGTATTCACCGCG -3' |

The PCR conditions are;




The amplicon obtained is around 320 pb.

The mix for the amplification is done in 50 µl of total volume containing,

| | |
|---|---|
| Primer with GC clamp (5 µM) | 1.5 µl |
| Primer without GC clamp (5 µM) | 1.5 µl |
| dNTPs (40 mM) | 0.1 µl |
| $MgCl_2$ (50 mM) (Ecogen) | 1 µl |
| Buffer Taq 10x, without Mg. (Ecogen) | 5 µl |
| *Taq* DNA polymerasa (Ecotag, Ecogen) (5 U/µl) | 0.25 µl |
| $H_2O$ milli-Q | 39.65 µl |
| DNA | 1 µl |

Once obtained the amplicons the next step is to construct and run the denaturing gradient gel electrophoresis.

### 6.4.1. Solutions for DGGE

| | | |
|---|---|---|
| TAE 50 X | Trizma base | 242 g/L distilled water |
| | Acetic acid glacial | 57.1 g/L |
| | EDTA 0.5 M (pH8) | 100 ml/L |
| | Autoclave 121ºC during 15 min | |
| EDTA 0.5 M pH 8 | EDTA 186.12 g/L distilled water | |
| | Adjust the pH at 8 with NaOH | |
| | Autoclave 121ºC during 15 min | |
| Dye solution | bromophenol blue | 0.05g |
| | xylene cyanol | 0.05g |
| | Buffer TAE 1 X | 10 ml |
| Gel loading dye | dye solution | 2.5ml |
| | glycerol | 7 ml |
| 0% denaturing solution | 40% Acrylamide/BisAcrylamide | 10 ml |
| | 50X TAE | 1 ml |
| | dH20 | to 50 ml |

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| | | |
|---|---|---|
| 100% denaturing solution | urea | 21 g |
| | formamide | 20 ml |
| | 40% Acrylamide/BisAcrylamide | 10 ml |
| | 50X TAE | 1 ml |
| | dH20 | to 50 ml |

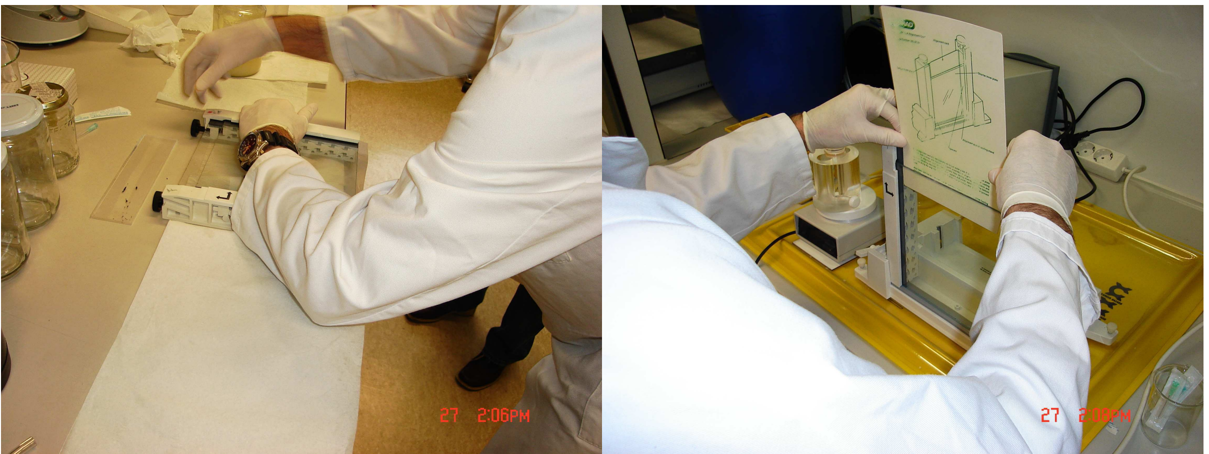
10% Ammonium persulphate (APS). Keep aliquoted and frozen

0.1 g ammonium persulphate in 1 ml dH20

TEMED (N,N,N,N'-tetra-methyl-ethylenediamine)

### 6.4.2. Building the gel assembly

First clean glass plates with ethanol and distilled water. Assemble the gel sandwich by placing the small glass plate on top of the large plate, being sure to correctly place a 1 mm spacer along each edge of the plate assembly. Attach the plate clamps (tight enough to hold everything together) and place the entire assembly into the rear slot of the pouring stand. Loosen the clamps slightly and use the spacing card to assure the proper spacer alignment. Tighten the plate clamps (snug, as if you were trying to prevent "leakage") and remove the plate assembly from the pouring stand. Inspect the plate assembly to ensure that the two glass plates and the spacers form a flush surface across bottom of the assembly. Place a foam gasket into one of the two front slots of pouring stand, insert the plate assembly, and clamp into place. Place the well comb firmly in between the plates.



The acrilamide gel is created with a mixer gradient pump. Both solutions 0 and 100% denaturing solutions are needed, around 20 ml of each solution keep in ice during the gel is built. In each 20 ml solution is added 20 μl TEMED and 200 μl APS. In the 100%

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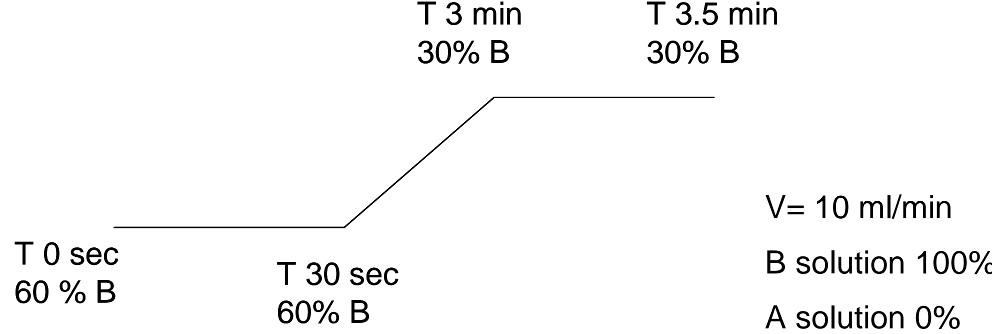
denaturing solution can be added 50 μl of dye solution to see the denaturing gradient when it is created. The gradients pump programme used was:




This denaturing gradient from 30% to 60% was optimitzate to separate different AAB. For LAB and yeasts species the better denaturing gradient was from 32.5% to 50%. Leave the gel for at least 1 hour to polymerize.

### 6.4.3. Run the gel

Add fresh 0.5 x TAE buffer to the buffer tank to the mark “Fill”. Switch on the DCode™ Universal Mutation Detection System (Bio-Rad) at least 60 minutes before electrophoresis, so that the buffer can heat up to 60℃. After polymerisation remove the comb carefully. Rinse the slots to remove non-polymerized gel with 0.5 x TAE buffer by using a syringe and needle. Click the sandwich in the sandwich-holder. There should always be a sandwich (gel or dummy) at the other side to get a closed upper buffer compartment. A dummy consists of a large and small glass plate stuck together with no spacers in between. Load the samples and the marker in the wells, both have to be mixed with the gel loading dye. In each hole can be charged a total volume of 30 μl,

20 μl DNA
10 μl gel loading dye

Take the super-sandwich and let it slide into the buffer tank, with the red dot of the kathode at the right side (buffer tank is positioned with the Bio-Rad mark towards you). The electrophoretic run was keep at a constant 170 V for 4 hours at a constant temperature of 60ºC in TAE buffer 0.5 X.

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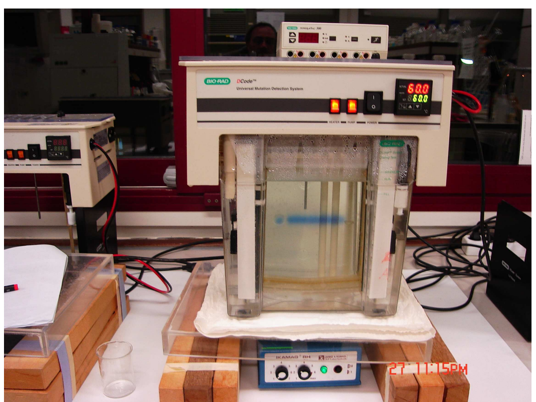

#### 6.4.4. Staining the gel and observation

Once the electrophoretic run is done, the gel is taken out of the tank and the sandwich. The acryamide gel is putted on a tank with ethidium monoazide solution for 15 min. Later on, the gel is visualized on a transillimunator UV and a picture was taken using a MiniBis Pro (DNR Bio-Imaging Systems Ltd.). The bands can be excised using a cutter. Each excised band was then transferred into 50 µl of sterile water and incubated overnight at 4ºC to allow diffusion of the DNA. Then, one microlitre of the eluted DNA can be re-amplified with the same primers although without the GC clamp. The PCR products were purified and sequenced by Macrogen Inc. facilities (Seoul, South Korea) using an ABI3730 XL automatic DNA sequencer.

### 6.5. Cloning 5.8S rDNA

#### 6.5.1. Solutions

| | | | |
|---|---|---|---|
| L- Broth | tryptone | 10 g/L | distilled water |
| | yeast extract | 5 g/L | |
| | NaCl | 5 g/L | |

Autoclave 121ºC during 15 min

LB-Agar plates containing ampicillin, X-gal and IPTG

Prepare the L-broth plus 1.5 % agar and autoclave.

Cool the bottle before add per 200 ml of LB-agar

200 µl of 3% X-gal in DMF (0.15 g in 5 ml DMF, store at -20ºC)

20 µl of 100 mM IPTG (0.12 g in 5 ml sterile distilled water)

200 µl of 100 mg/ml ampicillin (0.5 g in 5 ml sterile distilled water)

Dissolve and filter sterilize. Store aliquots at -20ºC.

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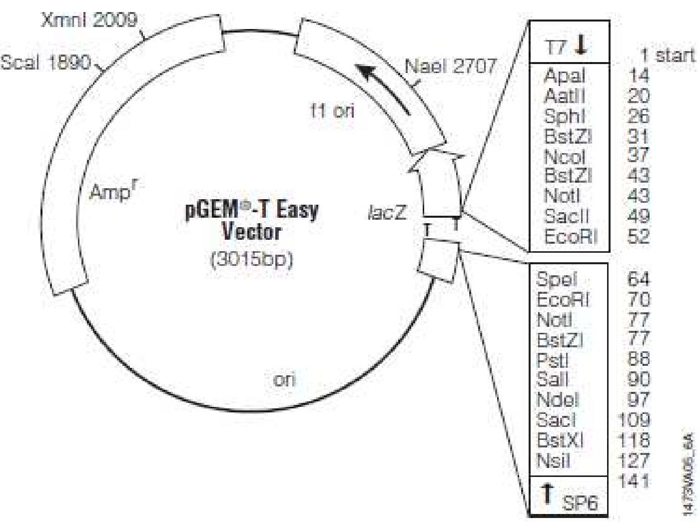
| SOC media | tryptone | 20 g/L | distilled water |
|---|---|---|---|
| | yeast extract | 5 g/L | |
| | NaCl | 0.5 g/L | |
| | KCl 1 M | 2.5 ml/L | |

Adjust pH to 7 with NaOH 10M. Autoclave to sterilize, add 20 ml of sterile 1 M glucose, 10 ml of 1 M $MgSO_4$ and 10 ml of 1 M $MgCl_2$ immediately before use.

### 6.5.2. Transformation *E. coli*

The plasmid used was PGEM-T Easy Vector (Promega Corporation, Madison, WI).




The pGEM®-T Easy Vector has been purchased linearized at base 60 with EcoRV and a T added to both 3´-ends. Then the amplicon have to be done with a Polymerase which generate fragments with 3´A-tailed fragments as the Ecotaq polymerase.

Binding the amplicon and the plasmid

5 µl binding buffer (2 x) (Promega)

1 µl PGEM vector (Promega)

1 µl T4 DNA ligase (Promega)

3 µl amplicon

Maintain 1 hour at room temperature

The amplicons are obtained from PCR amplification, this consist in the primers ITS1-ITS4 and the same procedure than RFLP rDNA. Then, a DNA library is formed containing different amplicons form all the microorganisms present in the sample or from a known DNA.

Previous to the electroporation

Transformate *E. coli* in electrocompetents

1. Inoculate 1ml *E. coli* in 200 ml LB. Growth until DO 0.5-0.6
2. Keep on ice during 15 min
3. Transfer to falcons of 50 ml. Centrifuge 10 min at 5400 rpm 2-4ºC
4. Discard the supernatant and resuspend the pellet in 2 ml of cold H2O
5. Centrifuge 10 min at 5400 rpm 4ºC. Discard the supernatant
6. Repeat the steps 4 and 5
7. Resuspend the pellet in 10 ml of cold glycerol 10%
8. Centrifuge 10 min at 54000 rpm 4ºC
9. Add the same volume of the glycerol 10% than the pellet, around 350 µl
10. Aliquota 45 µl of *E. coli* in eppendorfs
11. Frozen in $N_2$ liquid and maintain at -80ºC

Sterilize the cuvettes 5 min in the UV and maintain in ice until use

Maintain the SOC media in ice

Transformation the plasmid inside the *E.coli* through electroporation

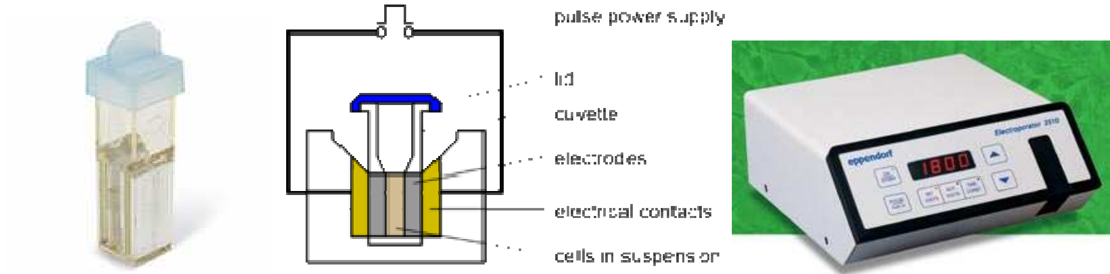
Add 2-3 µl of the plasmid plus the insert into *E. coli* electrocompetents

Electroporate with an Electroporator 2510 (Eppendorf, USA)

Conditions: 1700 Volts for 4 seconds

Resuspend the *E.coli* with 500 µl of SOC medium. Maintain 45 minuts at 37ºC.

Plate the solution into LB-ampicillin-IPTG-Xgal plates



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### 6.5.3. Plasmid extraction (Sambrook et al., 1989)

#### 6.5.3.1. Solutions

- Lysis buffer: Glucose 0.45 g/50 ml distilled water
  Tris-clorhidric 0.197 g/50 ml
  EDTA (0.5 M) 1 ml/50 ml
  Adjust the pH at 8 with NaOH

-0.2 M NaOH/1% SDS: NaOH 0.4 g/50 ml distilled water
  SDS (10 %) 5 ml/50 ml

- 3 M KAc pH 5.5: KAc 14.721 g/50 ml distilled water

- 70% ethanol

Except the ethanol solution, autoclave 121ºC during 15 min.

#### 6.5.3.2. Procedure

1. Inoculate 3 ml of LB (in a tube of 12 ml) with an isolate blue colony and incubate at 37ºC with shaking of 200 rmp
2. Centrifugate 1.5 ml of this cultura at 12000 rpm 5 minuts
3. Discard the supernatant and resuspend the pellet with 100 µl of lysis buffer by vortex
4. Add 200 µl of 0.2 M NaOH/1% SDS, mixture by inversion
5. Add 150 µl of 3 M KAc pH 5.5, mixture by inversion
6. Centrifugate at 12000 rpm during 15 min at 4ºC.
7. Precipitate the supernatant with 250 µl of isopropanol (-20ºC) and mixture
8. Centrifugate at 12000 rpm during 15 min at 4ºC.
9. Discard the supernatant and wash the pellet with 500 µl of etanol 70% (-20ºC)
10. Centrifugate at 12000 rpm during 15 min at 4ºC
11 Discard the supernatant and dry the pellet on the Speedy Vac
12. Resuspend the pellet in 40 µl of TE

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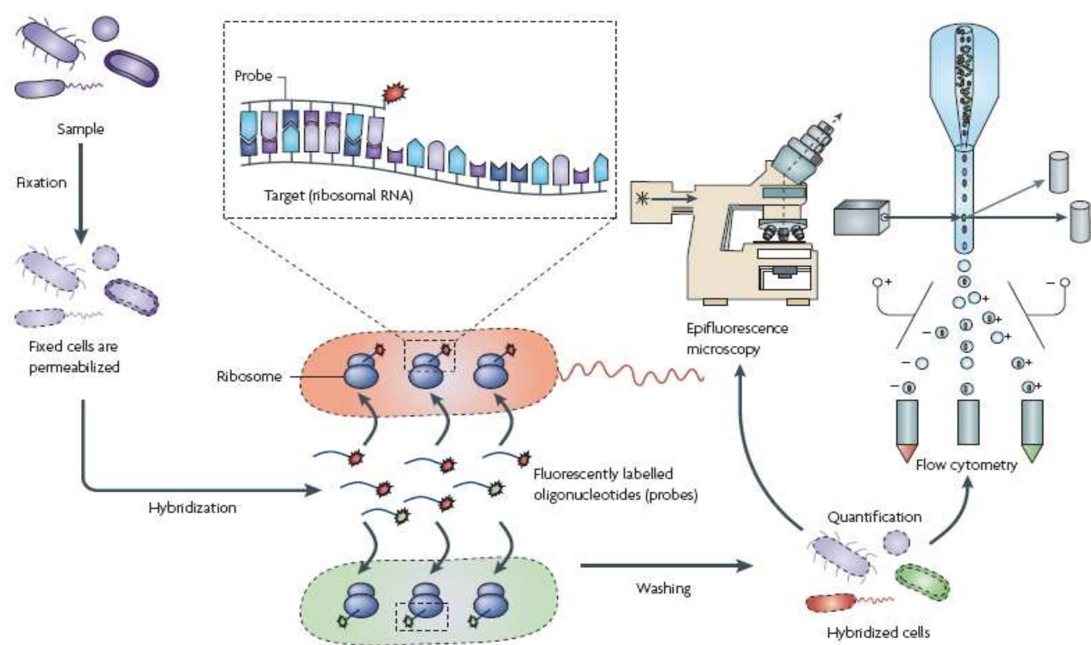
### 6.6. FISH

This technique consists in the direct hybridization of fluorescent labeled probes to RNA. First of all is done a fixation step, then a permeabilization, hybridization, some washing steps to remove the probes unbound and finally visualization using a fluorescence microscope.




#### 6.6.1. Solutions

| | | |
|---|---|---|
| PBS 10 X | $Na_2HPO_4$ | 1.424 g/100 ml distilled water |
| | $KH_2PO_4$ | 0.204 g/100 ml |
| | NaCl | 8.03 g/100 ml |
| | KCl | 0.201 g/100 ml |
| | Adjust the pH to 7.2 | |

Paraformaldehide 4 % (TOXIC) paraformaldehide 8 g/190 ml MiliQ water

Add 2 drops of NaOH 1 M (the solution become transparent)

Add 10 ml of PBS 10 X

Filtrate with sterile filters of 0.2 µm

Keep aliquots of 15 ml frozen

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| | | |
|---|---|---|
| Hibridization buffer | Formamide | 50 µl |
| | NaCl 5 M | 180 µl |
| | Tris-HCl 1M pH 8 | 20 µl |
| | SDS 10 % | 1 µl |
| | dH20 | 749 µl |

Keep the buffer frozen

| | | |
|---|---|---|
| Wash buffer | NaCl 1 M | 500 µl |
| | Tris-HCl 50 mM pH 8 | 500 µl |

Probes — concentration stock 500 ng/µl
solution work 50 ng/µl

Hg 5’-CAATCCCAGCTAGCAGTAT-3’
Sc 5’-TGACTTACGTCGCAGTCC-3’
EUK 516 5’-ACCAGACTTGCCCTCC-3’

DAPI — concentration stock 1mg/ml
solution work 10 µg/ml

### 6.6.2. Procedure

1. Collect 1 ml of a homogenous sample
2. Fixation
   - Centrifuge 5 minuts at 10000rpm. Discard the supernatant and add 100 µl of PBS 1 X
   - Add 300 µl paraformaldehid 4% and incubate minimum 4 hours at 4ºC with hard agitation
   - Centrifuge 2 minuts at 10000 rpm, discard the supernatant
   - Add 400 µl PBS 1 X and 400 µl ethanol (to maintain the sample and avoid the cellular permeabilitzation)
3. Hibridization
   - 100 µl of the step before, or dilution adequate to obtain around $10^6$ cells/ml
   - Centrifuge 5 minuts at 10000rpm, discard the supernatant
   - Add 50 µl of a solution with the probe and hibridization buffer in a ratio 1:4

- Incubate at 46ºC during 3 hours keep the sample out of the light
- centrifuge 5 minuts at 10000rpm, discard the supernatant
- resuspend the pellet with 100 µl of PBS 1X
- at this point can be added 10 µl of DAPI to colorate the nucleous DNA

4. Observation and quantification
- Mix 5 µl of Vecta Shield (Vector Laboratories, USA) with 5 µl of the sample
- Put the solution onto Neubauer Chamber
- Count the fluorescent cells using a fluorescence microscope.

## 7. Analysis of wine fermentation

### 7.1. Sugars consumption

During all the fermentation the sugars consumption was analysed by the density measure. Only at the end of fermentation the sugars was analysed by enzymatic assay (Roche Diagnostics, Germany).

### 7.2. Glycerol production

The glycerol content was analysed using commercial enzymatic kits (Roche Diagnostics, Germany)

### 7.3. Ethanol production

The ethanol production was analysed using commercial enzymatic kits (Roche Diagnostics, Germany).

### 7.4. Amino acids evolution

The simultaneous analysis of biogenic amines, amino acids and ammonium ion was determined by the method of Gómez-Alonso et al. (2007). Briefly, samples (400 µl) was derivatized by 15 µl of diethylethoxymethylenemalonate (Fluka, Steinhein, Germany) in presence of 700 µl of borate buffer 1 M (pH 9), 300 µl of methanol and 10 µl of L-2-aminoadipic acid (Internal Standard, 1 g/l) over 30 minuts in an ultrasound bath. Then the sample was treated at 80ºC for 2 hours. The analyses were performed on a Agilent 1100 Series HPLC (Agilent Technologies, Böblingen, Germany) comprising a quarternary pump, an autosampler and a multiple wavelength detector at 269, 280 and 300 nm. Nitrogen compound separation of sample (50 µl) was carried out using a 4.6 x 250 mm, 5 µm ACE C18-HL column (Symta, Madrid, Spain) with a guard column (ACE5

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C18-HL) through a binary gradient (Gómez-Alonso et al., 2007) at a flow of 0.9 ml/min. The target nitrogen compounds were identified according to the retention time of corresponding standards and were quantified using the internal standard method.

### 7.5. Organic acids

Organic acids were determined by HPLC using an Agilent 1100 Series connected to an Agilent multiple wavelength detector (Agilent Technologies, Wilmington, DE). Sample (450 µl) was mixed with 50 µl of formic acid (Internal Standard, 46.84 g/L) and 50 µl was injected into a 300 mm x 7.8 mm AMINEX HPX-87H columm (BioRad, Hercules, CA). The solvent used was sulfuric acid 2.5 mM at 0.5 ml/min. The analysis temperature was 70 ºC. The concentration of each metabolite was calculated using external and internal standards.

### 7.6. Aromas

A modified protocol of Ortega et al. (2001) was used to determine volatile fatty acids, ethyl esters of fatty acids, higher alcohol acetates and other volatile compounds. In 15-ml screw-capped tubes, 1.5 ml of wine, 3.5 ml of $(NH_4)_2SO_4$ (45% w/v), 20 µl of internal standard (4-methyl-2-pentanol (176 µg/ml), 1-nonanol (160 µg/ml) and heptanoic acid (150 µg/ml) in ethanol) and 200 µl of dichloromethane were added. The tube was shaken for 30 seconds (3x) and then centrifuged at 4000 rpm for 10 min. Once the phases were separated, the bottom phase (dichloromethane) was transferred to a glass vial insert. The extract (3 µl) was injected in split mode (10:1, 30 ml/min) into an Agilent 6850 equipped with a flame ionisation detector (Agilent Technologies, Böblingen, Germany). The extract (2 µl) was injected (split, 1 min) into an. The column (30 m x 0.25 mm, 0.25 µm phase thickness) was a HP-FFAP(Agilent) and the temperature program was as follows: 35ºC for 5 min, then raised at 3ºC/min up to 200 ºC and then at 8º C/min up to 220 ºC. Injector and detector temperatures were 180ºC and 280ºC. The carrier gas was helium at 3 ml/min. Volatile compounds were identified and quantified by comparison with standards.

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## 8. Online sources

### 8.1. Search nucleotide sequences

This tool was used to search the interest sequences for some species of yeasts, lactic and acetic acid bacteria

http://www.ncbi.nlm.nih.gov/nucleotide/

### 8.2. Aligment of specific regions on database

The Basic Local Alignment Search Tool (BLAST) finds regions of local similarity between sequences. This was used to compare the sequences obtained with databases.

http://blast.ncbi.nlm.nih.gov/

http://www.ebi.ac.uk/blastall/nucleotide.html

### 8.3. Sequence aligment

This tool is used to compare sequences.

http://www.ebi.ac.uk/Tools/emboss/align/

### 8.4. PCR *in silico*

This tool is used to simulate an amplification of a known fragment.

http://insilico.ehu.es/user_seqs/

General web for *in silico* simulations,

http://insilico.ehu.es/

### 8.5. Digestion *in silico*

This tood is used to simulate and digestion of a known fragment.

http://tools.neb.com/NEBcutter2/

### 8.6. Primers design

The primer design was done with the Primer Express 3.0 software (Applied Biosystems, USA).

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**Annex 2**

# Estudio mediante técnicas independientes de cultivo del efecto de la temperatura de fermentación sobre la población microbiana

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_ENOLOGÍA_

Técnicas independientes de cultivo

# La temperatura de fermentación sobre la población microbiana

POR I. ANDORRÀ, J.M. GUILLAMÓN, A. MAS, B. ESTEVE-ZARZOSO
*Universidad Rovira i Virgili*

La microbiota del vino se ve influenciada por múltiples factores, entre los que destacan las prácticas vitícolas y enológicas [1]. Dentro de las prácticas enológicas, la temperatura de fermentación influye directamente en la evolución de las poblaciones de microorganismos en el vino [2]. En concreto, a menor temperatura de fermentación mayor posibilidad de supervivencia de algunas levaduras no-Saccharomyces [3]. Por el contrario, la baja temperatura de fermentación parece reducir el crecimiento de bacterias lácticas y acéticas durante la fermentación alcohólica [4].

Todos estos cambios en la dinámica poblacional afectan directamente a la calidad organoléptica del vino. Así, se ha descrito que las bajas temperaturas mejoran el perfil organoléptico del vino, bien sea por éste cambio en poblaciones, por un cambio metabólico de Saccharomyces o por ambos [5]. Actualmente los estudios de dinámicas poblacionales se están realizando con técnicas independientes de cultivo, ya que se evita la limitación del crecimiento en placa de cultivo [6].

Además de ser técnicas más rápidas, con estas técnicas se solucionan varios problemas: (i) la selectividad de los medios de cultivo, (ii) la diferencia entre la tasa de crecimiento en medio de cultivo de los microorganismos y (iii) la presencia de los microorganismos viables pero no cultivables, que se ha demostrado que se encuentran presentes en una vinificación [7]. Así usando estas técnicas independientes de cultivo se tiene un conocimiento real de la población microbiana que interviene en la fermentación alcohólica, ya que con técnicas dependientes de cultivo sólo se analizan aquellos microorganismos capaces de crecer en un medio de cultivo.

El objetivo de este trabajo es analizar la evolución de la población de microorganismos durante dos fermentaciones alcohólicas, una a baja temperatura (13° C) y otra a la temperatura óptima de crecimiento de las levaduras vínicas (25° C) mediante tres técnicas independientes de cultivo: La PCR cuantitativa a tiempo real, la PCR-DGGE y una nueva técnica basada en la clonación de la región 5.8S-ITS del rDNA amplificada a partir de ADN purificado del vino. Los resultados obtenidos utilizando las tres técnicas independientes se compararon con el crecimiento en placa de cultivo y la posterior identificación de las colonias.

## Material y métodos

*Vinificación y muestreo*

Se utilizó la variedad Macabeo procedente de la bodega experimental Mas dels Frares de la Universidad Rovira i Virgili. Después del despalillado, estrujado y prensado, se añadieron 60 mg/l de $SO_2$ al mosto que se transfirió al tanque de desfangado. Una vez limpio el mosto (24 horas) se transfirió a dos tanques de fermentación con control de temperatura, uno a 13° C y el otro a 25° C. Las dos fermentaciones se hicieron espontáneamente, sin inoculación de levaduras seleccionadas. Se cogió muestra antes y después del desfangado del mosto (densidad 1095 g/l) al inicio de la fermentación (densidad 1090 g/l), a la mitad de fermentación (densidad 1070 g/l), mitad-tardía de fermentación (densidad 1040 g/l) y al final de fermentación (densidad 1000 g/l).

Estas muestras se sembraron en placas de YPD utilizando un sistema automatizado (Whitley Atomatic Spiral Plater), de cada punto se aislaron 50 colonias. De todas las cepas de referencia y muestras se realizó extracción de

DNA, utilizando el protocolo descrito por Hierro y cols. [8]. Se utilizó un único protocolo de extracción para todos los métodos y grupos microbianos analizados.

*Técnica dependiente de cultivo*

De cada colonia obtenida de la placa cultivo de YPD se amplificó el fragmento que corresponde a la región ITS-5.8S del rDNA, descrito por Guillamón y cols. [9]. El producto de la amplificación se digirió con enzimas de restricción HinfI y en los casos necesarios también se utilizaron CfoI, DraI o HaeIII para su posterior identificación.

*Técnicas independientes de cultivo*

A partir de la extracción directa del DNA de la muestra de fermentación, se amplificó la región 5.8S-ITS, así se obtuvo una librería de DNA de todas las especies de levaduras presentes en la fermentación, este amplicón fue clonado usando el vector pGEM-T Easy (Promega). Cincuenta colonias transformadas de *Escherichia coli* por punto muestreado fueron purificadas y sus plásmidos extraídos usando el procedimiento descrito por Sambrook y cols. [10]. El fragmento clonado fue digerido con la enzima de restricción HaeIII para su posterior identificación por comparación con las cepas de referencia. En las identificaciones dudosas se secuenció el producto de PCR. Previamente se hizo el mismo trabajo con cepas de referencia *C. zemplinina, H. uvarum* y *S. cerevisiae* para ver el perfil de restricción del plásmido con los fragmentos clonados que presentaban estas especies.

El procedimiento experimental tanto en la técnica de la QPCR como en la PCR-DGGE se hizo como esta descrito en Andorrà y cols. [11]. Para la cuantificación de *C. zemplinina* se diseñaron los cebadores AF (5'-CTAGCATTGACCTCATATAGG-3') y 200R (5'-GCATTCCCAAACAACTCGACTC-3'), en el dominio D1/D2 del gen 26S rDNA.

**Resultados y conclusiones**

La fermentación a baja temperatura necesitó el doble de tiempo que la fermentación a 25° C para finalizar, en cualquier caso el final de ambas fermentaciones fue más lento de lo esperado. Las muestras de mosto y mosto desfangado son las mismas para ambas fermentaciones (13° C y 25° C).

La técnica de la PCR-DGGE se ha descrito como una buena herramienta para el estudio de la diversidad microbiana en vinificaciones. El principal inconveniente de esta técnica es su baja sensibilidad, ya que cuando predomina una especie se dejan de detectar otras especies presentes en menor proporción. Así sólo se detectó una gran diversidad de especies de levaduras en el mosto y mosto desfangado, donde aún no hay una clara predominancia de ninguna especie. En estas muestras se detectaron tanto hongos filamentosos (*Aspergillus niger* y *Botriotinya fuckeliana*) como las especies de levaduras: *C. zemplinina, H. uvarum* y *S. cerevisiae*. Por lo que respecta a bacterias, sólo se detectaron bacterias acéticas y únicamente *Acetobacter aceti*, en todos los puntos analizados. A medida que avanza la fermentación no se encontraron más hongos filamentosos y la única diferencia detectada fue la rápida desaparición de H. uvarum en la fermentación de 25° C (tabla 1).

La técnica de la clonación del fragmento 5.8S-ITS detectó mayor diversidad de especies de levaduras que la identificación en placa. Con la técnica de la clonación, en el mosto se identificaron siete especies distintas (*A. niger, C. zemplinina, H. uvarum, H. viniae, S. cerevisiae, Saccharomycopsis vini* y *Zygosaccharomyces bailii*), pero la suma de dos especies

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| | 13 °C | 25 °C |
|---|---|---|
| **Mosto** | *A. niger*<br>*B. fuckeliana*<br>*C. zemplinina*<br>*H. uvarum*<br>*S. cerevisiae*<br>*Ac. aceti* | |
| **Mosto desfangado** | *A. niger*<br>*B. fuckeliana*<br>*C. zemplinina*<br>*H. uvarum*<br>*S. cerevisiae*<br>*Ac. aceti* | |
| **Inicio fermentación** | *C. zemplinina*<br>*H. uvarum*<br>*S. cerevisiae*<br>*Ac. aceti* | *C. zemplinina*<br>*S. cerevisiae*<br>*Ac. aceti* |
| **Mitad** | *C. zemplinina*<br>*S. cerevisiae*<br>*Ac. aceti* | *C. zemplinina*<br>*S. cerevisiae*<br>*Ac. aceti* |
| **Mitad-tardía** | *C. zemplinina*<br>*S. cerevisiae*<br>*Ac. aceti* | *C. zemplinina*<br>*S. cerevisiae*<br>*Ac. aceti* |
| **Final** | *C. zemplinina*<br>*S. cerevisiae*<br>*Ac. aceti* | *C. zemplinina*<br>*S. cerevisiae*<br>*Ac. aceti* |

Tabla 1. Diversidad microbiana analizada mediante la técnica PCR-DGGE. A.: *Aspergillus*, B.: *Botriotinya*, C.: *Candida*, H.: *Hanseniaspora*, S.: *Saccharomyces*, Ac.: *Acetobacter*.

(*S. cerevisiae* y *C. zemplinina*) representa el 95% del total de colonias analizadas; y a partir de mitad de fermentación sólo persisten *S. cerevisiae* y *C. zemplinina* (tabla 2). Esta última va disminuyendo su proporción conforme la fermentación avanza y *S. cerevisiae* va aumentando. El predominio de *S. cerevisiae* era más patente en el análisis de las colonias, donde encontramos, al final de la fermentación, que un 100% de las colonias analizadas corresponden a *S. cerevisiae* y en cambio cuando se analiza mediante la técnica independiente de cultivo se observa que la población de *S. cerevisiae* varía del 60 al 75% y por tanto *C. zemplinina* soporta las condiciones adversas del final de fermentación.

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En la fermentación a 25 °C se detectó *Hanseniaspora osmophila* mediante la técnica dependiente de cultivo. Según los datos obtenidos por la clonación del fragmento 5.8S-ITS, la fermentación a baja temperatura presenta mayor proporción de *C. zemplinina* al final de la fermentación, mientras que en la fermentación a 25 ° C desaparece más rápidamente. Esta técnica se ha visto que es más sensible que la técnica de la PCR-DGGE, pudiendo detectar la presencia de especies de levaduras que se encontraban en baja proporción, como es el caso de *H. vineae* detectada a 13° C a mitad de fermentación.

La cuantificación de las diferentes poblaciones de microorganismos se hizo mediante la técnica QPCR (figura 1). El mosto inicial tenía una población de aproximadamente 107 cel/ml y tras el desfangado disminuyó un 50%. La población máxima alcanzada durante la fermentación llegó a 5.107 cel/ml. Este máximo se alcanzó antes en la fermentación a 25° C, aunque la viabilidad del cultivo fue mayor en la fermentación a 13° C.

Las especies de levaduras no-Saccharomyces fueron las que se encontraron en mayor cantidad en el mosto, principalmente *C. zemplinina* y manteniéndose hasta final de la fermentación. *H. uvarum* mostró un declive evidente de su población a partir de mitad de fermentación. En referencia a las bacterias lácticas (figura 2), presentaban una baja población durante toda la fermentación (102 cel/ml). En ninguna de las fermentaciones empezó la fermentación maloláctica, en consonancia con niveles tan bajos de bacterias lácticas. Las bacterias acéticas partieron de 5.105 cel/ml y disminuyeron progresivamente hasta final de la fermentación, posiblemente

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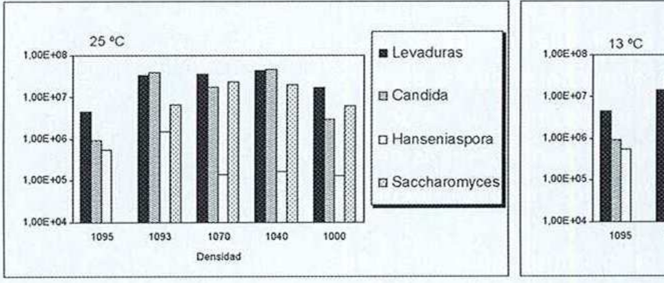
| | | | Mosto | Mosto desfangado | Inicio Fermentación | Mitad Fermentación | Mitad-tardía Fermentación | Final Fermentación |
|---|---|---|---|---|---|---|---|---|
| 13 °C | *A. niger* | Placa | - | - | - | - | - | - |
| | | Clonación | 4,55 | 5,26 | - | - | - | - |
| | *C. zemplinina* | Placa | 95 | 95 | 100 | - | - | - |
| | | Clonación | 63,64 | 78,95 | 61,90 | 17,39 | 28,57 | 37,50 |
| | *H. uvarum* | Placa | - | 5 | - | - | - | - |
| | | Clonación | 9,09 | 5,26 | - | - | - | - |
| | *H. vineae* | Placa | - | - | - | - | - | - |
| | | Clonación | 4,55 | - | - | 4,35 | - | - |
| | *S. cerevisiae* | Placa | 5 | - | - | 100 | 100 | 100 |
| | | Clonación | 4,55 | 10,53 | 38,10 | 78,27 | 71,43 | 62,50 |
| | *Smycopsis. vini* | Placa | - | - | - | - | - | - |
| | | Clonación | 9,09 | - | - | - | - | - |
| | *Z. bailii* | Placa | - | - | - | - | - | - |
| | | Clonación | 4,55 | - | - | - | - | - |
| 25 °C | *A. niger* | Placa | - | - | - | - | - | - |
| | | Clonación | 4,55 | 5,26 | - | - | - | - |
| | *C. zemplinina* | Placa | 95 | 95 | 75 | 30 | 10 | - |
| | | Clonación | 63,62 | 78,95 | 85,00 | 20,83 | 16,67 | 25,00 |
| | *H. osmophila* | Placa | - | - | 15 | - | - | - |
| | | Clonación | - | - | - | - | - | - |
| | *H. uvarum* | Placa | - | 5 | - | - | - | - |
| | | Clonación | 9,09 | 5,26 | - | - | - | - |
| | *H. vineae* | Placa | - | - | - | - | - | - |
| | | Clonación | 4,55 | - | - | - | - | - |
| | *S. cerevisiae* | Placa | 5 | - | 10 | 70 | 90 | 100 |
| | | Clonación | 4,55 | 10,53 | 15,00 | 79,17 | 83,33 | 75,00 |
| | *Smycopsis. vini* | Placa | - | - | - | - | - | - |
| | | Clonación | 9,09 | - | - | - | - | - |
| | *Z. bailii* | Placa | - | - | - | - | - | - |
| | | Clonación | 4,55 | - | - | - | - | - |

Tabla 2. Poblaciones de microorganismos analizados mediante la técnica de la clonación del fragmento 5.8S-ITS y la identificación de colonias aisladas en medio de cultivo. Los valores indican el porcentaje de colonias analizadas en cada punto de la fermentación. A.: *Aspergillus*, C.: *Candida*, H.: *Hanseniaspora*, S.: *Saccharomyces*, *Smycopsis*.: *Saccharomycopsis*, Z.: *Zygosaccharomyces*.



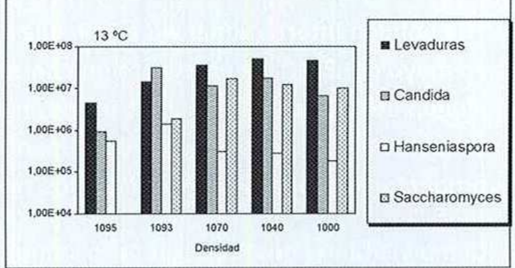




Figura 1.Cuantificación de las poblaciones de levaduras por QPCR en las fermentaciones (13° C y 25° C).

debido a las condiciones adversas durante la vinificación (figura 2). No se detectaron diferencias significativas en base a la temperatura.

En conclusión, las técnicas independientes de cultivo permiten detectar una mayor diversidad de especies que las técnicas clásicas, basadas en el crecimiento de los microorganismos en placas de cultivo. Entre las técnicas independientes de cultivo, la clonación es una técnica más sensible en la detección de especies minoritarias que la PCR-DGGE, ya que nos permitió detectar una mayor diversidad de especies que la PCR-DGGE. Además, esta técnica nos permite tener un análisis semicuantitativo de la población mientras que la PCR-DGGE es meramente cualitativo. La principal ventaja de la QPCR es que, a pesar de la presencia de especies predominantes, la especificidad de los cebadores diseñados nos permite cuantificar especies minoritarias, lo cual evidencia una alta presencia y permanencia de

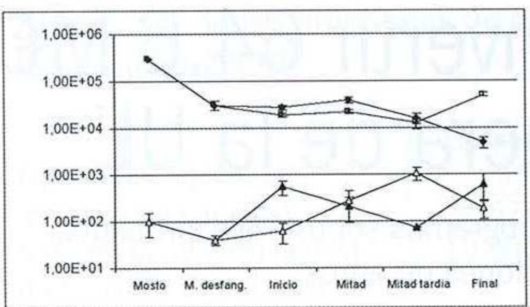



Figura 2. Evolución de las bacterias acéticas y lácticas analizadas mediante la técnica QPCR: Bacterias lácticas, 25° C (- -▲- -) y 13 °C (—Δ—). Bacterias acéticas, 25° C (- -■- -) y 13° C (—□—).

las especies no-Saccharomyces no solamente en los primeros días de fermentación.

En nuestras condiciones de trabajo, la temperatura de fermentación mostró una influencia limitada en la diversidad y distribución de los diferentes microorganismos vínicos. A pesar de las condiciones de anaerobiosis durante la fermentación se detectaron bacterias acéticas tanto mediante la técnica PCR-DGGE como de QPCR.

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