NEW APPROACHES TO ESTIMATE MICROBIAL DIVERSITY OF ALCOHOLIC FERMENTATION

Chunxiao Wang

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New approaches to estimate microbial diversity of alcoholic fermentation

DOCTORAL THESIS

Directed by Dr. Albert Mas and Dr. Braulio Esteve Zarzoso

For Ph. D. degree with International Mention

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Dr. Albert Mas   Dr. Braulio Esteve Zarzoso

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OUTLINE OF THESIS

As a young scientist interested in wine microbiology, I felt fascinated by the alcoholic fermentation, due to the differences in cell morphologies under microscope, different cell statues (culturable, viable but not-culturable and dead) and their different appearance time during alcoholic fermentation. A series of papers that I read is first authored by Imma Andorrà, who was working in Oenology and Biotechnology group of Universitat Rovira i Virgili. Therefore, with great enthusiasm, I joined the same group in October, 2012 and found that the platform of culture-independent techniques applied to wine microbiology such as quantitative PCR was well built here. My topic thus went to estimate and understand the yeast diversity during wine alcoholic fermentation, which contained the application of new and improved culture-independent techniques and the exploration on cell status and interaction mechanism between *Saccharomyces* and non-*Saccharomyces*.

My working hypothesis was that yeast population dynamic largely depends on the interaction between *S. cerevisiae* and non-*Saccharomyces* and that the combination of different culture-independent techniques could be the appropriate tools to understand that interaction. To verify the hypotheses, three main objectives were set up with the aim to better estimate yeast diversity and viable population dynamic during alcoholic fermentation, and to understand how the population dynamics formed by interaction among each other. The three objectives were listed as follows:

(1) To analyze the yeast diversity in wine fermentation through culture-dependent and culture-independent techniques.

To obtain a comprehensive investigation on yeast diversity in grape must from Priorat region, massive sequencing targeting D1/D2 region of rDNA was intended and introduced for the analysis. Other well-developed techniques such as quantitative PCR and DGGE were combined to analyze the same DNA sample from grape must. As a comparison, traditional isolation and identification (5.8S-ITS-RFLP and 26S-D1/D2 sequencing) was performed for the same grape must samples.

The results are shown in Chapter 1: **Fungal diversity in grape must and wine fermentation assessed by massive sequencing, quantitative PCR and DGGE**. Frontiers in Microbiology (2015) 6, 1156.

(2) To analyze the status of *S. cerevisiae* and two main non-*Saccharomyces* yeast in fermentation by different techniques.

To examine the status of the population of *S. cerevisiae* and two main non-*Saccharomyces* yeast (*Haseniaspora uvarum* and *Starmerella bacillaris*) in complex grape must, culture-independent techniques were developed at detection levels of both RNA and cell membrane. Fluorescence in situ hybridization (FISH) was used to target intracellular rRNA directly, with two primers newly designed for the non-*Saccharomyces* yeast studied. RNA was also extracted and analyzed by quantitative PCR and DGGE. Besides, quantitative PCR and DGGE were combined with ethidium monoazide bromide (EMA) treatment to exclude cells with compromised membrane.

The results are stated in Chapter 2 and 3.

Chapter 2: **Monitoring of *Saccharomyces cerevisiae*, *Hanseniaspora uvarum*, and *Starmerella bacillaris* (synonym *Candida zemplinina*) populations during alcoholic fermentation by fluorescence *in situ* hybridization**. International Journal of Food Microbiology (2014) 191, 1-9.

Chapter 3: **Viable and culturable populations of *Saccharomyces cerevisiae*, *Hanseniaspora uvarum* and *Starmerella bacillaris* (synonym *Candida zemplinina*) during Barbera must fermentation**. Food Research International (2015) 78, 195-200.

(3) To further analyze how *S. cerevisiae* interact with non-*Saccharomyces* yeast.

To understand the interaction between *S. cerevisiae* and non-*Saccharomyces* yeast, mixed alcoholic fermentation was conducted to analyze strains' culturability during the whole process. Then a series of trials were performed to discover the probable interaction mechanism affecting culturability of non-*Saccharomyces* yeast:

Firstly, to check if contact-mechanism exists in our *S. cerevisiae* strain against non-*Saccharomyces*, compartmented vessels using dialysis bags were designed, which allowed the molecule diffusion but prevented cell transfer between the compartments.

Secondly, to indicate the influence of environmental changes (depletion of sugar and nitrogen and ethanol production) on culturability of non-*Saccharomyces*

during wine fermentation, synthetic musts mimicking different stages of mixed fermentations were prepared. The culturability and viability of non-*Saccharomyces* strains was followed after their inoculation.

Thirdly, to find out the effect of some special metabolites from *S. cerevisiae* during wine fermentation, cell-free supernatants were derived from different stages of *S. cerevisiae* fermentations. In contrast, synthetic must mimicking the cell-free supernatants were designed. Finally, we compared the culturability and viability of non-*Saccharomyces* strains in both media.

The results are indicated in Chapter 4 and Chapter 5.

Chapter 4: **Interaction between *Hanseniaspora uvarum* and *Saccharomyces cerevisiae* during alcoholic fermentation**. International Journal of Food Microbiology (2015) 206, 67-74.

Chapter 5: **The interaction between *Saccharomyces cerevisiae* and non-*Saccharomyces* yeast during alcoholic fermentation is species and strain specific.** Frontiers in Microbiology (2016) 7, 502.

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Question 1: Which yeasts are relevant during alcoholic fermentation?

1. Yeast diversity associated with alcoholic fermentation

In late nineteenth century, Louis Pasteur indicated that wine microorganisms responsible for alcoholic fermentations were yeasts that exist on grape surface. After decades of investigations, today people know the surface of grape berry is colonized by filamentous fungi, yeast and bacteria. When grapes are broken or damaged, microbes on grape surface especially yeasts proliferates due to the available nutrient. Some yeast species can ferment sugar to alcohol and ultimately the first wine comes in history. Due to the relevance of yeast in winemaking, yeast diversity during grape must fermentations have been studied to find their traces and activities. Many techniques have been used to study it including the widely used culture-dependent and culture-independent techniques. However, the emergence of new techniques such as Next-Generation Sequencing (NGS) undoubtedly promotes corresponding research (Almeida et al. 2015).

1.1. Yeast on grape surface

Yeast quantity

Total yeast population on intact grape berries varies ranging 10^2 cfu/berry to 10^5 cfu/berry (Renouf et al. 2005). The wide range of values is regarded to be related with different factors especially grape soundness and ripeness. Damaged grape berries generally can increase one log cycle of population (i. e. 10^6 cfu/berry) because of the nutrient availability (Barata et al. 2012). Interestingly, population quantity also changes as the ripening of grape berries (Renouf et al. 2005; Clavijo et al. 2010). Yeast on berry set keep the level of 10^2 cfu/berry, increase obviously at the stage of veraison and reach to 10^5 cfu/berry at the harvest time (Figure 1). The increased population at the harvest time is due to nutrient availability, because the cuticle of intact berries become soften and probably bear some microfissures despite of the visual intactness (Barata et al. 2012).

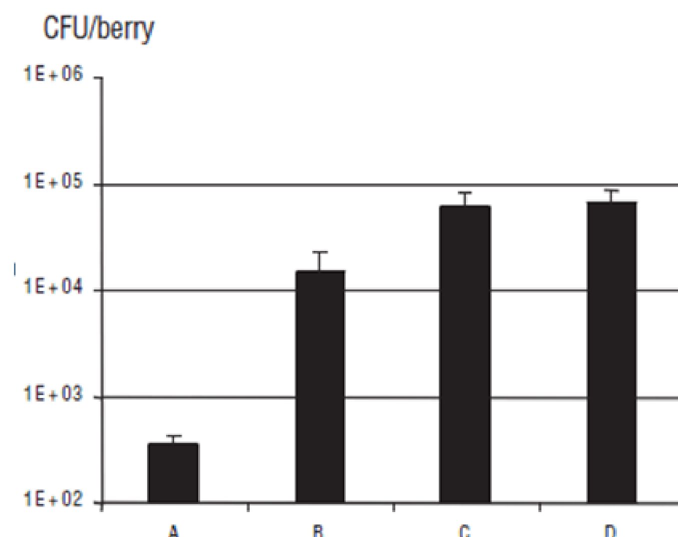

Figure 1 Total yeast (counted on TY plates) populations (CFU/berry) as a function of the stage of berry development (A=berry set, B=veraison, C=the end of the agrochemical applications and D=harvest). Data from Renouf et al. (2005).

Yeast diversity

Yeasts colonizing the grape surface are mainly composed of three types: *Ascomycetous* moulds (yeast-like), *Basidiomycetous* yeast and *Ascomycetous* yeast (Table 1). As representative specie of *Ascomycetous* moulds, *Aureobasidium pullulans* is a common yeast-like mould occupying grape surface. *Basidiomycetous* yeast is also predominant on grape surface and the common species are mainly from genera of *Cryptococcus*, *Rhodotorula* and *Sporodiobolus*. Although *Ascomycetous* yeasts generally colonize intact grape berries at a non-dominance state, a great diversity is found in the worldwide surveys (Table 1). Common *Ascomycetous* yeast on grape surface includes the genera of *Candida*, *Debaryomyces*, *Hanseniaspora*, *Issatchenkia*, *Metschnikowia* and *Pichia*. Species diversity of *Ascomycetous* yeast is even higher depending on a series of variations (climatic conditions, vineyard treatments, biotic factors, geographic location and vineyard factors including age, size, grape variety and vintage year) (Barata et al. 2012). However, some species from *Ascomycetous* show widespread distribution such as *Hanseniaspora uvarum*, *Issatchenkia orientalis*, *Issatchenkia terricola* and *Metschnikowia pulcherrima* (Table 1).

Table 1 Yeast or yeast-like species present on grape surface.

	Genera	Species	Countries*
Ascomycetous moulds	*Aureobasidium*	*A. pullulans*	France[3] Slovenia[4] Brazil[7] Italy[8]
	Bulleromyces	*B. albus*	France[3]
	Cryptococcus	*C. albidus*	France[3] Slovenia[4]
		C. carnescens	China[6] Italy[8]
		C. flavescens	France[3] China[6]
		C. Hungaricus	Slovenia[4]
		C. laurentii	France[3] Slovenia[4]
		C. magnus	China[6]
Basidiomycetous yeast	*Rhodosporidium*	*R. babjevae*	France[3]
	Rhodotorula	*R. aurantiaca*	Slovenia[4]
		R. glutinis	France[3] Italy[8]
		R. graminis	France[3]
		R. mucilaginosa	France[3]
	Sporodiobolus	*S. pararoseus*	France[3] China[6] Brazil[7]
		S. salmonicolor	France[3]
	Sporobolomyces	*S. roseus*	France[3] Slovenia[4]
	Candida	*C. azyma*	India[5]
		C. boidinii	France[3]
		C. colliculosa	Spain[1]
		C. fructus	France[3]
		C. inconpicua	China[6]
		C. intermedia	France[3]
		C. membranifaciens	France[3]
		C. quercitrusa	India[5]
		C. raghi	Argentina[2]
		C. stellata	Spain[1] Argentina[2] France[3]
		Starmerella bacillaris (*C. zemplinina*)	China[6]
	Debaryomyces	*D. hansenii*	France[3] Slovenia[4] India[5]
	Hanseniaspora	*H. guilliermondii*	France[3] India[5]
		H. opuntiae	France[3]
		H. uvarum	Spain[1] Argentina[2] France[3] Slovenia[4] India[5] China[6] Italy[8]
		H. viniae	India[5]
	Issatchenkia	*I. occidentalis*	Brazil[7]
Ascomycetous yeast		*I. orientalis*	Argentina[2] France[3] India[5] China[6] Brazil[7]
		I. terricola	France[3] India[5] Brazil[7] Italy[8]
	Kluyveromyces	*K. lactis*	France[3]
		K. thermotolerans	Spain[1]
	Lipomyces	*L. spencermartinsiae*	France[3]
	Metschnikowia	*M. pulcherrima*	Spain[1] Argentina[2] France[3] Slovenia[4] China[6]
		M. reukaufii	Slovenia[4]
	Pichia	*P. anomala*	France[3]
		P. fermentans	France[3] China[6]
		P. guilliermondii	China[6]
		P. kluyveri	Slovenia[4]
		P. membranifaciens	Argentina[2] India[5]
	Saccharomyces	*S. boulardii*	France[3]
		S. cerevisiae	France[3] India[5]
	Saccharomycodes	*S. ludwigii*	Argentina[2]
	Torulaspora	*T. delbrueckii*	Italy[8]
	Yarrowia	*Y. lipolytica*	France[3]
	Zygoascus	*Z. steatolyticus*	India[5]
	Zygosaccharomyces	*Z. bailii*	Spain[1] China[6]

**Data collected from eight surveys: [1] Torija et al. (2001), [2] Combina et al. (2005b), [3] Renouf et al. (2005), [4] Raspor et al. (2006), [5] Chavan et al. (2009), [6] Li et al. (2010), [7] Baffi et al. (2011), [8] Alessandria et al. (2015).*

Saccharomyces cerevisiae is found to be absent or present in low numbers from sound grape berries, similar to some spoilage species such as *Zygosaccharomyces bailii*. However, damaged or rotted berries can provide high nutrient to favor the growth of *Ascomycetous* yeast. When whole brunch is harvested, some damaged berries in the brunch may result in a high isolation of the *ascomycetous* yeast. Therefore, the isolation of *S. cerevisiae* and other spoilage species from grape berry is suspected to be related with grape health and sampling approach (Barata et al. 2012).

The origin

Ascomycetous moulds and *Basidiomycetous* yeast are considered as oligotrophic residents on grape berries and phylloplanes, because population quantity and dynamic of *Ascomycetous* moulds and *Basidiomycetous* yeast keeps a similar pace on both grape berries and phylloplanes. The oligotrophic residents are thought to be adapted to the environment with poor nutrient availability (Loureiro et al. 2012). However, *Ascomycetous* yeast is classified as copiotrophic opportunists, because they are rarely detected on immature grape berries but detected on grape berries with relative rich nutrient availability (verasion, harvest or damaged grape berries). This theory is supported by the finding of uneven distribution of *Ascomycetous* yeast: yeast microcolonies gather around the sites with more probable nutrient leaking from berry, such as pedicel insertion and stylar remnants (Loureiro et al. 2012).

Although all *Ascomycetous* yeast is copiotrophic opportunist, it is difficult to isolate some species on sound berries even at harvest time and the classical representative is *S. cerevisiae*. Considering its robust behavior in alcoholic fermentation, the rare detection of *S. cerevisiae* is, no doubt, irrelevant with its competitiveness. A more reliable explanation of this phenomenon comes from Loureiro et al. 2012: *S. cerevisiae* and its close relatives (other *Saccharomyces* yeast species) reside primarily in tree bark and soil as spores, where they are detected all year long. Only in the two months with grape growing from veraison to harvest or decay, the spores are dispersed onto grape berries by some vectors such as insects.

1.2. Yeast in grape must fermentation

Ascomycetous yeast existing on grape berries (Table 1) could survive and grow in grape must according to worldwide investigations of their isolations during spontaneous fermentation of grape must. The *Ascomycetous* yeast metabolizes main nutrients to ethanol and other volatile flavor and thus endues wine particular character. According to their fermentation capacity, competitiveness and contribution to wine, two main types of yeast are classified in spontaneous grape must fermentation: non-*Saccharomyces* yeast and *Saccharomyces* yeast. Non-*Saccharomyces* yeast is generally less fermentative and competitive than *Saccharomyces* but nowadays is regarded to be more related with wine complexity (Figure 2).

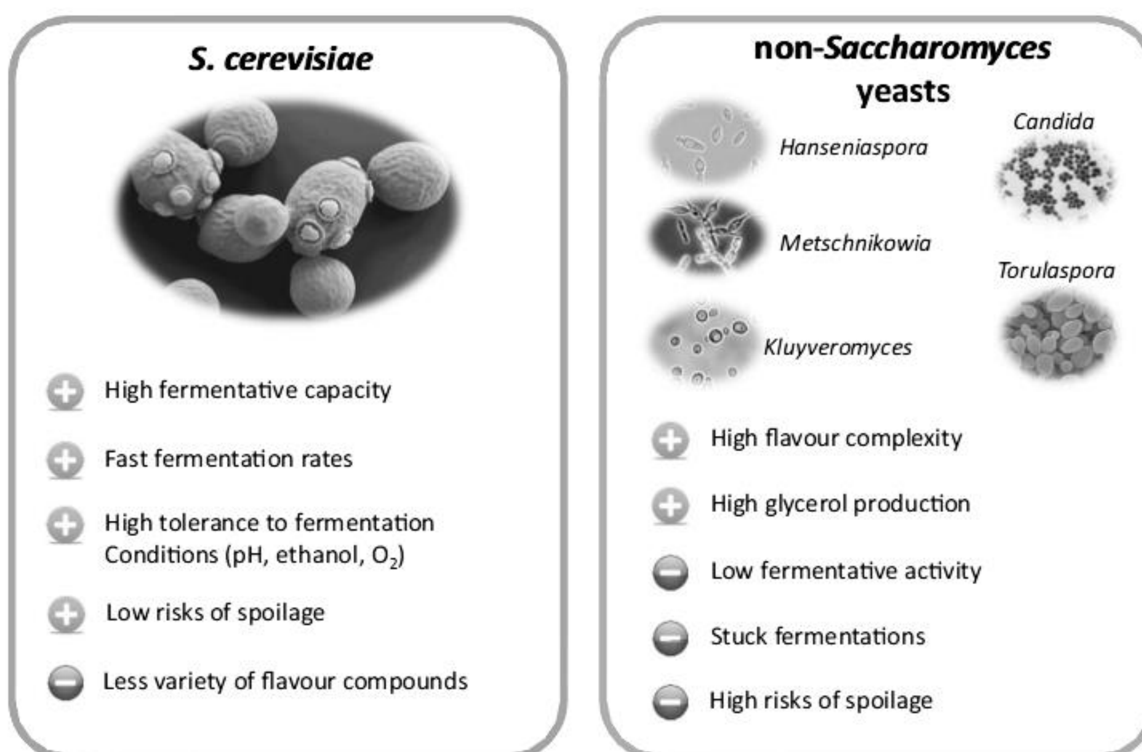

Figure 2 Main differences in enological properties of *Saccharomyces* and non-*Saccharomyces* yeast (Albergaria and Arneborg 2016).

1.2.1. non-Saccharomyces yeast

The term of non-*Saccharomyces* has little taxonomical significance, which contain not all genera from *Ascomycetous* yeast except *Saccharomyces*. According to Jolly et al. (2014), only yeast with a positive role in wine production is included in this description and yet spoilage yeasts such as *Dekkera*/*Brettanomyces* not.

Researchers have investigated the existence and their specific metabolisms of various non-*Saccharomyces* yeast species during alcoholic fermentation. The potential applications of non-*Saccharomyces* in wine industry are listed in Table 2. Some yeast species such as *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, *Pichia kluyveri* and *Lachancea thermotolerans* are currently used as commercial starters in alcoholic fermentation. The assessment on *Hanseniapsora uvarum*, *Starmerella bacillaris* (previously *Candida zemplinina*) and other species are still on the way to balance their positive contribution and negative impact on wine (Masneuf-Pomarede et al. 2016).

Table 2 non-*Saccharomyces* yeast with positive impact demonstrated. (Adapted according to Masneuf-Pomarede et al. 2016 and Jolly et al. 2014)

Features of interest in winemaking	Species/synonym
Acetate ester production	*Hanseniaspora guillermondii*
	Hanseniaspora vinae
	Lachancea thermotolerans/ *Kluyveromyces thermotolerans*
Aroma and complexity	*Hanseniaspora uvarum*
	Metschnikowia pulcherrima/*Torulopsis pulcherrima*
	Pichia anomala/ *Hanseluna anomala*
	Pichia fermentans
	Pichia Kluyveri/ *Hanseluna kluyveri*
	Starmerella bacillaris
	Torulaspora delbrueckii
Enzymatic activities	*Debaryomyces hansenii*/ *Pichia hansenii*
Ester production	*Metschnikowia pulcherrima*/ *Torulopsis pulcherrima*
	Pichia membranifaciens
Fructophily	*Candida stellata*/ *Torulopsis stellata*
	Starmerella bacillaris
	Zygosaccharomyces bailii
Glycerol production	*Candida stellata*
	Lachancea thermotolerans
	Starmerella bacillaris
Killer against *Dekkera*/*Brettanomyces*	*Pichia anomala*
Reduced ethanol production	*Starmerella bacillaris*
Reduced malic acid and total acid	*Schizosaccharomyces pombe*
Volatile acidity reduction	*Torulaspora delbrueckii*

The negative impact from non-*Saccharomyces* is mainly the low fermentative activity and high level of undesirable flavors. The low fermentative activity can be overcome by mixed fermentation with *Saccharomyces* yeasts. The undesirable

flavors are solved by a mass of olfactive perception experiments to screen acceptable or neutral strain (Bely et al. 2013). The genetic and phenotypic performance of 115 *H. uvarum* strains were fully assessed by Albertin et al. (2016), as well as 63 *Starm. bacillaris* strains by Englezos et al. (2015), both being designed for exploitation of the two common non-*Saccharomyces* yeast species isolated from grape must fermentation.

1.2.2. Saccharomyces yeasts

Saccharomyces yeasts are remarkably characteristics of their ability to produce and accumulate ethanol (Crabtree effect) even under aerobic conditions (Marsit and Dequin 2015). According to Barnett et al. (2000), Naumov et al. (2000) and Borneman and Pretorius (2015), *Saccharomyces* yeasts were taxonomically separated into three groups: *Saccharomyces sensu stricto* group, containing *S. cerevisiae*, *S. bayanus* (*S. bayanus* var. *bayanus*) / *S. uvarum* (*S. bayanus* var. *uvarum*), *S. paradoxus*, *S. eubayanus* (the parent of *S. pastorianus*), *S. cariocanus*, *S. mikatae*, *S. kudriavzevii* and *S. arboricolus*, *Saccharomyces sensu lato* group, including *S. dairensis*, *S. exiguus*, *S. unisporus*, *S. servazzi* and *S. castelli* and the third group with only *S. kluyveri*. So far, only species in *Saccharomyces sensu stricto* group have enological interest: *S. cerevisiae* is the primary yeast species in grape must fermentation, not only responsible for the metabolism of grape sugar to alcohol and carbon dioxide, but also relevant with formation of secondary metabolites and conversion of grape aroma precursors to varietal wine aromas (Jolly et al. 2014); *S. bayanus* also mediate grape must fermentation at low temperature since they are cryotolerant (Tamai et al. 1998); *S. uvarum* is proved to be a good starter culture due to its reduced ethanol production, psychrophilism and acetate ester production (Masneuf-Pomarede et al. 2010; Bely et al. 2013; Csernus et al. 2014). Rementeria et al. (2003) isolated strains of *S. kluyveri* from spontaneous fermentation, but their potential contribution to grape must fermentation is still unknown.

In addition, haploid cells or spores from the species in *Saccharomyces sensu stricto* group are able to mate with each other and form viable hybrids (Querol et al. 2003). Hybrid strains of *S. cerevisiae* and *S. bayanus* as well as *S. cerevisiae* and *S. kudriavzevii* have been found in fermentations (Gonzalez 2006). This phenomenon creates possibility for new species or strains, however, at the same time causes confusion about their taxonomy from the molecular and phenotypic classification

aspects. For example, *S. cerevisiae* and *S. bayanus* are thought to be either two separate species, or the same species, that differ slightly from the physiological aspect (Fugelsang and Edwards 2007). Researchers also noticed the physiological instability of strains belonging to *Saccharomyces sensu stricto* group (Ribéreau-Gayon et al. 2006).

1.3. Population dynamics of wine yeast during spontaneous fermentation

The contribution of yeasts to wine is affected by how they participate in the alcoholic fermentation (Comitini et al. 2011). Yeast species commonly found in spontaneous fermentation can be divided into three groups: aerobic yeast (*Pichia*, *Debaryomyces*, *Rhodotorula*, *Candida*, *Cryptococcus*), apiculate yeast (*Hanseniaspora*) and fermentative yeast (*Kluyveromyces*, *Torulaspora*, *Metschnikowia*, *Zygosaccharomyces* and *Saccharomyces*). Generally, the succession of yeast involves the initial domination of aerobic yeast and apiculate yeast which present on grape surface, the decrease of these yeasts and increase of fermentative yeast as fermentation progresses and final domination of *Saccharomyces* yeasts (Combina et al. 2005a, Ocón et al. 2010, Li et al. 2011, Milanović et al. 2013, Tristezza et al. 2013, de Ponzzes-Gomes et al. 2014, Sun and Liu 2014, Sun et al. 2014). Main yeast species isolated at the beginning of fermentation belong to *Hanseniaspora*, *Metschnikowia* and *Candida* genera.

The dominance of *S. cerevisiae* in fermentation is expected for completing grape must fermentation (Jolly et al. 2014). However, some distinct dynamics were still found depending on the fermentation conditions and relative levels of the major species present. In this sense, different studies found that: *Hanseniaspora* persisted longer in fermentation at low temperature (Andorrà et al. 2010b); *Z. bailii* governed botrytis-affected spontaneous fermentation (Nisiotou et al. 2007); *P. kudriavzevii* emerged along with *Saccharomyces* when relative low ethanol (9%) was obtained at the end of fermentation (Wang and Liu 2013); *Candida* has been reported to dominate late stage of fermentation (David et al. 2014) or to finish alcoholic fermentation (Clemente-Jimenez et al. 2004). Furthermore, recent studies applying culture-independent techniques have found that non-*Saccharomyces* populations persisted during fermentation process (Figure 3, Andorrà et al. 2011).

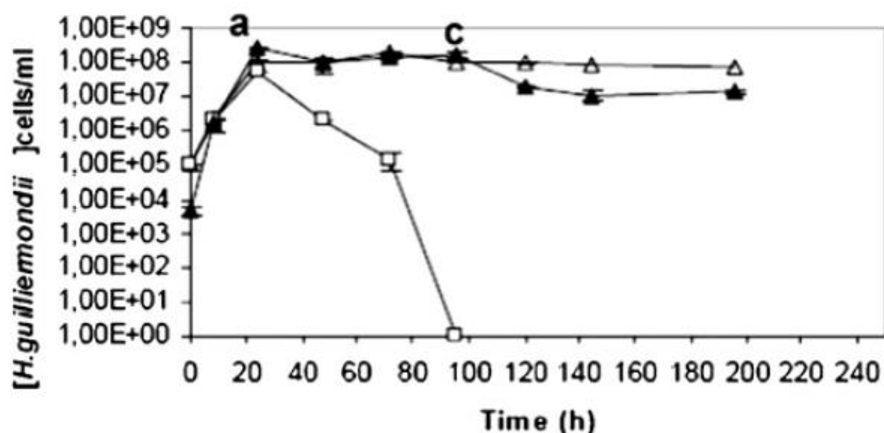

Figure 3 *H. guilliermondii* in mixed fermentation analyzed by FISH (Δ), QPCR (▲) and plating (□) (Andorrà et al. 2011). a: after 24h all plating values are statistically different from those of QPCR and FISH. b: after 96 h all QPCR values are statistically different from those of FISH.

In addition to the succession growth of different yeast species during grape must fermentation, dynamic change of strains within each species is also evident based on molecular techniques for strain differentiation (Fleet, 2003). For *S. cerevisiae*, some dominant or co-dominant strains have been found (Sabate et al. 1998; Torija et al. 2001), and in case where a single strain dominate shows killer phenotype (Schuller et al. 2005). Strain diversity of non-*Saccharomyces* species has also been reported but more focusing on their enological interest than their dynamic change (Capece et al. 2005, Masneuf-Pomarede et al. 2015, Albertin et al. 2016). These reports introduced the genetic and phenolic variation among strains of the same species and indicated that not all the strains of the same species showed the same physiological characteristics such as the different extracellular β-glucosidase activities shown by *H. uvarum* species.

1.4. Controlled mixed culture fermentation

A new tendency for winemaking, controlled mixed culture fermentation, has been highlighted in recent years (Ciani et al. 2010, Mas et al. 2016, Padilla et al. 2016). The main consideration of controlled mixed culture fermentation is to use mixed *Saccharomyces* strains (multi-strains) or mixed *S. cerevisiae* and non-*Saccharomyces* species (multi-species) as an alternative to single culture fermentation. Compared to single culture fermentation, mixed culture fermentation allows obtaining wines with greater flavor complexity. Proper inoculation of known

species can also reduce the risk of stuck fermentation or formation of defective flavor that is usually found from "uncontrolled" spontaneous fermentation.

Over the last few years, several non-*Saccharomyces* species have been commercialized as we mentioned before and designed for mixed fermentation with *S. cerevisiae*. Until now, two main inoculation approaches have been deploited including co-inoculation at high cell concentration with the same or different ratios between *S. cerevisiae* and non-*Saccharomyces*, and sequential inoculation with non-*Saccharomyces* firstly inoculated and delayed use of *S. cerevisiae* (Ciani et al. 2010, Padilla et al. 2016). The application of different inoculation strategies also encourages researchers to take advantage of "selected" indigenous yeast species from each wine region which could reproduce the natural yeast community and reduce the invasion of "foreign" yeast species by inoculation (Mas et al. 2016). All the inoculation strategies are feasible for winemaking practice and a real sense of "controlling" of mixed culture fermentation relies on interaction among inoculated yeast cultures.

Question 2: How can we analyze the states of yeast cells during alcoholic fermentation?

2. Yeast viability during alcoholic fermentation

The evolution of grape must fermentation is characterized by the complexity of yeast population, not only involving diverse yeast species and strains but also harboring different cell states. Millet and Lonvaud-Funel (2000) for the first time reported that some wine microorganisms existed in viable but not-culturable (VBNC) state. Since then, yeast viability in winemaking process has received more attention, because viable yeast and its potential metabolism in the process probably affect final wine quality. Thus, the analysis of yeast cell states during grape must fermentation is helpful for understanding their final contribution. Yeast viability analysis relies on the techniques that we use. In addition to traditional culture-dependent techniques, new techniques without the need of microbial cultivation have been developed in the last ten years. The application of these techniques in wine allows a more comprehensive observation and interpretation of yeast viability.

2.1. Culturable yeast and culture-dependent techniques

In a relative long time, yeast species in grape must fermentation were investigated by isolation on culture media and then classified. Until now, exploitation of yeast resources still depends on isolation of single colonies on solid media, because pure culture of yeast species or strains are necessary for storage, further laboratory analysis, screen and even commercialization in winemaking industry. The yeast which is able to form colony on solid growth media is designated as culturable yeast. Correspondingly, these techniques relying on identification yeast colonies on media is named culture-dependent techniques.

Modern culture-dependent techniques usually apply DNA-based techniques for an accurate identification due to the stability of DNA. Some culture-based techniques such as WL agar and lysine media are used as a combination with DNA-based techniques, largely due to special isolation requirement of some yeast species. WL agar permits differenciation of yeast species by their colony morphology and color (Cavazza et al. 1992) and lysine media is generally used for isolation of non-*Saccharomyces* where lysine serves as the sole nitrogen source (Angelo and Siebert, 1987).

These DNA-based techniques for species identification mostly targets ribosomal DNA, which includes 100 to 200 repeats of transcription units. Each transcription unit contains encoding units (18S rDNA, 5S rDNA, 5.8S rDNA and 26 rDNA), internal transcriber spacers (ITS) and external trascriber spacers (ETS) as shown in Figure 4. Information contained in these regions have been widely used in different techniques to identify yeast species, because the conserved sequences in these regions reveal greater sequence similarity within species than among different species (Kurtzman and Robnett 1998).

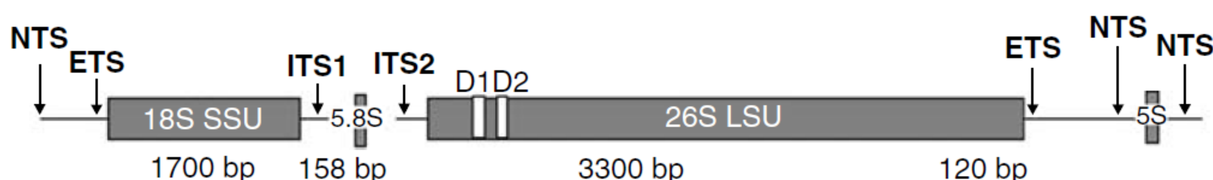

Figure 4. Structure of nuclear ribosomal DNA (Fernández-Espinar et al. 2006)

Culture-dependent techniques for species identification in grape must fermentation are rDNA sequencing analysis (5.8S ITS1/ITS4 or 26S D1/D2, Kurtzman and Robnett 1998, Beltran et al. 2002, Hierro et al. 2006a, Nisiotou et al. 2007, Clavijo et al. 2010, de Ponzzes-Gomes et al. 2014) and rDNA restriction analysis (5.8S-PCR-RFLP, Dlauchy et al. 1999, Esteve-Zarzoso et al. 1999, Granchi et al. 1999, Clavijo et al. 2010, Zott et al. 2008, Cordero-Bueso et al. 2011, David et al. 2014). There is also application of other techniques in wine yeast species identification such as Terminal-Restriction Fragment Length Polymorphism (T-RFLP, Sun and Liu 2014).

2.2. Culture-independent techniques

Wine samples or genetic material extracted from wine samples can be directly analyzed by culture-independent techniques. The emergence of culture-independent techniques could be due to two reasons. On the one hand, the outcome of identification based on culture-dependent techniques are obtained at least two days after sampling due to the necessity of growth on media. The lag-behind information commonly cannot provide instant guidance for grape must fermentation. On the other hand, the ability to grow on media might differ in different species and result in investigation bias (Chambers et al. 2015). In addition, intensive work is necessary for a relative accurate population composition study obtained from plating and isolation.

Conversely, the application of culture-independent methods allows a relative non-targeted, rapid, sensitive and comprehensive investigation of complex microbial communities in grape must fermentation.

Denaturing gradient gel electrophoresis (DGGE)

Polymerase chain reaction coupled with denaturing gradient gel electrophoresis (PCR-DGGE) was applied to differentiate wine yeast isolates (Manzano et al. 2004, Manzano et al. 2005, Di Maro et al. 2007, Nisiotou et al. 2007, Renouf et al. 2007), whereas now has been used as a common culture-independent method to investigate yeast diversity in grape must fermentation (Cocolin et al. 2000, Andorrà et al. 2010b, Cocolin et al. 2011, Milanović et al. 2013, David et al. 2014). The main advantage of this technique is the use of universal primers and the lack of specific primers for each species. Two sets of universal primers (NL1-LS2 and U1-U2) have been reported so far all targeting 26S rRNA (Cocolin et al. 2000, Andorrà et al. 2008). Yeast species identified covers nearly all common yeast species in grape must fermentation such as *S. cerevisiae*, *M. pulcherrima*, *H. guilliermondii*, *H. uvarum*, *Starm. bacillaris* and *T. delbrueckii*. Moreover, several DGGE profiles for *S. cerevisiae* strains were also observed, suggesting its potentiality for strain typing (Manzano et al. 2005).

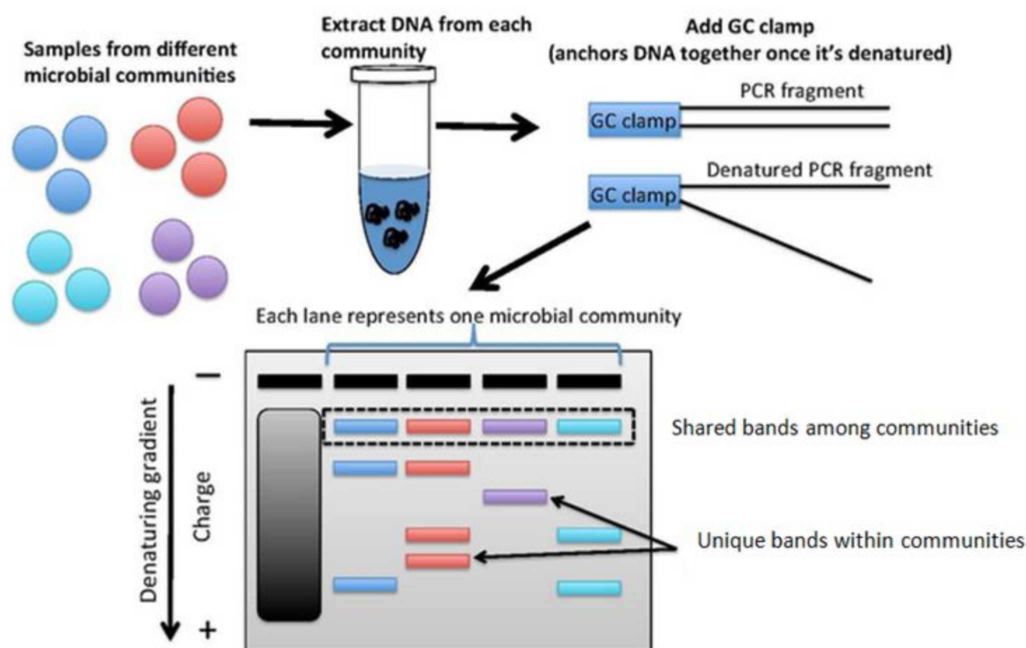

Figure 5 The principle of denaturing gradient gel electrophoresis (DGGE, adapted from Madigan et al. 2009)

A scheme for DGGE analysis is shown in Figure 5. In brief, DNA amplicons are obtained with the same length and a GC clamp at the 5' end. A polyacrylamide gel containing a linear gradient of DNA denaturants (a mixture of urea and formamide) is used to separate DNA amplicons with different sequences. The partially melted double-stranded DNA decreases its electrophoretic mobility in the polyacrylamide gel. And the final position in the gel varied due to different denaturing degree according to DNA sequences. Each band can be excised from gel for sequencing and further determine its identity.

The detection limits of DGGE is in the order of 10^3 cfu/mL (Cocolin et al. 2000), whereas Cocolin et al. (2011) emphasized that detection limit in DGGE analysis can change due to different affinity of primers to target species. Minor species are hardly detected when coexist with other overwhelming majority species (Mills et al. 2002, Prakitchaiwattana et al. 2004, Andorrà et al. 2008, Cocolin et al. 2011). Furthermore, multicopies of rRNA coding genes with small differences result in sequence heterogeneity within the same species. This is the reason why multi-bands can be found in the DGGE profiles of the same species. It makes the analysis of wine sample with many species more complicated (Cocolin et al. 2011).

Besides PCR-DGGE, RT-PCR-DGGE has also been applied to grape must fermentation. RNA is extracted directly from wine samples and subjected to reverse transcription. The cDNA obtained is further analyzed using the same operation for DNA. The combination use of PCR-DGGE and RT-PCR-DGGE helps to get a more complete and accurate yeast diversity, because the profile of RT-PCR-DGGE in some cases is richer than PCR-DGGE (Mills et al. 2002, Urso et al. 2008).

Massive sequencing

Massive sequencing, also named metagenomic sequencing, high-throughput sequencing or pyrosequencing, has been used very recently to determine the relative abundance of microorganism in vine, grape and grape must fermentations (Setati et al. 2012, Bokulich et al. 2014, David et al. 2014, Pinto et al. 2014, Taylor et al. 2014, Pinto et al. 2015, Setati et al. 2015). Massive sequencing owes high sensitivity due to the vast amount of sequence data collected by the techniques. Corresponding data generated highlights significant regional differences in vineyard biodiversity, and thus a hypothesis of "microbial terroir" is proposed mentioning the

possibility that distinct microbial diversity may be responsible for regional wine style (Bokulich et al. 2014, Capozzi et al. 2015, Setati et al. 2015).

Different platforms such as 454 Roche and illumina have been established for massive sequencing (reviewed by Mayo et al. 2014). Common workflow is shared by these platforms containing DNA library preparation, multiplex sequencing and raw data analysis. Massive sequencing has a high requirement of DNA quality out of the need of precision. However, compared to the operation, what is more important is the way of forming DNA library (reviewed in Huggett et al. 2013). Now the approach used in winemaking field is to use PCR to form library for each sample, referred to as targeted metasequencing. Then multiplex sequencing is used to analyze multiple samples simultaneously each containing thousands of molecules from the DNA library. Multiplexing sequencing is realized by using unique molecular identifiers (MIDs) which are tagged to samples during library construction (Figure 6). The successful application of massive sequencing also relies on the proper reading and interpretation of a host of high quality sequences collected, which largely rest on the database selected for the identification of genera or species if taxonomy-dependent methods are used. Prokaryotic microorganisms can be searched on ribosomal database project (RDP) database, whereas eukaryotic microorganisms can be identified on SILVA and GenBank databases (Pinto et al. 2014).

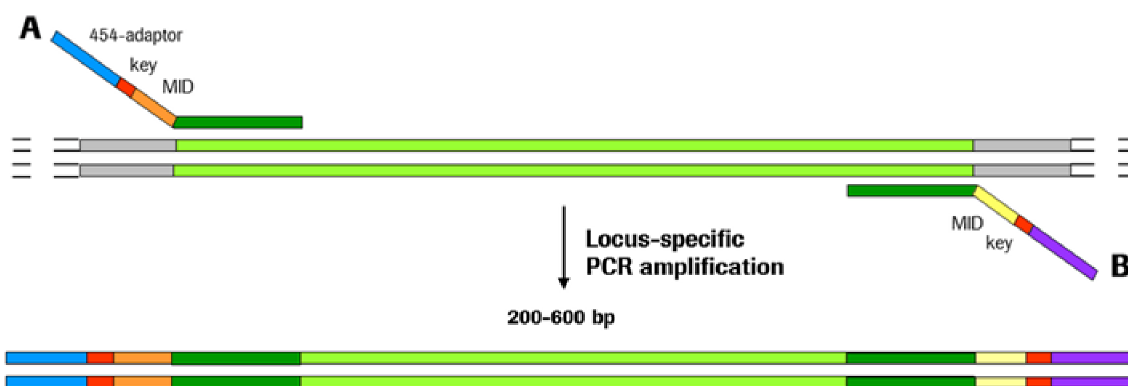

Figure 6 The addition of MIDs to target DNA by PCR in 454 Roche platform. A. improved forward primer. B. improved reverse primer.

For targeted metasequencing, the selection of primers determines analysis of target microorganisms, but all of them usually are designed on the ribosomal region. Recent studies mainly applied primers targeting D1/D2 region of 26S rDNA (Taylor et al. 2014, Pinto et al. 2014, Pinto et al. 2015), ITS region of 5.8S rDNA (Bokulich

and Mills 2013, Bokulich et al. 2013, Pinto et al. 2014, Pinto et al. 2015, Setati et al. 2015) and 18S rDNA (David et al. 2014). Bokulich and Mills (2013) suggested that no primer pair could exactly reconstruct microbial distribution from a complicated community. Taylor et al. (2014) chose D1/D2 region instead of ITS region because length polymorphism of ITS region may cause PCR and sequencing bias and D1/D2 region present a comprehensive reference database allowing species identification. However, other researchers (Pinto et al. 2014, Pinto et al. 2015) regarded that combination use of D1/D2 region and ITS region would allow for the highest coverage of eukaryotic organisms. According to their reports, the use of different primers generates different population structure in ecology investigation and yet the operational taxonomic units (OTUs) of microorganisms are similar (Figure 7).

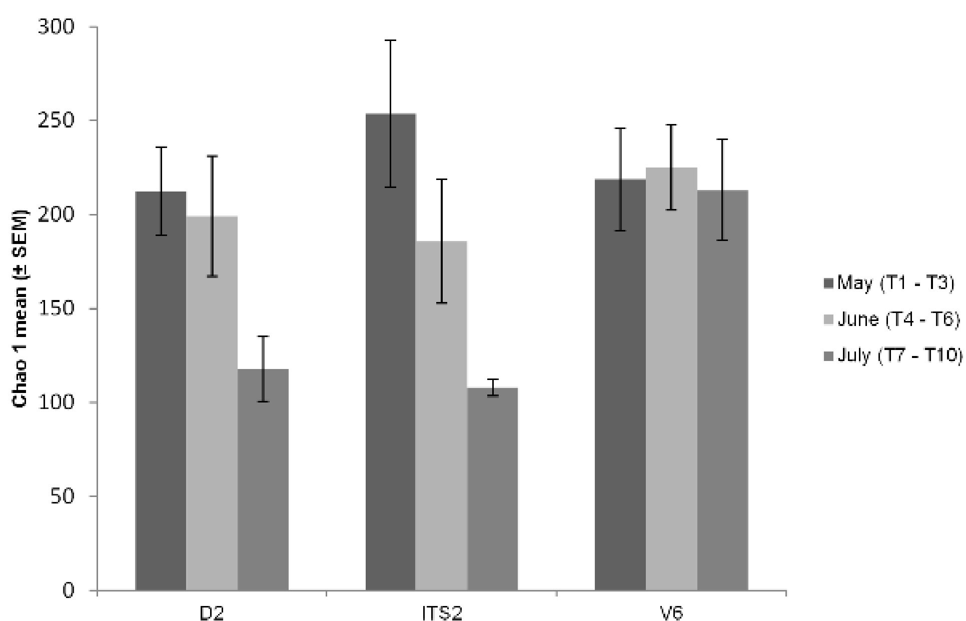

Figure 7 Biodiversity dynamics associated with D2 (fungi), ITS2 (fungi) and V6 (bacteria) region during the vegetative cycle of grapevine by massive sequencing (Pinto et al. 2014).

Quantitative PCR (qPCR)

Quantitative PCR (qPCR), also called real time PCR, applies fluorescent dye such as SYBR Green or fluorescently-labeled nucleotide probe to show the progress of a PCR reaction, because fluorescent signal increases in direct proportion to PCR product formed during reaction. The thermocycler used for qPCR has a detection system able to quantify signal at the end of each cycle and representing corresponding information into an amplification curve. The amplification curve provides a cycle number, called threshold cycle (Ct), at which fluorescent intensity

started exponential increase compared with the background signal. This technique permits quantification of cell concentrations, because good linearity can be built between the quantity of cells and threshold cycle (Ct). Therefore, qPCR can realize simultaneous identification and cell quantification on the basis of a well-built standard linearity curve (Figure 8).

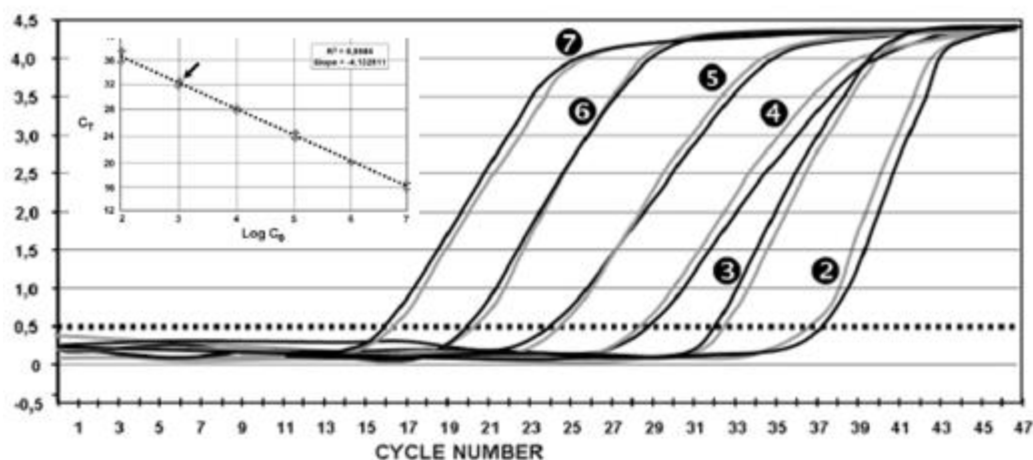

Figure 8 Fluoresence signal increase in the progress of qPCR reactions and a well-built standard linearity curve.

qPCR is a fast technique with high sensitivity and wide examination range of cell concentrations (from 1 or 10 cells/mL to 10^8 cells/mL depending on yeast species, Phister and Mills 2003, Rawsthorne and Phister 2006). These advantages make qPCR to be a useful technique for routine analysis (Andorrà et al. 2012, Albertin et al. 2014). To realize these advantages, the proper design of the primers or probes is very demanding. For wine yeast, different primer sets have been designed mainly targeting transcription units of rDNA such as ITS2, 5.8S rDNA and 26S rDNA (Figure 4). Therefore, several wine-related yeast genera or species can be identified and quantified during grape must fermentation, including *C. zeylanoides* (Díaz et al. 2013), *Hanseniaspora spp.* (Hierro et al. 2007, Phister et al. 2007, Zott et al. 2010), *H. uvarum* (Díaz et al. 2013), *M. pulcherrima* (Zott et al. 2010, Díaz et al. 2013), *P. angusta* (Díaz et al. 2013), *P. anomala* (Díaz et al. 2013), *P. kluyveri* (Díaz et al. 2013), *P. kudriavzevii* (Zott et al. 2010), *Rhodotorula mucilaginosa* (Díaz et al. 2013), *Saccharomyces spp.* (Hierro et al. 2007, Zott et al. 2010), *S. cerevisiae* (Díaz et al. 2013), *Starm. bacillaris* (Andorrà et al. 2010b, Zott et al. 2010), *T. delbrueckii* (Zott et al. 2010, Díaz et al. 2013), *Williopsis saturnus* (Díaz et al. 2013).

Although species-specific qPCR has been well developed, there are still no reports of strain-specific qPCR for wine yeast in alcoholic fermentation. The deep "reading" of massive genome sequences obtained by next generation sequencing is likely to promote the development of strain-specific qPCR (Bokulich and Mills 2012).

In addition, qPCR targeting cDNA after reverse transcription of rRNA (RT-qPCR) has also been proposed for yeast investigation in winemaking field. Based on RT-qPCR using universal primer tageting total yeast, standard curves could be established using yeast cells from wine sample and the detection limit could reach 10^3 cfu/mL (Hierro et al. 2006a). The application of RT-qPCR for specific yeast species has not been reported but will be meaningful for understanding the viability of cells (Postollec et al. 2011).

Techniques combined with fluorescence microscopy

An application of the fluorescence microscopy is the estimation of yeast identity and viability through some molecules, which absorb light of a specific wavelength and emit fluorescence of a lower energy and longer wavelength (Fugelsang and Edwards 2007). Some specific dyes from commercial kits can be used directly for discriminating viable and dead cells (Zhang and Fang 2004), whereas other methods including direct epifluorescence filter techniques (DEFT), fluorescence ratio imaging microscopy (FRIM) and fluorescence in situ hybridization (FISH) are combined with fluorescence microscopy (Divol and Lonvaud-Funel 2005, Andorrà et al. 2011, Branco et al. 2015). Techniques combined with fluorescence microscopy are suitable for routine analysis due to its simplicity. However, fluorescence microscopy has a relative low detection limit (10^4 cells/mL) as well as a low sensitivity which depends on the number of cells observed.

— Commercial kit for viability test

LIVE/DEAD BactLight™ Bacterial Viablility Kit is a commercial kit comprised of two stains: SYTO 9 and propidium iodide (PI). SYTO 9 is a green-fluorescent nucleic acid stain which can permeate into all cells, whereas PI is a red-fluorescent nucleic acid stain only penetrating cells with damaged membranes. Therefore, when both stains are combined used, cells with intact membranes emit green fluorescent and cells with damaged membranes emit red fluorescent. The kit has been used to distinguish membrane integrity of yeast cells (Zhang and Fang 2004, Hierro et al.

2006a, Andorrà et al. 2010a), but direct analysis of yeast in wine samples has not been applied since it is not species selective.

— Fluorescence in situ hybridization (FISH)

FISH uses short sequences of exogenous oligonucleotide (hybridization probe), which is fluorescently labeled, to hybridize with the complementary sequence of interest in DNA or RNA. DNA or RNA unwinds at elevated incubation temperature and thus helps the hybridization probe to combine with target complementary sequence in DNA or RNA. FISH for identification and enumeration of wine yeasts is designed targeting the rRNA of a species (Amann et al. 1995). The rRNA-targeted hybridization probe can diffuse to interior of yeast cells after the fixation in the first step, by which cell morphology is stabilized and cell membrane is permeabilized (Amann and Fuchs 2008). The probe further forms specific hybrids with its intracellular targets by hybridization (Figure 9). After washing away the excess probe, single-cell identification can be performed by fluorescence microscope (Bottari et al. 2006). Therefore, FISH can favor simultaneous identification and cell counting in one sample.

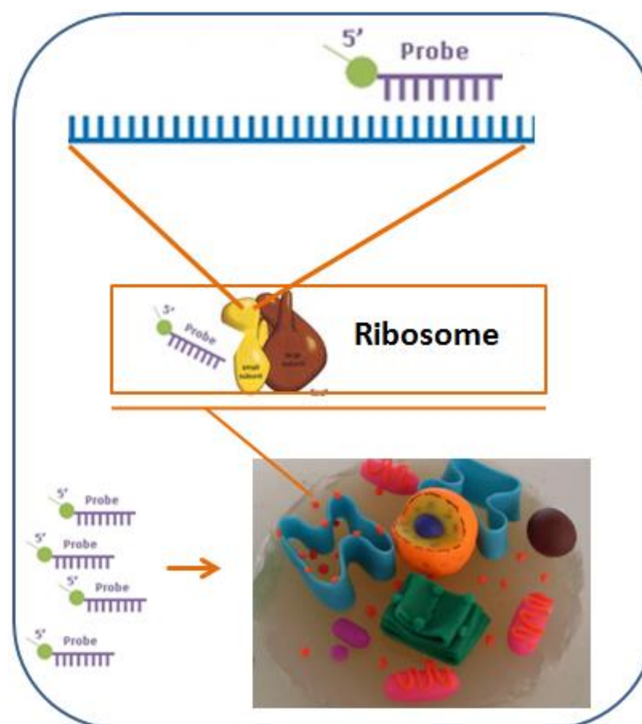

Figure 9. Hybridization process in FISH: probes enter permabilized cells, access to ribosome and hybridize with target rRNA sequences.

Early studies has reported FISH probes for several wine-related yeast species, containing *D. bruxellensis* (Stender et al. 2001), *S. cerevisiae* (Inácio et al. 2003,

Xufre et al. 2006), *C. stellata*, *H. uvarum*, *H. guilliermondii*, *K. thermotolerans* (now named L. *thermotolerans*), *K. marxianus*, *T. delbrueckii*, *P. membranaefaciens* and *P. anomala* (Xufre et al. 2006). However, all the early investigations on target species using FISH applied a preculture procedure to collect yeast cells. So far only probes for *D. bruxellensis* and *S. cerevisiae* have been successfully used for direct yeast identification in wine samples (Röder et al. 2007, Andorrà et al. 2011).

The difficulty for FISH application in wine samples lies in the poor signal-background ratio (Fröhlich et al. 2009). To overcome this difficulty, several factors should be considered if oligonucleotide probe is designed, containing specific target site, proper fluorescence dye used for labeling with the specific sequence and accessibility of probe binding site based on higher-order structure of ribosome (Inácio et al. 2003, Amann and Fuchs 2008, Yilmaz et al. 2011). The commonly used target site for yeast is D1/D2 region of 26S rRNA, whereas the identification of other sequence region with high interspecies variation beyond this region would provide new possibility for probe design (Röder et al., 2007). The most used labels in FISH for wine yeast are carbocyanine 3 (CY3) and fluorescein isothiocyanate (FITC) giving yellow and green emission when properly excited. The accessible binding sites were firstly investigated by Inácio et al. (2003), who found that most accessible sites do not own enough variation for species identification. Attempts have been performed focusing on developing helpers (Fuchs et al. 2000), catalyzed reporter deposition-FISH (Sekar et al. 2003, Amann and Fuchs 2008), side probes (Röder et al. 2007), peptide nucleic acid probes (Stender et al. 2001, Amann and Fuchs 2008, Almeida et al. 2010), locked nucleic acid probes (Kubota et al. 2006, Amann and Fuchs 2008) and double-labeled oligonucleotide probes (Behnam et al. 2012). However, no reports on these new trials have targeted *Saccharomyces* and non-*Saccharomyces* yeast in grape must fermentation.

Techniques combined with flow cytometry

Flow cytometry is a technology of analyzing cells or particles (0.2 to 50 μm) that are in liquid suspension. Multiple characteristics of single cells or particles including relative size, internal complexity and fluorescence intensity can be simultaneously measured as cells or particles pass through a beam of light in flow cytometer. A flow cytometer is composed of three main subsystems: fluidics system which brings particles to the interrogation point where they intersect with the

excitation light, optics systems providing light source and collecting light signals, and electronics system converting light signals to electronic signals which will be further processed to form data in the computer (Figure 10). Flow cytometry is a powerful technique for rapid analysis of single cells in a mixture which passes thousands of cells per second (Díaz et al. 2010). Flow cytometry also own a high sensitivity because it can detect one target cell in one to ten millions of cells depending on the cytometer.

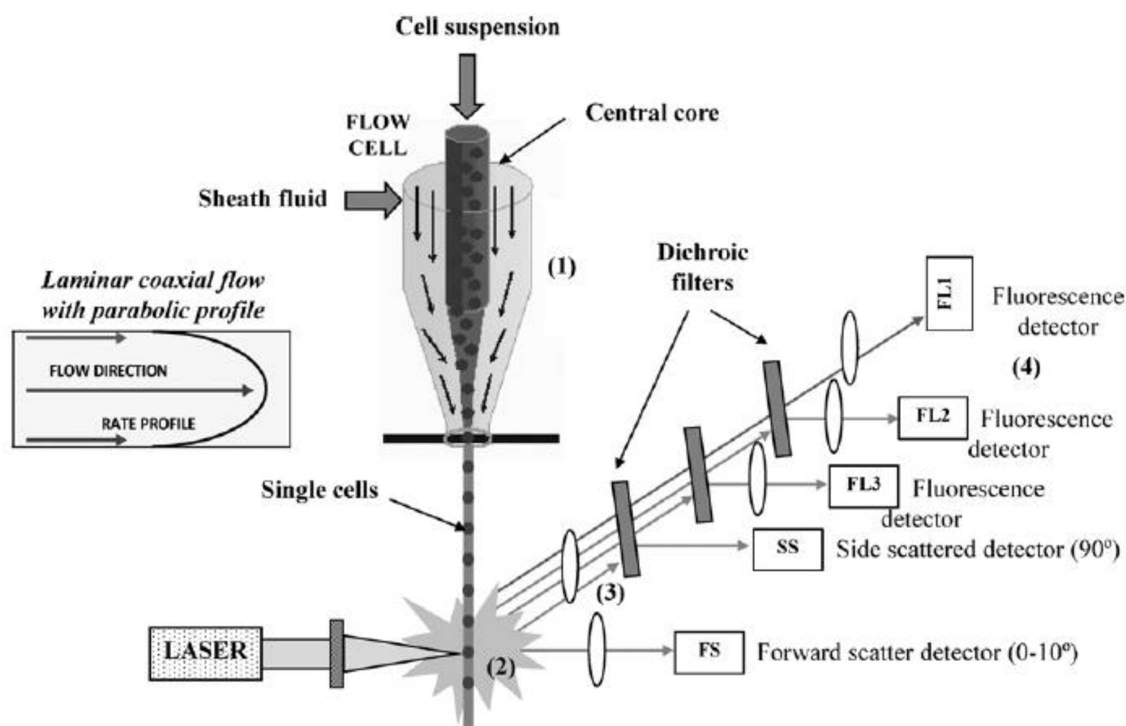

Figure 10 Scheme of a typical flow cytometer: (1) The formation of a single stream of particles in the flow cell by hydrodynamic focusing, (2) cells impact with laser beam and emit signals related with cell parameters, (3) Scattered and fluorescence signals emited by each cells are separated by filters and mirrors according to wavelengths, (4) These signals are seperactely collected by different detectors and sent to a computer for further data processing (Díaz et al. 2010).

Most reports on application of flow cytometry in wine alcoholic fermentation focus on the cell state of *S. cerevisiae* due to its important significance for completing fermentation (Bruetschy et al. 1994, Attfield et al. 2000, Malacrino et al. 2001, Bouchez et al. 2004, Chaney et al. 2006, Farthing et al. 2007, Rodriquez and Thornton 2008). Membrane integrity, DNA, esterase activity, enzymatic activity and microbial interactions were analyzed in these reports (Reviewed in Díaz et al. 2010). Recently, populations of *S. cerevisiae* and *H. guilliermondii* during grape must

fermentation were followed by combination of FISH and flow cytometry (Andorrà et al. 2011, Branco et al. 2012). The design of species-specific probes will enable simultaneous analysis of different wine yeast by flow cytometry in the future.

Development of culture-independent techniques

In spite of their advances, none of the culture-independent techniques is perfect. Techniques based on DNA analysis cannot distinguish between viable and nonviable cells; Techniques using specific primers or probes are limited to finding yeast species which have previously been identified. If a new species is unexpected in grape must or fermentation, although the chance is low nowadays, it may not be detected using techniques with high specificity (Bisson and Joseph 2009). Researchers also complained that no available culture-independent techniques for monitoring yeast populations at strain level (Fröhlich et al. 2009). Due to these disadvantages, improvements of corresponding techniques have been explored such as the combination of qPCR with treatment of ethidium monoazide bromide or propidium monoazide bromide (EMA-qPCR or PMA-qPCR, Andorrà et al. 2010a) and live/dead staining with FISH (Branco et al. 2012). Furthermore, combined use of more techniques is usually taken in a yeast resources investigation in grape must fermentation (Andorrà et al. 2010b, Andorrà et al. 2011, Cocolin et al. 2011, Milanović et al. 2013, David et al. 2014, Branco et al. 2015).

2.3. Different states of viable cells

During the process of grape must fermentation, yeast cell survival is paramount to ensure efficient bioconversion of biomass and metabolite from nutritional substrate. However, alcoholic fermentation indeed provides a stressful and competitive environment, where some yeast might be stressed, damaged and die off when confronting with harsh condition out of their tolerance. Culture-independent techniques offer the possibility to observe different cell states beyond its culturability and characterize cell population by structural and functional cell properties such as metabolic activity (Díaz et al. 2010). Therefore, in addition to reproductively viable cells and dead cells, there are several intermediate cell states including metabolically active cells, membrane integral cells, rRNA stable cells and DNA stable cells (Figure 11). Viability of these intermediate cell states cannot be simply justified based on some cell property, and generally membrane integrity and

rRNA stability are recognized to be more related with final justification. Furthermore, most information on detailed states of yeast is obtained from *S. cerevisiae*. Although there is increasing concern on viability of non-*Saccharomyces* yeast, more studies on different species are still required for full investigation.

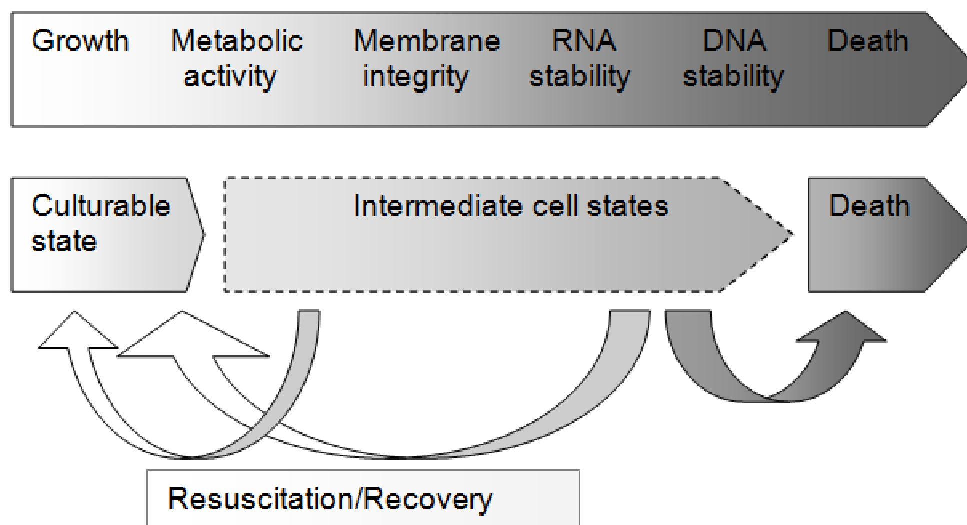

Figure 11 Structural and functional criteria to determine different levels of cell viability (adapted from Díaz et al. 2010).

Culturable state

Culturable states of yeast cells refer to viable and healthy cells which have the capacity of growing and reproducing on microbiological media. For sure, cells in culturable state keep metabolic activity, membrane integrity and DNA/RNA stability. In early stages of grape must fermentation, most yeast cells stay in culturable states, whereas at late stages non-*Saccharomyces* species cannot maintain the culturable states showing “disappearance” based on culture-dependent techniques (Andorrà et al. 2011).

Intermediate cell states

Cells in intermediate states probably lose their culturability temporally due to cell damage or a higher requirement for culture conditions (Díaz et al. 2010). However, their viability can still be measured by metabolic activity, membrane integrity, rRNA stability and DNA stability.

Metabolic activity of cells can be presented by enzymatic activity, substrate transportation of cell pumps or biosynthesis of macromolecules. Divol and Lonvaud-Funel (2005) applied DEFT followed esterase activity of yeast cells under SO_2 stress

during grape must fermentation. They found that the fluorescence intensity marking esterase activity decreased rapidly to nearly a half of original intensity and maintained this level until the stress was removed. After removal of SO_2 stress, fluorescence intensity could recover to original level. In spite of the low fluorescence intensity observed from cells in transition state, the intensity is still higher than dead cells. Díaz et al. (2010) regarded that activity of cell pumps and biosynthesis of macromolecules could reflect metabolic activity of a cell more instantly and accurately than enzymatic activity. However, few reports on the two aspects have been given on wine yeast during alcoholic fermentation.

Cells with intact membrane, which is referred to as membrane integrity, can generate different ion level inside and outside of the cells which is linked to ATP formation (Shapiro et al. 2000). The difference decreases to zero when membrane is structurally damaged allowing ions pass the membrane freely. Membrane integrity can be detected by pH gradient inside and outside of cells and dye exclusion methods. Branco et al. (2015) measured pH gradient of *H. guilliermondii* cells through FRIM and found that non-culturable cells showed a decreased or even no difference of extracellular and intracellular pH. PI is the most commonly used dye which can passes compromised membrane (Haugland 2002). In commercial kit, PI is usually combined used with SYTO 9 to double confirm permeability of cell membrane (Zhang and Fang 2004, Hierro et al. 2006a, Andorrà et al. 2010a). In addition, DNA binding dyes such as EMA and PMA are also demonstrated to be able to penetrate injured membranes which will further hamper DNA amplification by covalent combination with DNA (Rudi et al. 2005). The treatment of EMA or PMA has been combined used with qPCR to quantify cells with membrane impermeable to EMA or PMA (Rawsthorne and Phister 2009, Andorrà et al. 2010a, Shi et al. 2012, Vendrame et al. 2014).

RNA has a relative stability compared to plasma membrane and yet is thought to be degraded rapidly after cell death (Hierro et al. 2006a). As a consequence, culture-independent techniques based on RNA analysis are considered to be able to distinguish live and dead cells. Furthermore, these techniques can perform simultaneous identification at the species level, and thus have been paid more attention in yeast investigations during alcoholic fermentation. No matter if RNA extraction is needed or not, relying on techniques based on RNA analysis such as RT-PCR-DGGE, FISH and flow cytometry, reported similar results: non-

Saccharomyces yeast could maintain high levels of RNA after losing culturability (Cocolin and Mills 2003, Andorrà et al. 2011). Furthermore, Andorrà et al. (2011) doubted that RNA degradation speed can be varied due to different death treatments and yeast species involved. Thus more studies are needed to fully explain the relationship between cell death and RNA degradation during late stage of alcoholic fermentation.

DNA is much more stable than RNA, and is regarded able to persist long time after the cell death (Cangelosi and Meschke 2014). Thus, in spite of their powerful identification function, direct DNA analysis such as qPCR and massive sequencing is doubted of overestimating non-*Saccharomyces* populations at late stage of fermentation. Recently, EMA-qPCR and PMA-qPCR has been used to follow viable yeast population in grape must fermentation to avoid quantifying DNA from dead yeast cells. One log unit less population was observed in EMA/PMA-qPCR than in direct qPCR, verifying viability of these non-*Saccharomyces* yeast (Andorrà et al. 2010a).

2.4. Viable but not-culturable yeast

Viable but not-culturable (VBNC) is a term describing cells that are not able to grow on culture media yet still presents measurable viability (Oliver, 2005). Theoretically, all cells in intermediate states could be named VBNC cells as long as they are not culturable. The existence of VBNC state for wine yeast in alcoholic fermentation has been demonstrated by different techniques as noted in 2.3. However, it is still difficult to precise evaluate all VBNC cells due to several intermediate states probably involved. Díaz et al. (2010) recommended combining structural and functional strategies in the applications such as using fluorescein diactetate for detecting esterase activity and PI for checking membrane integrity (Divol and Lonvaud-Funel 2005, Schenk et al. 2011). In addition, recovery ability of VBNC cells are also investigated considering that cells in VBNC state may be resuscitated if favorable condition is provided, because VBNC state is generally induced by stressful environmental condition (Divol and Lonvaud-Funel 2005, Salma et al. 2013, Branco et al. 2015). The free entry into the VBNC state when stress is set and exit from the VBNC state when stress is removed could illustrate the existence of VBNC yeast cells. Salma et al. (2013) reported that once removed the

limitation factor *S. cerevisiae* cells could recover from VBNC state even if these cells have kept the VBNC state over a long period of time (21 days, Figure 12).

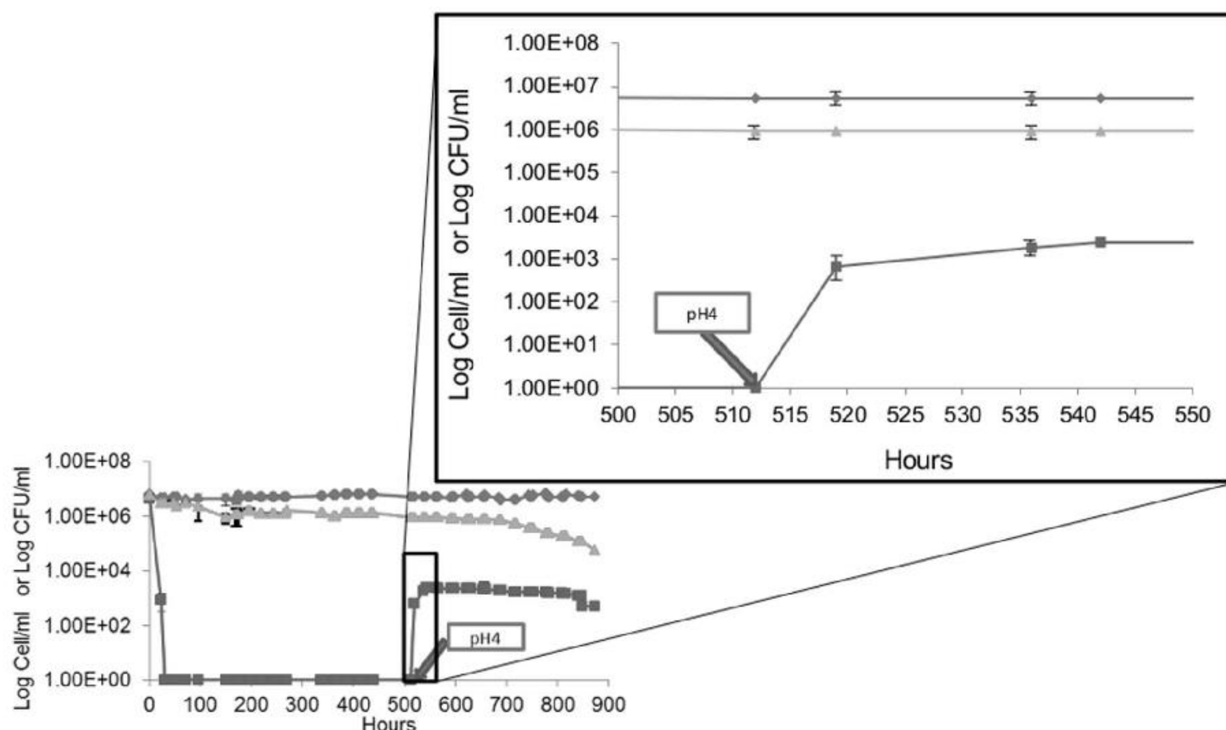

Figure 12 The induction of VBNC state and resuscitation from VBNC state for *S. cerevisiae* S288C in synthetic wine (Salma et al. 2013). The addition of 4.5 mg/L molecular SO_2 is performed at time 0 and the removal of SO_2 effect by increasing pH is carried out at 500h. Total cell counts (♦), culturable counts (■), and viable counts by fluorescein diacetate and FUN-1(▲).

The contribution of VBNC cells during grape must fermentation is still not clear. However, the existence of these cells in aging stage is probably a potential threat to wine stability (Divol and Lonvaud-Funel 2005, Salma et al. 2013).

Question 3: What limits the growth of non-Saccharomyces during alcoholic fermentation?

3. Factors related with yeast growth during alcoholic fermentation

Marsit and Dequin (2015) adopt “fluctuating environment” to describe grape must fermentation, which exposes yeast to various stress (Figure 13): osmotic pressure, anaerobic condition, ethanol, nutrient depletion, temperature and SO_2. The specific environmental conditions in the must are thought to play a role in determining which species can survive and grow, referred to as physiological fitness. In addition to the influence of environment, the growth of specific yeast species in grape must fermentation is also affected by the presence of other yeast species. Recent studies have pointed out the culturability loss of non-*Saccharomyces* at late stage of fermentation is associated with their interaction with *Saccharomyces* and two main mechanisms are involved in the interaction: contact-dependent mechanism and chemical interaction such as production of antimicrobial peptides (Albergaria and Arneborg 2016). More knowledge on yeast interactions and related mechanisms are important for a successful alcoholic fermentation especially when we consider the use of mixed starter cultures (Ivey et al. 2013).

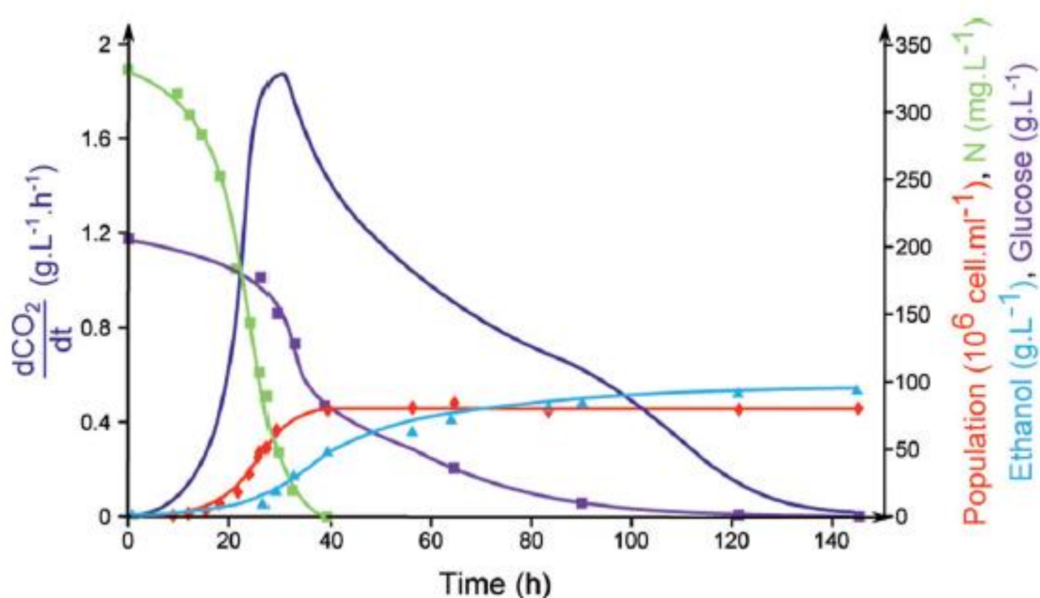

Figure 13. Evolution of the main fermentation parameters during wine fermentation (Marsit and Dequin 2015). A synthetic medium was fermented by strain EC1118 at 24°C. Dark blue: fermentation rate; light blue: ethanol; red: cell number; green: nitrogen; purple: sugars.

3.1. Environmental changes

Osmotic pressure

Osmotic pressure is the first stress that yeast encounters after inoculation. It is formed by high sugar concentration in grape must (> 200g/L). Yeast cells need a lag-phase to adapt and then start to reproduce rapidly. In the adaptation process, yeast cells accumulate intracellular glycerol to counteract osmotic pressure (Heinisch and Rodicio 2009). Generally, both *Saccharomyces* and non-*Saccharomyces* yeast can propagate to 10^7 to 10^8 cells/mL after adaptation. In addition, Divol and Lonvaud-Funel (2005) indicated high osmotic pressure affected the resuscitation of yeast cells from VBNC state to culturable state. Thus half-diluted must with sugar concentration of 75 g/L was used to avoid the influence of osmotic pressure on fragile VBNC cells.

Low oxygen availability

Sugars are converted to carbon dioxide and ethanol as fermentation goes on. Continual production of carbon dioxide limits the growth of aerobic species due to the removal of oxygen (Visser et al. 1990, Fleet, 2003, Brandam et al. 2013). Yeast metabolism during winemaking requires oxygen for the synthesis of sterols and fatty acids and thus the amount of available oxygen is crucial for yeast growth and fermentation performance in the process (Hanl et al. 2005). Hansen et al. (2001) mentioned that *S. cerevisiae* is more tolerant to low oxygen levels compared to other yeast. Nissen et al. (2004) further explained how oxygen increased coexistence of *L. thermotolerans* and *T. delbrueckii* with *S. cerevisiae* by the theory of relative glucose uptake abilities. Morales et al. (2015) also indicated that *M. pulcherrima* grew and consumed sugar better under aerobic conditions than anaerobiosis. In mixed fermentation with *S. cerevisiae*, culturable *M. pulcherrima* population started to decrease on day five with only nitrogen as provided, on day six when 10% air is supplied and on day seven with 25% air. Although the population decrease of non-*Saccharomyces* was postponed with more air addition, there are no conclusive proofs that the low oxygen availability forces non-*Saccharomyces* yeast to enter VBNC state.

Ethanol

Upon ethanol production, yeast cells stop breeding and enter stationary phase. Marks et al. (2008) postulated that this transition is caused by ethanol concentrations above 2%. Conversion of must sugars to ethanol (> 10%) is reported to increase membrane permeability and change membrane fluidity. Protons and protein unfolding in plasma membrane are especially affected (Heinisch and Rodicio 2009). Therefore, high ethanol concentration at late stage of fermentation is considered a major hurdle for yeast growth, especially for non-*Saccharomyces* (Fleet, 2003).

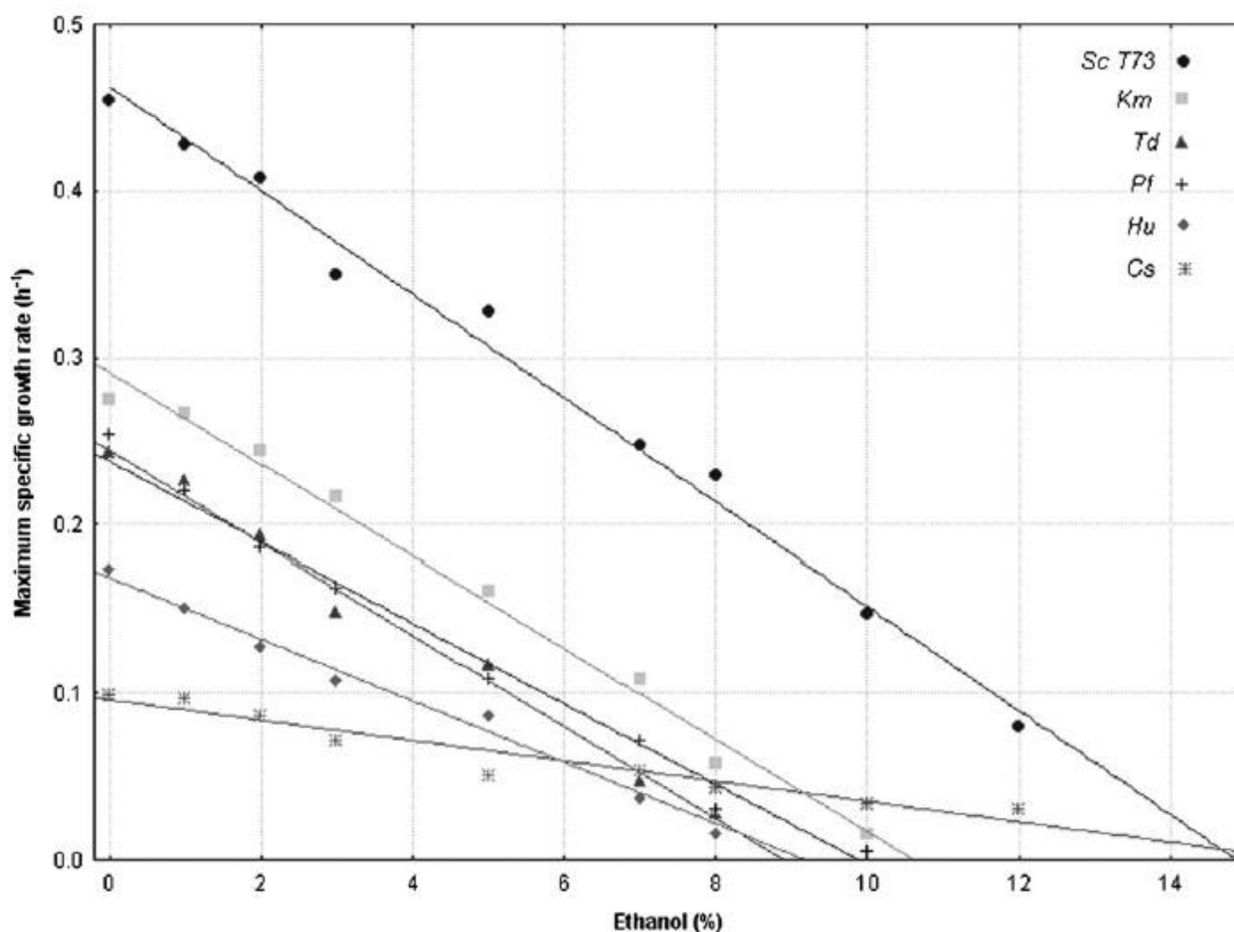

Figure 14 The influence of ethanol concentration on maximum specific growth rates of the non-*Saccharomyces* species *H. uvarum* (Hu), *C. zemplinina* (Cs), *T. delbrueckii* (Td), *P. fermentans* (Pf), *K. marxianus* and *S. cerevisiae* (ScT73). Data from Salvadó et al. (2011).

Noteworthy, some ethanol tolerant strains of non-*Saccharomyces* were isolated at late or end of spontaneous fermentation (Nurgel et al. 2005; Wang and Liu 2013). Recent studies also found that some non-*Saccharomyces* strains of *H. uvarum*, *H. guilliermondii*, *T. delbrueckii* and *Starm. bacillaris* could tolerate ethanol of 10% (Pina et al. 2004, Pérez-Nevado et al. 2006), which is higher of those

concentrations considered in the past around 5-7% (Heard and Fleet 1988, Gao and Fleet 1988). Salvadó et al. 2011 reported that maximum specific growth rate of yeast decreased linearly as the increase of ethanol from 0% to 25% and no growth was found for all yeast when ethanol was higher than 16%. The most resistant yeast was *Starm. bacillaris* (synonym *C. zemplinina*) the minimum inhibitory concentration for which is 15.6%. The second was *S. cerevisiae*, which was followed by *K. marxianus*, *P. fermentans* and *H. uvarum*. *T. delbrueckii* was the least resistant yeast which was not able to tolerant 8.9% of ethanol. Although *S. cerevisiae* presented the highest specific growth rate, the reduction of the growth rate as ethanol increase was also the fastest showing that *S. cerevisiae* was the most influenced species by the increase of ethanol (Figure 14, Salvadó et al. 2011).

Why some yeast species such as *Saccharomyces* can cope with the stress from ethanol? Ethanol stress may trigger multiple cells responses, containing (1) inhibition of cell cycle and propagation, (2) accumulation of trehalose and glycogen, (3) increase in heat shock proteins, (4) increased activity of plasma membrane ATPase and levels of oleic acid and ergosterol in membrane, (5) induction of genes encoding vacuolar proteases and their inhibitors and (6) increased activation of genes related with unfolded protein response and its transcription factor (Heinisch and Rodicio 2009, Navarro-Tapia et al. 2016). These responses make wine yeast to endure ethanol, whereas most mechanisms are checked in the model species *S. cerevisiae*. Little is known about the mechanism related with ethanol tolerance in non-*Saccharomyces* yeast.

Interestingly, Marsit and Dequin (2015) reviewed a “make-accumulate-consume” strategy used by *Saccharomyces* yeast: they rapidly consume a high quantity of sugars, transform these carbohydrates into ethanol, which help them to establish competitive dominance in the ecological niche, and then catabolize ethanol for energy. However, whether ethanol directly induces culturability loss of non-*Saccharomyces* yeast or not still need more evidences (Salvadó et al. 2011).

Nitrogen limitation

Yeast assimilable nitrogen (YAN) is mainly made up of ammonium ions and amino acids, which is present in limited amounts in grape must and yet is directly associated with yeast biomass and thus fermentation rate. Amino acids are directly utilizable by yeast, whereas ammonium is firstly changed into glutamate by yeast

cells and glutamate can be further used to yield other amino acids such as glutamine, asparagine, histamine, arginine and tryptophan (Fugelsang and Edwards 2007). It is generally accepted that 140 mg N/L of YAN is necessary for fermenting 200 g/L of sugar (Martínez-Moreno et al. 2012), whereas 300 mg N/L of YAN is needed for an optimized fermentation (Marsit and Dequin 2015).

As fermentations progress, nutrient supplies become limited or exhausted and become source of stress. Preliminary findings indicated that less nitrogen was consumed in mixed fermentation of *S. cerevisiae* and *H. uvarum* than in pure culture and thus people hypothesized that YAN competition did not exist between *S. cerevisiae* and apiculate yeast (Ciani and Comitini 2015). However, Fleet (2003) insisted that non-*Saccharomyces* present in early stage of fermentation probably deplete amino acids and lead to the deficient growth of *S. cerevisiae*. Evidence has been provided by Bisson (1999) and Taillandier et al. (2014) that the early growth of *K. apiculata* (now named *H. uvarum*) and *T. delbrueckii* lead to nitrogen exhaustion and further sluggish fermentation. Evident competition for nitrogen has also been shown in sequential fermentation inoculated with *S. cerevisiae* and *M. pulcherrima* or *H. vinae* (Medina et al. 2012). Therefore, in wine industry nitrogen is generally added to secure fermentation with low initial nitrogen level. Combined addition of oxygen and nitrogen is regarded to be able to efficiently prevent sluggish alcoholic fermentation (Sablayrolles et al. 1996).

Nitrogen limitation probably impacts on yeast growth pattern during grape must fermentation which depends on the different nitrogen requirements from different wine yeast species (Ciani and Comitini 2015, Albergaria and Arneborg 2016). The nitrogen requirement, metabolic mechanism and genetic basis have been well studied for *S. cerevisiae* in recent reports (Gutiérrez et al. 2012, Martínez-Moreno et al. 2012, Gutiérrez et al. 2013, Brice et al. 2014, Brice et al. 2015). However, specific nitrogen requirement of non-*Saccharomyces* yeast have not yet been fully characterized especially in grape must fermentation. Albergaria (2007) reported that the addition of complex nutrient such as yeast extract and peptone partially improved the fermentation capacity of *H. uvarum*, *H. guilliermondii* and *Starm. bacillaris*. Andorrà et al. (2012) studied nitrogen consumption of *S. cerevisiae*, *H. uvarum* and *Starm. bacillaris* in pure and mixed fermentation and indicated that the two non-*Saccharomyces* yeast were less effective at converting amino acid to biomass than *S. cerevisiae* but contributed more to aromatic compounds formation.

Kemsawasd et al. (2015b) described the nitrogen sources (nineteen amino acids, ammonium sulphate and two multiple nitrogen sources) beneficially impacting on growth, glucose consumption and ethanol production of *S. cerevisiae*, *L. thermotolerans*, *M. pulcherrima*, *H. uvarum* and *T. delbrueckii*. In addition to the single amino acids preference, multiple nitrogen sources had a positive effect on *S. cerevisiae* and *T. delbrueckii*, whereas showed a similar effect as single amino acids on the other three non-*Saccharomyces*.

Temperature

High temperature stress disrupts hydrogen bonding and denatures proteins and nucleic acids, which causes damage of yeast cells (Walker and Dijck 2006). Vigorous alcoholic fermentation can first cause a temperature rise and then a significant drop, which can provoke excessive rigidity in yeast membranes (Zamora 2009). Nevertheless, the temperature change caused by fermentation itself is tailored by temperature control not only in wine industry but also in laboratory. Fleet (2003) indicated that low temperature decreased the sensitivity of non-*Saccharomyces* to ethanol and thus more non-*Saccharomyces* species could be found in fermentation at 15 °C than at higher temperature. In the work of Andorrà et al. (2010b), although a limited influence of temperature on yeast diversity was found, *Hanseniaspora* showed a rapid disappearance at 25 °C when compared to 13 °C. Salvadó et al. (2011) analyzed growth rates of six yeast species under temperature from 4 °C to 46 °C and found that optimal growth temperature for *H. uvarum*, *C. zemplinina*, *T. delbrueckii* and *P. fermentans was* around 25 °C, for *K. marxianus* was around 39 °C and for *S. cerevisiae* was around 32 °C. When temperature went down to around 15 °C, *S. cerevisiae* started to lose its growth superiority to other non-*Saccharomyces* species (Figure 15). Consistent with Salvadó et al. (2011), other studies on *S. cerevisiae* dominance also highlight the decisive role of temperature (Goddard et al. 2008, Williams et al. 2015).

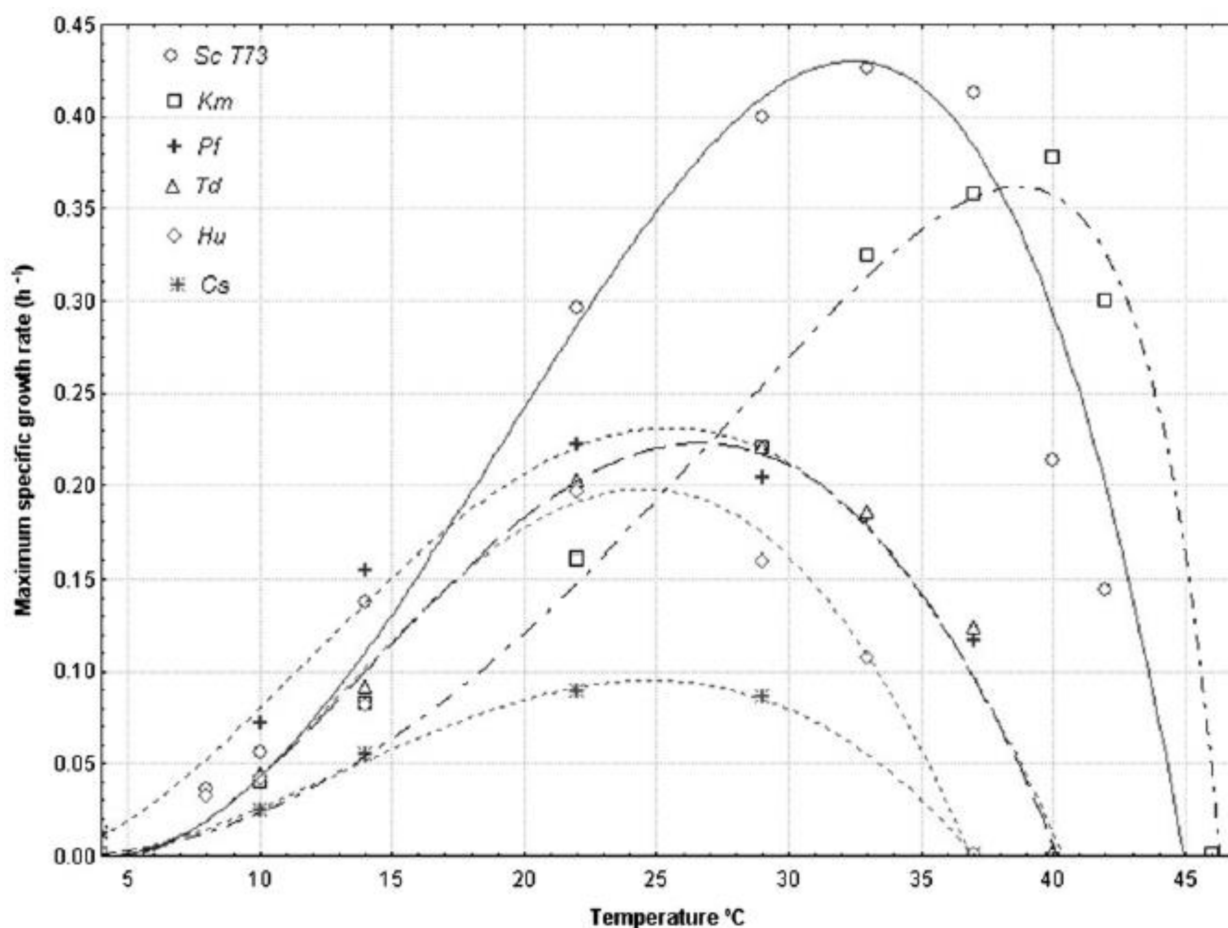

Figure 15 The influence of temperature on maximum specific growth rates of the non-*Saccharomyces* species *H. uvarum* (Hu), *C. zemplinina* (Cs), *T. delbrueckii* (Td), *P. fermentans* (Pf), *K. marxianus* and *S. cerevisiae* (ScT73). Data from Salvadó et al. (2011).

The maximal population of non-*Saccharomyces* can also be affected by prefermentation temperature (Zott et al. 2008). The growth of *T. delbrueckii* and *Hanseniaspora* is favored by 15 °C, whereas for *Starm. bacillaris* 10 °C is better (Albertin et al. 2014). Cold maceration before fermentation (4 °C for seven days) increases the diversity of non-*Saccharomyces* species (Hierro et al. 2006b). Maturano et al. (2015) indicated that prefermentative cold soak at 14 °C favored the growth of non-*Saccharomyces* more than 8 °C and 2 °C.

SO_2

Yeast need to assimilate sulfur dioxide or sulfate to synthesize sulfur-containing amino acids (methionine or cysteine) by sulfate reduction sequence. However, in wine industry, SO_2 is generally used to control the growth of undesirable microorganisms and prevent oxidation. Sulfur dioxide exists in equilibrium among

molecular SO_2, bisulfate and sulfite after dissolving in water and the dominant form depends on pH as shown in Figure 16. Molecular SO_2 is believed to be the antimicrobial form by various mechanisms including rupture disulfide bridges in proteins, reaction with NAD+ and ATP, deamination of cytosine to uracil and reduction of crucial nutrients such as thiamin (Fugelsang and Edwards 2007). Salma et al. (2013) reported that increase pH in the medium from 3.5 to 4.0 can reduce the toxicity of SO_2, allowing resuscitation of VBNC cells.

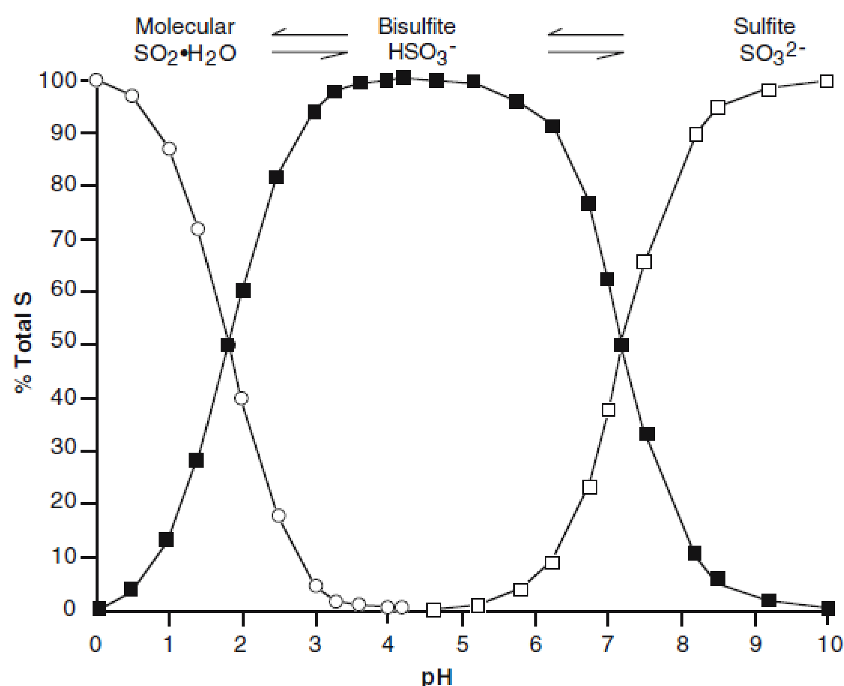

Figure 16. Relative abundance of molecular SO_2, bisulfate, and sulfite at different pH values (Fugelsang and Edwards 2007).

Non-*Saccharomyces* yeast is known to be more sensitive to the combined toxicity of the SO_2 and ethanol than *Saccharomyces* (Jolly et al. 2014). The inhibitory effect of SO_2 on growth of non-*Saccharomyces* has been investigated by culture-dependent and culture independent techniques including PCR, DGGE and DEFT (Cocolin et al. 2003, Divol and Lonvaud-Funel 2005, Andorrà et al. 2008, Takahashi et al. 2014). For example, Divol and Lonvaud-Funel (2005) used DEFT to observe metabolic activity of yeast cells under SO_2 stress and found that *C. stellata* is more sensitive to SO_2 than *Z. bailii* and *S. cerevisiae*.

However, SO_2 addition after crush has small effect to some yeast species from genera of *Pichia*, *Saccharomycodes*, *Schizosaccharomyces* and *Zygosaccharomyces* (Fugelsang and Edwards 2007). Bokulich et al. (2015) used

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massive sequencing to investigate the impacts of SO_2 treatment at a broad range from 0 to 150 mg/L on microbial communities in grape must fermentations. They found that sulfite addition did not affect fungal populations significantly but indeed affected bacterial diversity throughout the course of fermentations.

3.2. Contact-dependent mechanism

Contact-dependent mechanism means that microorganisms interact with each other by direct cell contact and ultimately limit the growth or survival of some microbes in the community. Some researchers believe that the culturability loss of non-*Saccharomyces* induced by contact-dependent mechanism happens between *Saccharomyces* and non-*Saccharomyces* cells (Nissen and Arneborg 2003, Nissen et al. 2003, Renault et al. 2013). In their work, physically separated fermentors were prepared with dialysis tube or filtration membrane, which allowed the free flow of medium but avoided cell contact between the separated compartments. When *Saccharomyces* and non-*Saccharomyces* (*L. thermotolerans* or *T. delbruekii*) were inoculated separately to different compartments, non-*Saccharomyces* yeast grew well in the fermentors. However, when they coexisted in a mixed culture or fermentation where direct cell contact is allowed, non-*Saccharomyces* yeast lost their culturability gradually. As a consequence, a contact-dependent mechanism was concluded in these studies. In addition to use physically separated fermentors, Arneborg et al. (2005) applied an interactive optical trapping system to bring *S. cerevisiae* cells to surround an individual *H. uvarum* cell, forming “confinement” (Figure 17). Surrounded *H. uvarum* cells need longer time to produce new generation than non-surrounded cells by budding reproduction. Therefore, Arneborg and other coworkers believe that confinement stress affects microbial growth and the mechanism involved is probably contact-dependent.

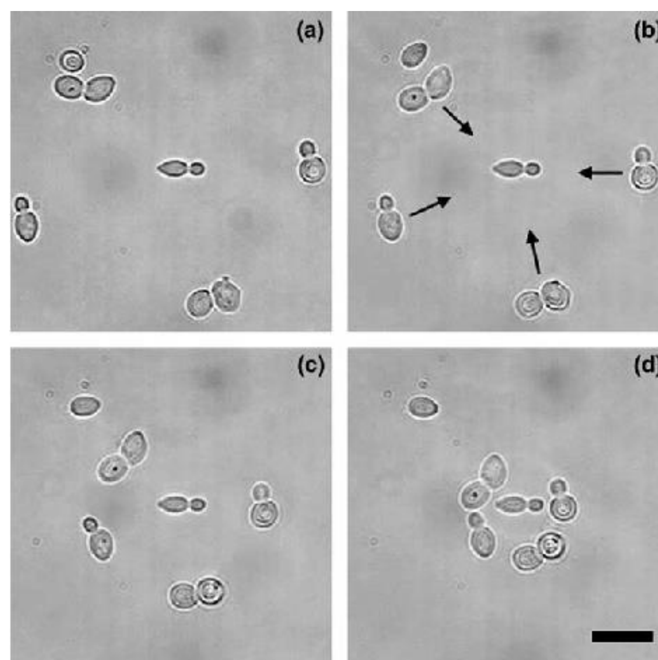

Figure 17 Preparation of *H. uvarum* and *S. cerevisiae* cells for micro-scale, mixed-culture fermentation by user-interactive optical trapping system (Arneborg et al. 2005). The optical traps bring *S. cerevisiae* cells to surround an individual *H. uvarum* cell with arrows showing the direction. Images were recorded at (a) 0 s, (b) 30 s, (c) 60 s and (d) 90 s. Scale bar represents 10 μm.

Although all the studies former stated point to contact-dependent mechanism, the underlying theory on how the mechanism mediates the interaction between *Saccharomyces* and non-*Saccharomyces* remain unclear. Most cell contact examples found in mammalian cells and bacteria applied cell contact to mediate signals, which arrested growth at high cell densities instead of regulating cell death (Caveda et al. 1996, Fiore and Degrassi 1999, Shimkets and Kaiser 1999, Hirano et al. 2001). Cell death regulated by contact only exists in the mammalian immune system using antigen response (Krammer 2000). Therefore, Renault et al. (2013) hypothesized that a receptor/ligand-like interaction maybe involved in the contact-dependent mechanism because it is unidirectional to non-*Saccharomyces* cells. More proofs are required to illustrate the contact-dependent mechanism in wine yeast interaction.

In addition, contact-dependent mechanism seems to be dependent on the strains of *S. cerevisiae* used (Kemsawasd et al. 2015a). When *S. cerevisiae* S101 was used, the contact-dependent mechanism could be observed (Nissen and Arneborg 2003, Nissen et al. 2003, Renault et al. 2013). However, when other *S.*

cerevisiae strains were used such as CCMI 885 and QA23, non-*Saccharomyces* cells always lost their culturability at late stage of fermentation even if a separated fermenter was used (Pérez-Nevado et al. 2006, Taillandier et al. 2014). Furthermore, the culturability loss of non-*Saccharomyces* is universal phenomenon, which is not limited to some *S. cerevisiae* strains. Therefore, Kemsawasd et al. (2015a) rechecked the interaction induced by *S. cerevisiae* S101 and found that chemical interaction was also involved in the process and played a combined role with contact-dependent mechanism in the culturability loss of non-*Saccharomyces*.

Recently, Rossouw et al. 2015 found that *S. cerevisiae* was able to form mixed species aggregates with some non-*Saccharomyces* yeast, referred to as co-flocculation. The co-flocculation was thought to be a possible mechanism governing population dynamics, because the flocculation efficiency in mixed inocula was higher than single culture. However, as an important adhesion-dependent phenotype, flocculation is mainly proposed as a protective mechanism to resist environmental stresses (Honigberg 2011). The relevance of co-flocculation with yeast dynamics during grape must fermentation still need more research to demonstrate it.

3.3. Chemical interaction: production of killer toxins

Yeasts are well-known to produce and secrete so-called killer toxins containing proteins, glycoproteins and polypeptide, which are lethal to sensitive cells of their own species, or those of other yeast species (Jacobs and van Vuuren 1991, van Vuuren and Jacobs 1992, Shimizu 1993). The ability to produce killer toxins is widespread among yeasts including *Saccharomyces*, *Hansenula*, *Pichia*, *Kluyveromyces*, *Candida*, *Hanseniaspora*, *Rhodotorula*, *Trichosporon*, *Debaryomyces* and *Cryptococcus* (Shimizu, 1993, Zagorc et al. 2001, de Ullivarri et al. 2011). The production of killer toxins affects yeast evolution and even fermentation progress. Therefore, the selection of indigenous yeast with killer phenotype was studied in Slovenia and Argentina for well-controlled grape must fermentations, especially killer S. *cerevisiae* (Zagorc et al. 2001, de Ullivarri et al. 2011).

Yeasts which excrete metabolites able to prevent yeast growth are called killer yeasts. The killer toxins which have been identified from grape must fermentation are shown in Table 3. The yeasts, which react to the killer toxin secreted by killer yeast are named sensitive strains. Sensitive strains involve

Saccharomyces, non-*Saccharomyces* or spoilage yeasts depending on the type of killer toxin. Firstly, sensitive *Saccharomyces* strains were mainly affected by K2 type killer toxin in grape must fermentation (Shimizu, 1993). K2 type killer toxin show a narrow spectrum of action, only acting against strains belonging to the same species. Wine isolates of *Candida*, *Pichia* and *Hanseniaspora* could exert their killer activity against *S. cerevisiae* (Radler et al. 1985, Fleet and Heard 1993). However, little is known about corresponding killer toxin. Secondly, two types of killer toxins have been reported to act on non-*Saccharomyces* as shown in Table 3. Kpkt has extensive anti-*Hanseniaspora*/*Kloeckera* activity under winemaking conditions (Oro et al. 2014). Antimicrobial peptides have wide action spectrum of several yeast species, although it just shows fungistatic effect on some species (Branco et al. 2014). Due to its correlation with culturability loss of non-*Saccharomyces*, antimicrobial peptides will be stated in detail in next section. Thirdly, several killer toxins have been reported to act against *Brettamomyces*/*Dekkera*, which is regarded as one of the most damaging species for wine quality (Comitini et al. 2004, Santos et al. 2011). The development of these killer toxins becomes a potential strategy for biocontrol due to limited tools for controlling growth of *Brettamomyces*/*Dekkera* (Wedral et al. 2010, Mahlomakulu et al. 2015).

Table 3 Killer toxins involved in wine yeast interaction (adapted from Ciani and Comitini 2015).

Killer yeast	Killer toxin	Sensitive strain	Reference
S. cerevisiae	K2 type	*S. cerevisiae*	Shimizu 1993
S. cerevisiae	antimicrobial peptides	*D. bruxellensis*	Branco et al. 2014
		K. marxianus	
		H. guilliermondii	
		L. thermotolerans	
		T. delbrueckii	
Tetrapisispora phaffii	Kpkt	*H. uvarum*	Oro et al. 2014
C. pyralidae	CpKT1, CpKT2	*B. bruxellensis*	Mehlomakulu et al. 2014
K. wickerhamii	Kwkt	*Brettanomyces/Dekkera*	Comitini et al. 2004
P. membranifaciens	PMKT2	*B. bruxellensis*	Santos et al. 2009
Ustilago maydis	KP6	*B. bruxellensis*	Santos et al. 2011
Wickerhamomyces anomalus	Pikt	*Brettanomyces/Dekkera*	Comitini et al. 2004

Furthermore, Mostert and Divol (2014) investigated the extracellular proteins released by yeasts in synthetic wine fermentations, finding that the proteome of *S. cerevisiae* changes depending on the presence of *M. pulcherrima* or *L.*

thermotolerans in mixed fermentations. Identies of these diverse proteins revealed proprable influence of yeast interactions but still need further studies to illustrate.

Killer toxins ── antimicrobial peptides

The well-known species evolution in grape must fermentation, which is culturability loss of non-*Saccharomyces*, is recently regarded to be correlated with antimicrobial peptides secreted by *S. cerevisiae*. The discovery and analysis of antimicrobial peptides in *S. cerevisiae* CCMI 885 has experienced almost ten years. In 2006, Pérez-Nevado et al. revealed that some toxic compounds produced by *S. cerevisiae* caused culturability loss of *H. guilliermondii* cells, because cell-free supernatants from late stage of mixed fermentation could trigger the culturability loss of *H. guilliermondii* population. Subsequently, Albergaria et al. (2010) further analyzed the nature of toxic compounds in supernatants of mixed fermentation by protease treatment and protein electrophoresis. The toxic compounds were proved to be peptides of 2-10 kDa, which exhibited antimicrobial effect against *H. guilliermondii*, *K. marxianus*, *L. thermotolerans* and *T. delbrueckii*. Then a new question arose: what are these peptides? Branco et al. (2014) reported that these peptides corresponded to fragments of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein by mass spectrometry analysis. The purified fragments showed wide fungicide or fungistatic effect on five wine yeast species and also *Oenococcus oeni* and thus were referred to as antimicrobial peptides. Further analysis using *S. cerevisiae* mutant strains demonstrated that the secretion of antimicrobial peptides was related with GAPDH encoding genes of *TDH1*, *TDH2* and *TDH3*, which codified three GAPDH isoenzymes.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein is a key enzyme involved in glycolysis, which is responsible for the transformation of glyceraldehyde-3-P to 1,3-Diphosphoglycerate (Figure 18). It is surprising finding that GAPDH protein correlates with these antimicrobial peptides (Branco et al. 2014). This finding can initially explain why the amount of sugar metabolized by *S. cerevisiae* had a pronounced effect on the culturability of *H. guilliermondii*. Pérez-Nevado et al. (2006) indicated that the culturability loss of *H. guilliermondii* happened several days later in medium with 100 g/L of initial sugar than in medium with 200 g/L of initial sugar. When YPD medium with 20 g/L of sugar was used, no evidence

of *S. cerevisiae* dominance over non-*Saccharomyces* yeast by killer toxins was found (Williams et al. 2015).

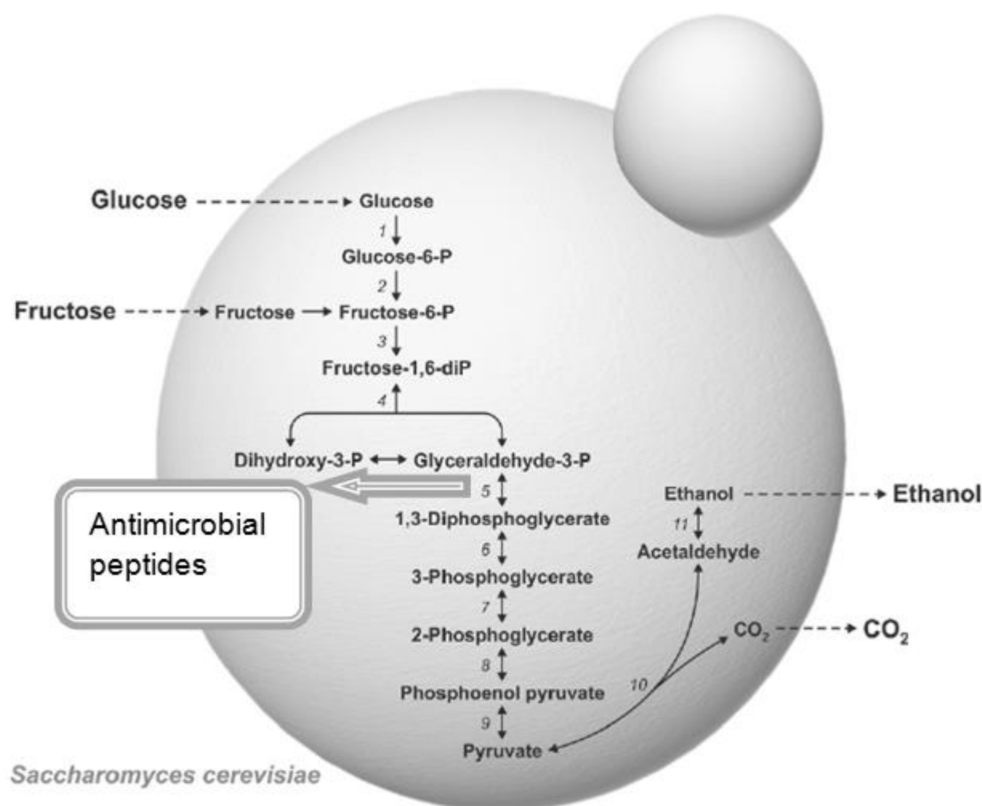

Figure 18. Pathway of glycolysis and alcoholic fermentation in *S. cerevisiae* (adapted from Bartowsky and Pretorius 2009). Enzymes for the different stages in the pathway: 1, hexokinase; 2, phosphoglucose isomerase; 3, phosphofructokinase; 4, aldolase; 5, glyceraldehyde-3-phosphate dehydrogenase; 6, phosphoglycerate kinase; 7, phosphoglycero mutase; 8, enolase; 9, pyruvate kinase; 10, pyruvate decarboxylase; 11, alcohol dehydrogenase.

In order to further explain how these antimicrobial peptides affect the viability of non-*Saccharomyces*, Branco et al. (2015) evaluated physiological changes induced by them. After 24 h of exposure to antimicrobial peptides, all *H. guilliermondii* cells lost their culturability and 77% of cells lost the membrane integrity and pH gradient. Therefore, antimicrobial peptides could disturb intracellular pH homeostasis, compromise plasma membrane, induce culturability loss and finally kill the cells.

Until now, most studies on antimicrobial peptides have been tested by *S. cerevisiae* CCMI 885, but it seems like not only limited to this strain. The secretion of antimicrobial peptides was also tested on *S. cerevisiae* BY4741 and BY4742 (Branco et al. 2014). In addition, *S. cerevisiae* S101 which was considered to act on

non-*Saccharomyces* by contact-dependent mechanism, was demonstrated to be able to secret antimicrobial peptides. Nevertheless, 2-3 times lower concentration of antimicrobial peptides was secreted by S101 than CCMI885, which probably explain a slow influence of antimicrobial peptides from S101 (Kemsawasd et al. 2015a). The secretion ability from other *S. cerevisiae* strains is still needed for a complete understanding. Furthermore, the effect of antimicrobial peptides on different yeast species is also required to fully explain the culturability loss of non-*Saccharomyces*.

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Chapter 1

Fungal diversity in grape must and wine fermentation assessed by massive sequencing, quantitative PCR and DGGE

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Abstract

The diversity of fungi in grape must and during wine fermentation was investigated in this study by culture-dependent and culture-independent techniques. Carignan and Grenache grapes were harvested from three vineyards in the Priorat region (Spain) in 2012, and nine samples were selected from the grape must after crushing and during wine fermentation. From culture-dependent techniques, 362 isolates were randomly selected and identified by 5.8S-ITS-RFLP and 26S-D1/D2 sequencing. Meanwhile, genomic DNA was extracted directly from the nine samples and analyzed by qPCR, DGGE and massive sequencing. The results indicated that grape must after crushing harbored a high species richness of fungi with *Aspergillus tubingensis*, *Aureobasidium pullulans*, or *Starmerella bacillaris* as the dominant species. As fermentation proceeded, the species richness decreased, and yeasts such as *Hanseniaspora uvarum*, *Starmerella bacillaris* and *Saccharomyces cerevisiae* successively occupied the must samples. The “terroir” characteristics of the fungus population are more related to the location of the vineyard than to grape variety. Sulfur dioxide treatment caused a low effect on yeast diversity by similarity analysis. Because of the existence of large population of fungi on grape berries, massive sequencing was more appropriate to understand the fungal community in grape must after crushing than the other techniques used in this study. Suitable target sequences and databases were necessary for accurate evaluation of the community and the identification of species by the 454 pyrosequencing of amplicons.

Keywords: culture-independent techniques, pyrosequencing, SO_2 treatment, community diversity and composition, wine yeast

1. Introduction

Investigating the fungal community in grape must and wine fermentation is relevant for understanding its relationship with the grape sanitary status and the final wine characteristics (Bokulich et al., 2014). Recently, the development of next-generation sequencing provided a useful tool for the description of prokaryotic and eukaryotic microbial communities that exist in grape leaves, berries, must and wineries (Bokulich et al., 2013; Bokulich et al., 2014; David et al., 2014; Pinto et al., 2014; Taylor et al., 2014; Valera et al., 2015). The common approach used in these studies was targeted metasequencing: generic target sequences were amplified by PCR to establish a library; then amplicons were sequenced; and identification was performed by comparison with known sequences in databases (Huggett et al., 2013; Mayo et al., 2014). These studies indicated advances relative to the traditional culture-dependent techniques: a greater abundance of bacteria and fungi found in grape leaves and berries and higher sensitivity to minor species due to the possibility of massive sequencing in a short time. Moreover, other culture-independent techniques have played important roles in monitoring the main yeast dynamics during wine fermentation for the last 10 years (Mills et al., 2002; Hierro et al., 2006; Andorrà et al., 2010). Thus, the main aim of this study was to apply these techniques to interpret the fungal communities in grape must and wine fermentation from the Priorat region in Spain.

The Priorat region, the second qualified DOC (Denominación de Origen Calificada) wine region in Spain, is located in southwest Catalonia. This region is characterized by its own "*terroir*" (French word widely use in the wine industry and wine marketing that means specific place character): a topsoil of reddish and black slate with small particles of mica, a hot and dry summer climate with different micro-climates due to the hilly landform (average annual rainfall is 400-600 mm), and vineyards on terraced slopes at altitudes between 100 m and 700 m above sea level (Robinson, 2006; Hudin and Serra, 2013). However, few studies have reported on the native microbial ecology of grapes in this region. Torija et al. (2001) investigated the yeast population in spontaneous fermentation from this region over three years and reported a unique ecology of yeast species and *Saccharomyces* strains. To investigate the probable fungal "*terroir*" of this region, grapes from slopes at altitudes 400 m above sea level in the villages of Poboleda, Escaladei and Porrera were crushed into must and fermented in this study. The fungal diversity from grape must

and fermentation samples was analyzed by culture-dependent techniques and culture-independent techniques and compared among different samples. The effect of SO_2 treatment on fungal diversity was also evaluated by low-dosage addition to two grape must varieties from Porrera.

2. Materials and methods

2.1. Spontaneous fermentations

Mature grapes (Carignan and Grenache) were randomly taken from vineyards in three villages (Poboleda, Escaladei and Porrera) of the Priorat region (Spain) in 2012. The grapes were hand-harvested from the plants with gloves and kept in sterile bags in an ice box for transportation. Approximately 1.8 L of grape must was obtained from each two kilograms of grapes at different locations, which were crushed sterilely in the same plastic bag by hand and put into 2 L bottles for spontaneous fermentation. The fermentations were performed at 24 °C with 120 rpm agitation speed, and 30 ppm of SO_2 was added at 24 h in the form of potassium metabisulfite. The fermentation proceeded in semianaerobic conditions as the bottles are not tightly closed and some gas exchange is allowed. All the fermentations were monitored daily using a Densito 30PX Portable Density Meter (Mettler Toledo, Spain), and samples were taken at five different fermentation stages: 0 h (grape must after crush), 24 h (before SO_2 treatment), 48 h (24 h after SO_2 treatment), middle stage (density approx. 1040-1060 g/L) and end stage (stable density less than 1000 g/L). Fresh samples were directly analyzed by culture-dependent techniques; cell pellets from 1 mL of samples at each fermentation stage were collected by centrifugation after washing with sterile water and kept at -20 °C for further culture-independent analysis by qPCR, DGGE and massive sequencing techniques.

2.2. Culture-dependent techniques

One milliliter of sample at each fermentation stage was diluted in series and spread onto YPD medium (2 % glucose, 2 % peptone, 1 % yeast extract and 1.7 % agar) and Lysine medium (Oxoid, USA) for incubation at 25 °C for 2-3 days. For plating, a Whitley Automatic Spiral Plater (AES Laboratoire, France) was used, and the viable yeast quantification was performed using a ProtoColHr automatic colony counter (Microbiology International, USA). For further colony identification, 25 colonies were selected randomly from YPD and Lysine plates of each sample (50

colonies in total for each sample) and identified by 5.8S-ITS-RFLP analysis and 26S rDNA D1/D2 domain sequencing. In 5.8S-ITS-RFLP analysis, colony amplifications were first performed by primer pairs of ITS1/ITS4 as described by Esteve-Zarzoso et al. (1999). The amplification products were digested by five restriction enzymes (*Hinf*I, *Hae*III, *Cfo*I, *Dde*I and *Mbo*I), and corresponding restriction profiles were identified according to Esteve-Zarzoso et al. (1999) and Csoma and Sipiczki (2008). Then, 26S rDNA D1/D2 domain sequencing was used to confirm the colony identification. Each PCR reaction was performed with primer pairs of NL1/NL4 and the program described by Kurtzman and Robnett (1998). An ABI3730 XL DNA sequencer (Macrogen, Korea) was used for the sequencing process, and corresponding sequence alignment was performed by BLAST from the NCBI database (http://blast.ncbi.nlm.nih.gov/).

2.3. DNA extraction

DNA was extracted from the cell pellets stored at -20 °C using the DNeasy Plant minikit (Qiagen, USA) as described in Hierro et al. (2006). The same extraction protocol was used for DGGE, qPCR and massive sequencing analyses.

2.4. DGGE analysis

The PCR reactions were performed using a Gene Amp PCR System 2720 (Applied Biosystems, USA) with Primers $U1^{GC}$ and U2 (Meroth et al., 2003). The DGGE procedures followed the description in Andorrà et al. (2008) with a modified DGGE gel using a denaturing gradient from 35 % to 55 % urea and formamide.

2.5. qPCR analysis

The qPCR reactions were performed using an Applied Biosystems 7300 Fast Real-Time PCR System (Applied Biosystems, USA) with primers for total yeast, *Saccharomyces*, *Hanseniaspora* and *Starmerella bacillaris* as described in Andorrà et al. (2010). Standard curves were built for each yeast species in triplicate using 10-fold serial dilutions of fresh cultures.

2.6. Massive sequencing analysis

A fragment of approximately 600 nt from D1/D2 of 26S rDNA was amplified using modified NL1/NL4 primers, which were designed with adaptor and molecular

identifier (MID) sequences specially for massive sequencing (Invitrogen, USA). The whole sequencing process was performed using a 454 Roche platform with the Genome Sequencing FLX System (LifeSequencing S.L., Spain): DNA libraries with specific MID sequences were built for each sample by target PCR with the improved primers, and then a primer-dimer removal protocol was applied to each PCR product to increase the sequencing throughput. An equimolecular pool was generated by quantification of the clean PCR products using the Quan-IT™ PicoGreen® kit (Invitrogen), and sequencing of the pooled samples was performed using a 454 FLX Roche sequencer (LifeScience, USA).

The bioinformatics analysis of each sample was conducted by Life Sequencing S.L. (Spain). Quality control of all sequences was first performed by removing sequences with low quality or length lower than 300 nt and the PCR primers. An updated database of 26S rDNA sequences obtained from GenBank of NCBI was constructed for local alignment comparison. By local alignment comparison, each read was assigned to the most probable operational taxonomic unit (OTU) at different taxonomical levels (family, genera and species) with a confidence cutoff value of 80 % and an e-value of 10^{-5}. Sequences with identity value lower than 80 % and e-value lower than 10^{-5} were assigned as “no hit”.

The fungal community in each sample was analyzed by different biodiversity and similarity metrics at the species level using Estimate S v9.1.0 (Colwell, 2013). Both Shannon diversity and Simpson diversity were used to evaluate species diversity because Simpson diversity is less sensitive to richness and more sensitive to evenness than Shannon diversity (Colwell, 2009). The estimated species richness was also calculated by a nonparametric estimator, Chao1, which depends on the observed number of singletons and doubletons in a sample. Similarities were evaluated using Jaccard Classic and Bray-Curtis because we focused on comparing community compositions.

3. Results

Nine samples were obtained from different stages of fermentations; the details are described in Table 1. They were analyzed by culture-dependent techniques (YPD and Lysine plating) and three different culture-independent techniques (qPCR, DGGE and massive sequencing).

Table 1 Details of nine samples from grape must fermentations. The middle and end stages of fermentation were determined by density analysis.

Samples	Fermentation stages	Grape varieties	Locations	Coordinates
I	0 h grape must	Grenache	Poboleda	41.227148, 0.844750
II	0 h grape must	Carignan	Escaladei	41.258156, 0.808214
III	24 h grape must (before SO_2 treatment)	Carignan	Porrera	41.179651, 0.860334
IV	48 h grape must (24 h after SO_2 treatment)			
V	0 h grape must	Grenache	Porrera	41.176748, 0.860619
VI	24 h grape must (before SO_2 adding)			
VII	48 h grape must (24 h after SO_2 treatment)			
VIII	Middle stage of fermentation (day 3)			
IX	Final stage of fermentation (day 11)			

3.1. Yeast diversity analysis by culture-dependent techniques

The 183 isolates from YPD plates were identified as five different species by 5.8S-ITS-RFLP analysis and 26S-D1/D2 sequencing (Table 2). *Hanseniaspora uvarum* was the most frequently isolated species in all samples except sample IX (the end of fermentation, when *Saccharomyces cerevisiae* dominated). *Starmerella bacillaris* was the second most common species, isolated in samples III, IV, V, VIII and IX. *Issatchenkia terricola* was mainly isolated from fresh grape must after crushing (sample I, III, V and VI). *Hanseniaspora valbyensis* and *S. cerevisiae* only appeared in a single sample.

The 179 non-*Saccharomyces* isolates from Lysine medium were identified. Only three species were recovered, with *H. uvarum* as the main species (Table 2). *I. terricola* was only isolated from grape must after crushing (sample I and V), and *Starm. bacillaris* was present in grape must after crushing and also at the end of fermentation.

Table 2 The fungal diversity of nine different grape must and fermentation samples evaluated by culture-dependent and culture-independent techniques.

Techniques	Yeast	I	II	III	IV	V	VI	VII	VIII	IX
Culture-dependent techniques by YPD plating	Total yeast	*	4.80×10^{3}	*	1.51×10^{8}	3.58×10^{6}	1.06×10^{7}	2.10×10^{7}	3.00×10^{7}	9.10×10^{5}
	H. uvarum	7/9	*	9/25	19/24	14/25	24/25	25/25	22/25	nd
	H. valbyensis	nd	*	6/25	nd	nd	nd	nd	nd	nd
	I. terricola	2/9	*	8/25	nd	3/25	1/25	nd	nd	nd
	S. cerevisiae	nd	*	nd	nd	nd	nd	nd	nd	18/25
	Starm. bacillaris	nd	*	2/25	5/24	8/25	nd	nd	3/25	7/25
Culture-dependent techniques by Lysine plating	Total yeast	*	2.70×10^{3}	4.88×10^{6}	1.41×10^{7}	1.75×10^{6}	*	1.33×10^{7}	3.10×10^{8}	1.10×10^{5}
	H. uvarum	13/25	8/9	25/25	25/25	13/20	*	25/25	25/25	nd
	I. terricola	9/25	nd	nd	nd	7/20	*	nd	nd	nd
	Starm. bacillaris	3/25	1/9	nd	nd	nd	*	nd	nd	25/25
qPCR	Total yeast	7.62×10^{2}	2.85×10^{5}	6.07×10^{7}	1.13×10^{8}	1.82×10^{4}	6.06×10^{6}	2.93×10^{6}	4.35×10^{5}	2.31×10^{5}
	Hanseniaspora	nd	9.95×10^{3}	2.46×10^{7}	1.44×10^{7}	6.70×10^{2}	2.64×10^{6}	3.42×10^{5}	2.45×10^{5}	1.94×10^{4}
	Saccharomyces	nd	nd	nd	nd	nd	nd	nd	nd	5.98×10^{4}
	Starm. bacillaris	nd	nd	1.85×10^{6}	2.90×10^{5}	8.93×10^{2}	1.49×10^{6}	3.54×10^{4}	4.67×10^{2}	nd
DGGE	*Aureobasidium pullulans*	–	+	nd	nd	+	nd	nd	nd	nd
	Botryosphaeria dothidea	nd	+	nd	nd	nd	nd	nd	nd	nd
	H. opuntiae	nd	+	nd	nd	+	+	+	+	+
	H. uvarum	nd	+	+	+	+	+	+	+	+
	S. cerevisiae	nd	nd	nd	nd	nd	nd	nd	nd	+
	Starm. bacillaris	nd	nd	+	+	nd	+	+	+	+
Massive sequencing	*Aspergillus tubingensis*	55.80 %	18.18 %	<	<	<	<	nd	nd	nd
	Aureo. pullulans	<	18.63 %	<	<	<	<	nd	nd	nd
	B. dothidea	nd	<	nd	nd	nd	nd	nd	nd	nd
	H. thailandica	nd	<	5.25 %	5.00 %	<	<	<	<	<
	H. opuntiae	nd	6.05 %	<	<	<	<	<	<	nd
	H. uvarum	nd	<	60.78 %	56.68 %	<	13.57 %	11.80 %	<	<
	uncultured Hanseniaspora	nd	<	12.37 %	13.44 %	<	<	<	<	<
	I. terricola	nd	nd	<	<	<	<	<	<	nd
	Penicillium brevicompactum	<	5.47 %	nd	nd	<	nd	nd	nd	nd
	P. crustosum	<	5.56 %	nd	nd	<	nd	nd	nd	nd
	P. glabrum	8.64 %	nd	nd	nd	<	nd	nd	nd	nd
	S. cerevisiae	nd	<	nd	nd	nd	<	nd	nd	25.98 %
	uncultured Saccharomyces	nd	<	nd	<	nd	<	nd	nd	<
	Starm. bacillaris	nd	<	17.19 %	20.22 %	87.86 %	79.61 %	80.22 %	98.10 %	71.19 %
	uncultured soil fungus	14.45 %	<	nd	<	<	<	<	nd	nd

The results from culture-dependent techniques are shown as specie colony numbers compared with total colony numbers, with total yeast concentration also shown (cfu/ml). Grape must samples with moulds mainly found on plates, resulting in hard quantification or isolation, are labeled with "". The qPCR results are shown as cells concentration (cells/ml), the species detectable by DGGE are represented by "+", and the results from massive sequencing are shown as percentages (%). Because of the rich diversity of the massive sequencing results, only the major species with percentages higher than 5% are shown in the table, and the species with lower percentages in some samples, if listed, are marked with "<". The symbol of "nd" represents the species with lower concentrations below the detection limit (100 cells/ml) by qPCR and species undetectable by the other three techniques.*

3.2. Yeast population diversity by qPCR analysis

The population levels of total yeast, *Hanseniaspora* spp., *Starm. bacillaris* and *Saccharomyces* spp., were separately quantified (Table 2). The total yeast population in grape must after crushing (sample I, II and V) was lower than 10^6 cells/mL, but the yeast population then increased to 10^5 to 10^8 cells/mL. *Hanseniaspora* was the main genus detected in almost all samples, ranging from 10^2 to 10^7 cells/mL. *Starm. bacillaris* mainly appeared in grape must from Porrera (10^2 to 10^6 cells/mL), although it was not detected at the end of fermentation. Surprisingly, the *Saccharomyces* population was only detected by this technique at the end of fermentation. The total yeast population size was not affected by the SO_2 treatment; however, the *Starm. bacillaris* population was reduced by approximately tenfold after SO_2 addition. This observation was made in the two samples analyzed before and after SO_2 addition.

3.3. DGGE analysis of grape must samples

The bands obtained in DGGE profiles were assigned to six species by sequencing, as indicated in Table 2. No species were observed from sample I, and in the remaining eight samples, *H. uvarum* appeared in each sample, *Hanseniaspora opuntiae* and *Starm. bacillaris* in six samples, *Aureobasidum* (*Aureo.*) *pullulans* in sample II and V, and *Botryosphaeria dothidea* and *S. cerevisiae* in only one sample.

3.4. Fungal diversity analysis by massive sequencing

3.4.1 Species diversity and similarity of grape must samples

A total of 120081 original sequences were obtained from nine samples, of which 106095 sequences passed the quality control filter. As shown in Table 3, approximately 10000 high quality reads were obtained from each sample, and the average sequence length was approximately 500 nt. The similar level of read numbers from each sample established comparability among samples. The analysis of massive sequencing was performed based on taxonomy-dependent methods, by which query sequences were compared with known sequences deposited in annotated databases. After alignment, 247 OTUs were identified at the species level from the 105541 hit reads, and 554 reads were not assigned an identity in the current eukaryotic database of NCBI (0.5 % of no hit reads).

Table 3 Total sequences obtained from massive sequencing and fungal community metrics of all samples.

Metrics	I	II	III	IV	V	VI	VII	VIII	IX
High quality reads	10033	9301	10255	12798	10503	18162	11529	12559	10955
Average length (nt)	499	471	545	539	505	512	510	511	486
Number of OTUs at species level	86	186	22	21	64	25	15	11	15
Number of no hit reads	30	165	55	85	69	61	29	10	50
Estimated species richness	152	329	32	22	96	32	16	11	20
confidence intervals	113-248	263-451	24-76	21-33	75-153	26-59	15-30	11-17	16-44
Shannon exponential species diversity	5.57	20.61	3.32	3.49	1.99	2.04	1.90	1.12	2.03
Simpson inverse species diversity	2.88	10.94	2.37	2.57	1.28	1.52	1.43	1.04	1.72

Estimated species richness was calculated using the Chao1 richness estimator, with log-linear 95 % confidence intervals. OTU, operational taxonomic unit.

Rich OTUs were found in the three grape must samples after crushing (I, II and V). However, the fermentation samples showed a lower OTU richness. The species richness of each sample was estimated by Chao 1, and more OTUs were expected from the three grape must samples after crushing; however, in the other six fermentation samples, the observed OTUs were similar to the estimated species richness. Thus, both observed and estimated species richness decreased as fermentation proceeded, as we expected. The Shannon (exponential form) and Simpson (inverse form) diversity indices were used to evaluate the community diversity, in which both richness and evenness were integrated. The diversity values were no less than 1 due to the corresponding forms used, and higher values meant higher diversity. Thus, sample II presented the highest diversity and the best evenness of the nine samples. Although sample V had a higher value of richness than some fermentation samples (III, IV, VI, IX), its diversity by both indexes was lower, mainly due to its poor evenness.

The community similarity in nine samples was pairwise analyzed using the Jaccard Classic and Bray-Curtis indices (Table 4). Values from both indices range from 0 to 1, with 0 representing no similarity between two samples and 1 meaning no differentiation. Samples I and V showed similarities of 0.271 by Jaccard Classic and 0.066 by Bray-Curtis, which were lower values than the similarities between III and VI (0.516 Jaccard Classic and 0.376 Bray-Curtis) or IV and VII (0.565 Jaccard Classic and 0.377 Bray-Curtis). As noted in Table 1, samples I and V were from the same grape variety (Grenache) but different locations (Poboleda, Porrera), while III/IV and VI/VII were from the same location (Porrera) but from two different grape

varieties (Carignan and Grenache). Therefore, the location seemed to contribute more to the dissimilarities between two samples than the grape variety.

Table 4 Community similarity metrics (Jaccard Classic and Bray-Curtis) by pairwise multivariate analysis of all samples (I-IX).

		Bray-Curtis								
		I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**
Jaccard	**I**		0.357	0.004	0.002	0.066	0.001	0.000	0.000	0.000
Classic	**II**	0.242		0.087	0.078	0.118	0.062	0.079	0.040	0.045
	III	0.049	0.072		0.887	0.217	0.376	0.343	0.173	0.174
	IV	0.059	0.078	0.593		0.263	0.400	0.377	0.221	0.226
	V	0.271	0.185	0.284	0.288		0.677	0.880	0.822	0.738
	VI	0.078	0.093	0.516	0.704	0.290		0.777	0.819	0.543
	VII	0.020	0.052	0.423	0.565	0.197	0.538		0.809	0.703
	VIII	0.000	0.037	0.375	0.391	0.154	0.333	0.625		0.672
	IX	0.020	0.052	0.276	0.385	0.145	0.379	0.364	0.444	

3.4.2 Fungal community composition at different phylogenetic levels

The fungal communities of the grape must were mainly characterized by high amounts of OTUs from the *Ascomycota* phylum (more than 95 % in each sample). Forty-six of the 247 OTUs were present at 0.1 % to 5 % in each sample, and 189 OTUs presented a minor proportion (lower than 0.1 %). Only 12 species were higher than 5 % in each sample, as shown in Table 2. The dominant species were *Aspergillus* (*Asper.*) *tubingensis* in sample I, *Aureo. pullulans* in sample II, *H. uvarum* in samples III and IV, and *Starm. bacillaris* in samples V - IX. Species from the *Eurotiomycetes* and/or *Dothideomycetes* class mainly occupied the grape must after crushing (sample I and II), and most of the species found in grape fermentation must (sample III - IX) were from the *Saccharomycetes* class. At the genus level, the eight most abundant genera in nine samples are listed in Figure 1. Their sum accounts for more than 80 % in each sample. The fungal community composition at different phylogenetic levels was more obviously affected by region and grape variety than the SO_2 treatment, as the latter only caused small percentage changes in some non-*Saccharomyces* species, mainly in the *Hanseniaspora* yeast genus.

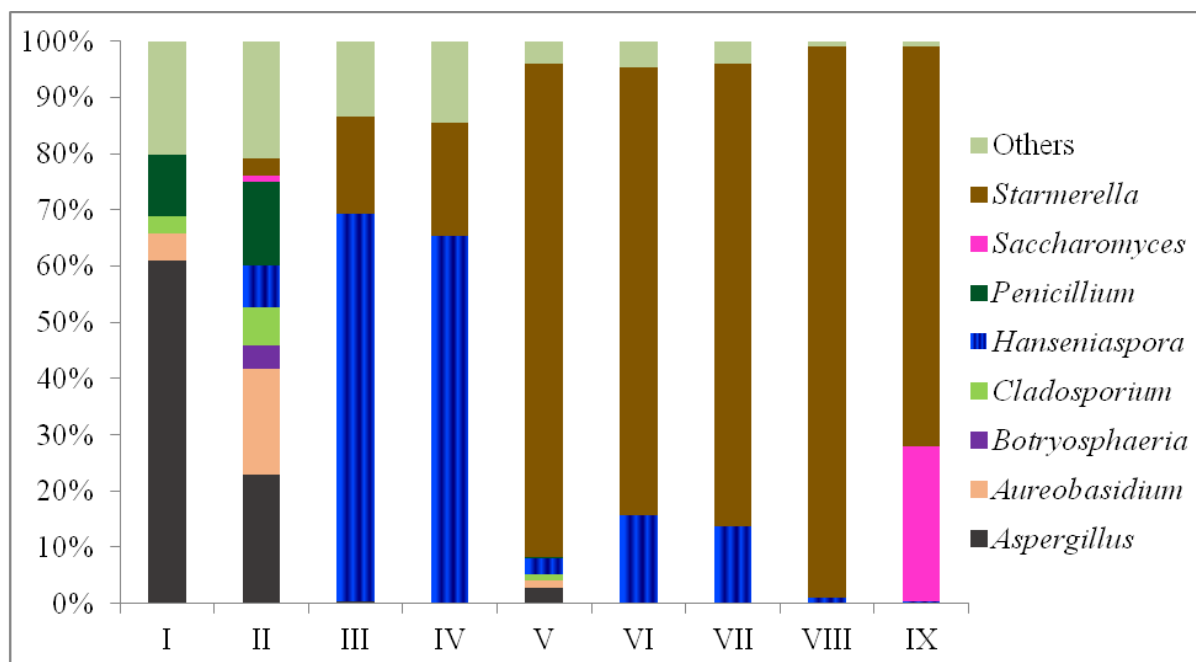

Figure 1 Community distribution of the eight most abundant genera of nine samples (I - IX) using massive sequencing analysis.

3.5. Comparison among culture-dependent techniques and different culture-independent techniques

Comparing the results from different techniques, all the species detected by culture-dependent techniques, qPCR and DGGE were also found by massive sequencing except for sample I; however, the quantity or percentage of some species from the *Hanseniaspora* and *Starmerella* genera varied depending on the techniques used. *Saccharomyces* was found only in sample IX by culture-dependent techniques, qPCR and DGGE, while a minor population was also found in samples II, IV and VI by massive sequencing. Most of the fungi from the non-*Saccharomycetes* class were detectable by massive sequencing, whereas only dominant species could be found by DGGE. Although they were also observed on YPD or Lysine plates, it was difficult to perform identification and quantification by culture-dependent techniques. Furthermore, non-culturable cells at the end of fermentation, such as *H. uvarum*, were quantifiable or detectable by the three culture-independent techniques.

4. Discussion

The nine samples from different locations, grape varieties and corresponding fermentation stages allowed the analysis of yeast diversity and ecology in the Priorat

wine region of Spain. However, our study went beyond descriptive analysis and focused on the comparison between culture-dependent techniques and culture-independent techniques to evaluate the fungal diversity based on rDNA-PCR polymorphism. Recent studies have mentioned drawbacks of rDNA-PCR-based methods, especially for culture-independent techniques, such as preferential annealing of the primers, the representativity and quality of DNA, and variable gene copy numbers in different species, and these drawbacks might lead to overestimation/underestimation of the proportion of some species in the overall fungal community (Andorrà et al., 2008; Angly et al., 2014; Valera et al., 2015). Although, it was also observed in this study that massive sequencing, culture-dependent techniques and qPCR detected different percentages of *Starm. bacillaris*, these methods were all necessary for yeast identification and quantification analysis. Culture-dependent techniques and culture-independent techniques such as qPCR, DGGE and massive sequencing were used in this study to weigh the biases introduced by the techniques in an effort to estimate the true fungal community diversity, similarity and composition.

4.1. Fungal community in grape must after crushing

The main fungi in grape must from the three vineyards of the Priorat region were *Eurotiomycetes*, *Dothideomycetes* and *Saccharomycetes*, all in the *Ascomycota* phylum. These fungi are commonly found in grape berries or grape must after crushing in various world wine regions (Bokulich et al., 2014; David et al., 2014; Taylor et al., 2014). The dominant species in a single vineyard were *Asper. tubingensis* (Grenache from Poboleda), *Aureo. pullulans* (Carignan from Escaladei) and *Starm. bacillaris* (Grenache from Porrera). None of these three species are plant pathogens. The high population of *Starm. bacillaris* in grape must after crushing is unexpected but understandable: approximately 31 % of *Candida* (previous denomination of *Starm. bacillaris*) was found in Chardonnay grapes of Burgundy (France) (David et al., 2014), indicating the possibility of dominance of this yeast over other fungi in grape must. Moreover, some species that are considered common plant pathogens, such as *Alternaria alternate*, *Aspergillus niger*, *B. dothidea*, *Cladosporium cladosporioides* and *Cytospora sacculus*, were found in low percentages (0.1 % to 5 % according to massive sequencing results). Only one sequence of *Botrytis cinerea* was found in Carignan from the Escaladei vineyard and

Grenache from Porrera. No other common grape pathogen was detected. As noted by Taylor et al. (2014), the presence of DNA from these species does not necessarily mean that the grapes or plants have an infection. Fungal diseases are rare in the Priorat region because of the high temperature and low level of rainfall in the summer (Robinson, 2006). Some reads of *S. cerevisiae* (1.03 %) were found in Carignan from the Escaladei vineyard but did not appear in the other two grape must samples. The low or absent evidence of DNA from *Saccharomyces* was consistent with other reports based on high-throughput sequence analysis, and with the presence of other non-dominant non-*Saccharomyces* yeasts such as *Hanseniaspora*, *Issatchenkia* or *Pichia* in this study (Bokulich et al., 2014; David et al., 2014; Taylor et al., 2014).

Regional microbial "*terroir*" was proposed by Bokulich et al. (2014) as a probable explanation for the regional characteristics of final wine quality, as the fungal community was more resistant to vintage variation than regional or even vineyard variation. Our results also showed that the fungal community was more affected by geographical location than by grape variety, even though the three vineyards were all located in the Priorat region with similar altitudes and were geographically close (approx. from 5 to 12 km to each other). Interestingly, Torija et al. (2001) found that *Candida stellata* (currently renamed *Starm. bacillaris*) was the only species isolated from grape must at the same location (Porrera) in 1996. Nevertheless, the formation of grape-surface communities by vineyard or region needed more proof to be established. Furthermore, the fungal community analysis in grape must after crushing was more reliable when estimated by massive sequencing than other techniques used in this study because of the "deep community sequencing" due to the larger number of sequences analyzed (Taylor et al., 2014).

4.2. Fungal community in grape must during wine fermentation

Fungal community dynamics during wine fermentation involve the decline of non-yeast fungi during the first 24 h, the simultaneous increase of *Hanseniaspora* species and the increase of *S. cerevisiae* at the end of fermentation. The non-yeast fungi seemed to be less tolerant of environmental change from grape skin to grape must, as few sequences were detected in grape must at 24 h, and only one sequence of *Aspergillus niger* was found in grape must at 48 h. The massive decline in non-yeast fungi contributed directly to the decreased biodiversity in grape must

during fermentation. Although the lack of detection of non-yeast fungi in grape must after 48 h resulted partly from their reduction in grape must after crushing, the decrease in non-yeast fungi could also be correlated with the dominance of *Hanseniaspora* species. A clear increase in *S. cerevisiae* appeared at the end of fermentation, which was expected (Ribéreau-Gayon et al., 2006) and was consistent in all the results with all the techniques used in this study. Only one sequence of *S. cerevisiae* was occasionally detected in grape must at 24 h, which is also consistent with the consolidated knowledge. This low percentage of *Saccharomyces* species was also observed by David et al. (2014), and in their studies, when fermentations had reached two-thirds of the process (late stages), *Saccharomyces* species were detected at lower levels. The high representation of non-*Saccharomyces* yeast in grape must (*Starmerella* in this study, and *Candida* in David et al. 2014, which could be equivalent) can account for this late detection of *S. cerevisiae* as a main species during fermentation. This competition between *Starmerella*/*Candida* and *Saccharomyces* needs further investigation.

Regardless of regional and varietal factors, fungal diversity decreased as fermentation proceeded, with the disappearance of non-yeast fungi and the predominance of non-*Saccharomyces* yeast (*Hanseniaspora*). Thus, the grape must changes during wine fermentation also seemed to affect the fungal community. However, the analysis of similarity during wine fermentation showed a high value, likely resulting from the dominance of *Starm. bacillaris* throughout the process. Moreover, the influence of SO_2 did not change the community similarity and composition. This result was consistent with the conclusions from former studies based on culture-dependent and culture-independent techniques (Andorrà et al., 2008; Wang and Liu 2013). However, more studies are necessary to explain how the fungal community is formed in the vineyard, the changes during wine fermentation, and the relationship between the fungal communities and regional wine characteristics.

The results from different techniques were more comparable during fermentation than in grape must. Massive sequencing was still the most comprehensive technique used in this study, as the detection of fungi is based on few sequences. For these results from massive sequencing analysis, it was important to accurately compare and search for information in the appropriate databases. To analyze the fungal community in this study, primers targeting the

D1/D2 region of 26S rDNA were used due to lower differences in the sequence length and more comprehensive reference databases than for the ITS region (Taylor et al., 2014). Some other authors used different approaches based on massive sequencing: Pinto et al. (2014) analyzed sequences from both regions (D1/D2 region of 26S rDNA and ITS) to analyze the whole community, and the results indicated some variations but no significant differences were found. David et al. (2014) used amplicons of 18S rDNA for yeast diversity analysis, and the yeast dynamics trend was basically consistent with our study here. Bokulin and Mills (2013) analyzed very short amplicons from the ITS region to improve the accuracy of high-throughput sequencing, and this approach decreased the bias caused by the differences in length of conventional ITS amplicons. The amplification of different regions might provide results with fewer biases, but databases for corresponding identification are also essential if taxonomy-dependent methods are used. RDP, SILVA and GenBank were used to assign an identity to all the sequences here (data not shown), and GenBank provided the most complete databases, with which identification at a lower taxonomical level (species) with a high confidence value of identity was achieved (Taylor et al., 2014).

In conclusion, this work indicated different fungal community diversities in grape must after crushing Grenache or Carignan grapes from three vineyards in the Priorat region of Spain. The massive sequencing analysis of grape must could provide information on the presence of plant pathogens and the species able to successfully ferment grape must. The community dynamics during wine fermentation as analyzed by qPCR, DGGE and massive sequencing showed consistent results, especially for detecting non-culturable yeast at the end of fermentation. The population changes from grape skin to grape must are related with the presence of non-*Saccharomyces* yeast on the grapes. The changes during fermentation including ethanol, nutrition or even some yeast metabolites, introduce the appropriate conditions for the imposition of *S. cerevisiae*, which conducts the final part of the alcoholic fermentation.

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Chapter 2

Monitoring of *Saccharomyces cerevisiae*, *Hanseniaspora uvarum*, and *Starmerella bacillaris* (synonym *Candida zemplinina*) populations during alcoholic fermentation by fluorescence *in situ* hybridization

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Abstract

Various molecular approaches have been applied as culture-independent techniques to monitor wine fermentations over the last decade. Among them, those based on RNA detection have been widely used for yeast cell detection, assuming that RNA only exists in live cells. Fluorescence in situ hybridization (FISH) targeting intracellular rRNA is considered a promising technique for the investigation of wine ecology. For the present study, we applied the FISH techniques in combination with epifluorescence microscopy and flow cytometry to directly quantify population of *Saccharomyces cerevisiae*, *Hanseniaspora uvarum*, and *Starmerella bacillaris* during alcoholic fermentations. A new specific probe that hybridizes with eight species of *Hanseniaspora* genus and a second probe specific for *Starm. bacillaris* were designed, and the conditions for their application to pure cultures, mixed cultures, and wine samples were optimized. Single and mixed fermentations were performed with natural, concentrated must at two different temperatures, 15 °C and 25 °C. The population dynamics revealed that the *S. cerevisiae* population increased to 10^7-10^8 cells/mL during all fermentations, whereas *H. uvarum* and *Starm. bacillaris* tended to increase in single fermentations but remained at levels similar to their inoculations at 10^6 cells/mL in mixed fermentations. Temperature mainly affected the fermentation duration (slower at the lower temperature) but did not affect the population sizes of the different species. The use of these probes in natural wine fermentations has been validated.

Keywords: Culture-independent techniques, Wine ecology, FISH, Epifluorescence microscopy, Flow cytometry.

1. Introduction

Wine fermentation is an ecologically complex process, and it is widely accepted that the yeast population changes as the fermentation proceeds (Fleet, 2008; Wang and Liu, 2013). Yeast species play important roles in the wine-making processes: transforming sugar to ethanol, producing specific secondary metabolites, and finally, contributing to wine flavor characteristics. The main yeast involved in this transformation process belongs to the *Saccharomyces* genus, but other wine yeasts can also be isolated during the process, producing varying impacts on the wine composition (Andorrà et al., 2012; Fleet, 2003). Most of the knowledge generated concerning wine yeast ecology has been derived from plating, which has incorporated molecular analysis in the past 25 years (Ribéreau-Gayon et al., 2006). However, in the past 15 years, culture-independent techniques have provided a new view of microbial ecology during the wine production process.

The culture-independent molecular techniques employed to study the ecology of wine yeasts have mainly been based on the detection of DNA in wine samples (Andorrà et al., 2010a; Cocolin et al., 2000; Hierro et al., 2006). However, it is possible to detect DNA from dead cells when using these techniques (Andorrà et al., 2010b; Hierro et al., 2006). Furthermore, the presence of viable but non-culturable (VBNC) or injured cells in wine (Divol and Lonvaud-Funel, 2005; Millet and Lonvaud-Funel, 2000) introduces a new bias into the analysis of the wine yeast ecosystem. More recently, a small modification of the same culture-independent methods, QPCR coupled with ethidium monoazide (EMA)/PMA (Andorrà et al., 2010b; Rawsthorne and Phister, 2009), has been proposed to reveal the actual viable yeast population structure and resolve the differences observed between plate counting and molecular results. Other methodologies based on RNA detection, such as RT-PCR-DGGE (Mills et al., 2002) and RT-QPCR (Hierro et al., 2006), have also been proposed. A main hurdle of these techniques is that all of these methods rely on a high-quality DNA/RNA extraction and PCR/QPCR operation to provide accurate results. Fluorescence *in situ* hybridization (FISH) probes directly hybridize with intracellular ribosomal RNA at specific sites, and the target cells with fluorescent signals can be easily observed and recorded by epifluorescence microscopy and flow cytometry (Amann and Fuchs, 2008). In addition to its simplicity and rapidity, the ability to observe the cell morphology by a microscope and the high sensitivity obtained using a flow cytometer (ten fluorescent cells can be detected among ten

million non-fluorescent cells, as the flow cytometer can detect up to ten million events) make FISH a very promising technique for wine ecology studies (Andorrà et al., 2011; Branco et al., 2012; Díaz et al., 2010; Röder et al., 2007).

To our knowledge, only a few wine ecology studies have reported on the detection of yeast populations by FISH, mainly because of the difficulty of designing probes. Although early studies (Inácio et al., 2003; Stender et al., 2001; Xufre et al., 2006) were based on culture-dependent techniques, probes for several wine-related yeast species have been proposed, and the data can be used to design new probes for other species as well. Xufre et al. (2006) developed nine different probes for the detection of *Saccharomyces* and non-*Saccharomyces* wine yeasts. The first approximation of population monitoring during wine fermentation accomplished using FISH combined with flow cytometry was proposed by Andorrà et al. (2011). These authors found that the *Hanseniaspora* (*H.*) *guilliermondii* probe demonstrated a low fluorescent intensity compared to the *Saccharomyces cerevisiae* probe, whereas *H. guilliermondii* cells could be differentiated easily from *S. cerevisiae* cells by flow cytometry based on the cell size and granularity. Upon analyzing the changes in the fluorescence intensity of the stained cells during the fermentation, the intensity of the *S. cerevisiae* probes decreased while *H. guilliermondii* maintained a similar intensity as that of the initial conditions (Andorrà et al., 2011). Because of the high background fluorescence in wine, only high-intensity signal probes, which have only been applied for detecting *Dekkera bruxellensis* and *S. cerevisiae* in direct wine sample analysis, can be used (Andorrà et al., 2011; Röder et al., 2007). Therefore, the development of specific probes with sufficient fluorescence is still required for further assays of yeast populations directly from wine samples by FISH.

Yeast diversity studies of red and white musts from our faculty cellar (Tarragona, Spain) have been performed for several years, with *Saccharomyces*, *Hanseniaspora*, and *Candida* (*C.*) comprising the main yeast genera detected. Thus, the present study aims to analyze the applicability of culture-independent FISH techniques coupled with epifluorescence microscopy and flow cytometry in determining changes in these yeast populations during alcoholic fermentations. Sterile musts were inoculated with *S. cerevisiae*, *Hanseniaspora uvarum*, and *Starmerella (Starm.) bacillaris* (synonym *Candida zemplinina***)** to perform single and mixed fermentations. In this study, we have detailed the design of new probes with improved fluorescence intensity for monitoring *Hanseniaspora* and *Starm. bacillaris*

in wine fermentations. The fermentations were performed at two temperatures that are relevant from the wine-making point of view.

2. Materials and methods

2.1. Yeasts strains

The yeast strains and species used in this study are listed in Table 1. Yeasts were grown overnight in YPD (1% yeast extract, 2% peptone, and 2% glucose, w/v) medium at 28°C before use. All yeasts were identified by 5.8S-ITS-RFLP analysis according to Csoma and Sipiczki (2008) and Esteve-Zarzoso et al. (1999) and by sequences analysis of the D1/D2 domain of the 26S rDNA (Kurtzman and Robnett, 1998).

2.2. Design of oligonucleotide probe

Prior to designing the new probe, the published probes of *S. cerevisiae*, *H. guilliermondii*, and *Candida stellata* (Xufre et al., 2006) were synthesized and checked by hybridization with target species. Because of the undesirable results of the probes for *H. guilliermondii* and *C. stellata* in our initial assays, new oligonucleotide probes for *Hanseniaspora* and *Starm. bacillaris* that target within the D1/D2 domain of the 26S rRNA were designed (sequences were obtained from the GenBank database, accession numbers are shown in Table 2). Species-specific sequences were selected according to the alignment by Clustal Omega (EMBL-EBI) and the accessibility map of *S. cerevisiae* (Inácio et al., 2003). Subsequently, general and mismatch analyses with mathFISH (Yilmaz et al., 2011) were used to evaluate the sequences of these probes. To overcome a potential unspecific hybridization site, a competitor was designed to combine with the mismatched species to block the probe hybridization site on the non-target species. Competitor analysis from mathFISH (Yilmaz et al., 2011) was used to evaluate the effect of the competitor. Finally, the probes and the competitor were synthesized (Table 2) and tested first by hybridization with target species, and then, the fluorescence intensity and specificity of each probe were evaluated by FISH tests in pure cultures, mixed cultures, and wine samples using both methodologies, epifluorescence microscopy and flow cytometry. During the entire evaluation process, a multifactor trial of FISH techniques was performed until suitable probe and experimental conditions were found. All probes were labeled with fluorescein isothiocyanate (FITC).

Table 2. Sequences of oligonucleotide probes labeled with FITC at the 5' end and the non-labeled competitor used in this study.

Target species	Probe	Position[a]	Sequence (5'-3')	$\Delta G°_{overall}$[b] (kcal/mol)
C. stellata	Cst-FITC[c]	D133	CTCTATGGCGTTTCTTTC	-11.9
Eight species of	H8a-FITC	D402	TGAGAGGCCCAAGCCCAC	-15.8
Hanseniaspora[d]	H8b-FITC	D2	AGGTAATCCCAGTTGGTT	-14.3
S. cerevisiae	H8b-Com[e]	D2	AGGCAATCCCGGTTGGTT	
H. guilliermondii	Hgu-FITC[c]	D506	CAATCCCAGCTAGCAGTAT	-10.0
H. uvarum	Huv-FITC[c]	D507	TCAATCCCGGCTAACAGTA	-9.3
S. cerevisiae	Sce-FITC[c]	D526	TGACTTACGTCGCAGTCC	-13.9
Starm. bacillaris[f]	Sba-FITC	D133	CTCCATGGCGCTCCTTTC	-15.0

[a] *Sequence positions refer to the D1/D2 domain of the 26S rRNA gene of S. cerevisiae (U44806).*
[b] *The data rely on the general analysis of mathFISH (Yilmaz et al., 2011) under the same conditions of temperature (46°C), [Na^+] (0.9 M), and probe concentration (1000 nM).*
[c] *Probes designed by Xufre et al. (2006)*
[d] *H. clermontiae (sequences accession numbers: AJ512456, AJ512452), H guilliermondii (AB618029, EF449520, U84230), H. lachancei (AJ512457, AJ512459), H. meyeri (AJ512454, AJ512458, AJ512461), H. opuntiae (AJ512453, AJ512451, FM180532), H. pseudoguilliermondii (AJ512455, AB525689), H. uvarum (EU807899, JX103173, U84229), and H. valbyensis (U73596, JQ689026, JN938929).*
[e] *Oligonucleotide competitor used together with probe H8b-FITC to increase the specificity.*
[f] *Sequences accession numbers from GenBank database were AY160761, JX103187, EF452193, EF452215.*

2.3. Specificity test of probes

The specificity of each probe was tested in pure cultures, mixed cultures, and wine samples. All of the samples were collected by centrifugation (1 ml sample, 10,000 rpm for 5 min) and then hybridized separately with each probe. For pure cultures, the cells of each strain in Table 1 were collected directly from their YPD media. Mixed cultures were inoculated by mixing the same order of cells (2-5 x 10^7 cells/ml) from overnight cultures of *S. cerevisiae*, *H. uvarum*, *Starm. bacillaris*, *Torulaspora delbrueckii*, and *Metschnikowia pulcherrima*. Six different wine samples from Macabeo (white) and Garnatxa Negra (red) varieties were prepared in this study. Grapes were picked and fermented in two different vats of the experimental cellar of the Oenology Faculty in Tarragona (Spain) separately, with *S. cerevisiae* inoculated at the beginning. Two wine samples, W and R, were sampled at the end of the two fermentations. Samples MW1 and MR1 were created by adding an *H. uvarum* pure culture at the end of the two fermentations, and MW2 and MR2 were

created by adding *Starm. bacillaris* pure cultures. These additions were necessary because no *H. uvarum* or *Starm. bacillaris* was observed at the end of fermentation.

Table 1. Yeast strains and species used in this study and the specificity results for the three probes in pure cultures (sources: CECT, Spanish Type Culture Collection, Universitat de València; CBS, Centralalbureau voor Schimmelcultures, Delft, Netherlands; MCYC, Microbiology Collection of Yeast Cultures, Universidad Politécnica de Madrid; NS, natural isolates from our group collection; NCYC, National Collection of Yeast Cultures, Norwich, United Kingdom). Positive (+) or negative (-) hybridization is indicated.

Strains	CECT designation	Other designation	Isolation source	Specificity results for each probe		
				Sce-FITC	H8b-FITC + H8b-Com	Sba-FITC
Candida bodinii	11168	CBS 6990	Tepache	-	-	-
	1014[T]	CBS 2428	Tanning fluid	-	-	-
Candida mesenterica	1025	CBS 602	Brewery	-	-	-
Candida sake	10034	MCYC 123	Feces of sheep	-	-	-
	1044	CBS 617	Lambic beer	-	-	-
Candida stellata	11918[T]	CBS 157	Wine grapes	-	-	-
Starmerella bacillaris (Synonym *Candida zemplinina*)[a]	11046	CBS 2649	Grape juice	-	-	+
	11109	CBS 1713	Wine	-	-	+
	-	NS c	Grape must	-	-	+
	-	NS d	Grape must	-	-	+
Hanseniaspora opuntiae	11027[b]	NCYC 2380	Grape must	-	+	-
Hanseniaspora guilliermondii	11029	CBS 465	Infected nail	-	+	-
	11102	CBS 1972	Grape juice	-	+	-
Hanseniaspora osmophila	11206	CBS 313	Ripe Riesling grape	-	-	-
Hanseniaspora uvarum	1444[T]	CBS 314	Muscadet grape	-	+	-
	10389	MCYC 1857	Grape juice	-	+	-
	11105	CBS 2589	Grape must	-	+	-
	11106	CBS 5073	Wine grape	-	+	-
	11107	CBS 8130	Grapes	-	+	-
	-	NS b	Grape must	-	+	-
Hanseniaspora vineae	1471	CBS 6555	Grape juice	-	-	-
Metschnikowia pulcherrima	-	NS f	Grape must	-	-	-
Saccharomyces cerevisiae	1942[T]	CBS 1171	Beer	+	-	-
	-	NS a	Grape must	+	-	-
Torulaspora delbrueckii	-	NS e	Grape must	-	-	-

[T] means Type strain.
[a]A new name change according to Duarte et al. (2012); the two strains CECT 11046 and CECT 11109 were preserved as C. stellata until November 2012 by CECT.
[b]CECT11027, which is recorded as H. guilliermondii by CECT, was identified as H. opuntiae in this study.

Natural must from the Macabeo variety from our experimental facility was used to validate the probes. After settling, the must was left for 48 h prior to its

simultaneous inoculation with 3 different strains of *S. cerevisiae* (1 x 10^6 cells/ml from each strain). Samples were collected from the initial must after settling (time 0), at 12, 48, 60, and 84 h and at the end of fermentation. The samples were plated on YPD (1% yeast extract, 2% peptone, 2% glucose and 2% Agar, w/v) and 25 colonies were sampled for species identification by sequencing their D1/D2 26S rRNA coding region. The fermentations were performed in triplicates.

2.4. FISH procedure

The multifactor trial for each probe focused on four different parameters: fixation time (1 h, 2 h, 3 h, and overnight), percentage of formamide in the hybridization buffer (0%, 1%, 2%, 5%, 10%, and 20%), hybridization temperature (37°C, 42°C, and 46°C), and time (3 h and overnight), which were evaluated to optimize the hybridization potential of each probe. The effect of each parameter was assessed by performing single-factor tests based on the procedure of Andorrà et al. (2011).

The published FISH procedure used in Andorrà et al. (2011) was adjusted as follows. Collected cells were suspended with 1× phosphate-buffered saline (PBS) and incubated with 4% (v/v) of paraformaldehyde for 1 h at 4ºC and 1000 rpm agitation in a shaker. Fixed cells were centrifuged at 10,000 rpm for 2 min, resuspended with a 1:1 solution of 1× PBS and absolute ethanol, and then stored at −20°C until required. Approximately 10^6 cells were hybridized at 46°C for 3 h in 50 µl of hybridization buffer (0.9 M NaCl, 0.01% w/v SDS, 20 mM Tris-HCl, and 1% v/v formamide) with 10 ng/µl of probe and 10 ng/µl of competitor (when it was required). After hybridization, cells were centrifuged at 10,000 rpm for 5 min, resuspended in 100 µl of washing buffer (25 mM Tris/HCl and 0.5 M NaCl), and incubated at 48°C for 30 min. Then, the cells were centrifuged again, resuspended in 1× PBS, and analyzed immediately if possible but always within 4 h. A Neubauer chamber and epifluorescence microscope (Leica DM4000B, Wetzlar, Germany) equipped with filter I3 were used for cell observation and enumeration.

2.5. Flow cytometry

The fluorescence of the cell suspension in PBS (the same cells indicated in Section 2.4) was quantified using a BD FACSAria III flow cytometer (BD Biosciences, California, USA) equipped with a 15 mW, 488 nm argon-ion laser. Fluorescence was

detected using a 530 (±30) nm band pass filter. Daily instrument quality control, including fluorescence standardization, linearity assessment, and spectral compensation were performed to ensure operational consistency from day to day. At least 10,000 events were acquired and recorded in the linear mode for side scatter and in the log mode for fluorescent signals. The data were collected and analyzed using the FACSDiva software (BD Biosciences, California, USA).

2.6. Alcoholic fermentations

Fermentations were performed in triplicate in 50 ml conical tubes filled with 40 ml of natural concentrated must diluted to a sugar concentration of 240 g/l, the common concentration of local grape must (DOQ Priorat). After plating, no colonies were recovered; hence, we considered this must to be sterile. Single fermentations of Sc (*S. cerevisiae*), Hu (*H. uvarum*), and Sb (*Starm. bacillaris*) and mixed fermentations of ScHu (1:1 of *S. cerevisiae* and *H. uvarum*), ScSb (1:1 of *S. cerevisiae* and *Starm. bacillaris*), and ScHuSb (1:1:1 of *S. cerevisiae*, *H. uvarum*, and *Starm. bacillaris*) were performed separately with 2×10^6 cells/ml of total yeast inocula obtained from overnight YPD cultures. Fermentations were conducted with constant agitation (120 rpm) at two different temperatures, 15°C and 25°C, and the entire process was monitored by weight every 12 h until there was no weight loss. Samples (1 ml) were collected at five points (1, 2, 3, 4, and 5) according to the CO_2 releases of 0% (starting point), 25%, 50%, 75%, and 100%, respectively. Cells from each sample were collected after centrifugation at 10,000 rpm for 5 min. Each sample was fixed and then hybridized separately with each species-specific probe. Species were monitored by epifluorescence microscopy and flow cytometry using the same sampling process for both methodologies.

3. Results

3.1. Development of oligonucleotide probes

Four published probes and three new probes were tested in this study (Table 2) to find suitable probes for wine-related yeast species of *Saccharomyces*, *Hanseniaspora*, and *Starmerella*. The published probe Sce-FITC (Xufre et al., 2006) worked well in our initial assays, whereas Hgu-FITC and Cst-FITC (Xufre et al., 2006) exhibited low fluorescence intensities when hybridized with target species and were considered unsuitable.

The three new probes targeting the D1/D2 domain of the 26S rRNA were designed, with two probes (H8a-FITC and H8b-FITC) for eight species of the *Hanseniaspora* genus (*Hanseniaspora clermondiae*, *H. guilliermondii*, *Hanseniaspora lachancei*, *Hansenisapora meyeri*, *Hanseniaspora opuntiae*, *Hanseniaspora pseudoguilliermondii*, *H. uvarum*, and *Hanseniaspora valbyensis*) and one (Sba-FITC) for *Starm. bacillaris*. H8a-FITC and H8b-FITC were the only two possible probes for *Hanseniaspora* according to an *in silico* analysis. Both probes could hybridize with the eight species of this genus because the D1/D2 sequences of these eight species are closely related phylogenetically (Jindamorakot et al., 2009). However, both probes exhibited some limitations according to the *in silico* analysis: the target site of H8a-FITC incorporated a complex second structure according to the accessibility map; for H8b-FITC, there were only two mismatch bases between the target sequences of this probe and the corresponding sequence of *S. cerevisiae*. The *in silico* analysis was not sufficient for selecting between H8a-FITC and H8b-FITC; thus, both probes were synthesized and tested. H8a-FITC exhibited a low fluorescence intensity when hybridized with *Hanseniaspora* species, while H8b-FITC presented a positive signal when hybridized with *S. cerevisiae*. Because of the high intensity exhibited by H8b-FITC, this probe was selected and a competitor (H8b-Com) was designed to block the hybridization of this probe with *S. cerevisiae*, resulting in the removal of the unspecific signal from *S. cerevisiae*.

Furthermore, after the optimization of the protocol, the results revealed that 1 h of fixation was sufficient for sample preparation. One percent of formamide in the hybridization buffer was necessary for the optimal hybridization efficiency of H8b-FITC, whereas Sce-FITC and Sba-FITC also performed well with 5% and 10% formamide. The results obtained from varying the hybridization temperature and time did not reveal any improvements. However, the percentage of formamide in the hybridization buffer was more critical determining the hybridization efficiency of H8b-FITC when using the competitor.

3.2 Specificity tests of probes in pure cultures, mixed cultures, and wine samples

For pure cultures, as indicated in Table 1, the probes provided positive signals only when hybridized with strains of the target species. To consider any potential background signal interference from non-target species, a mixed culture was created

to provide the same background for all of the species. Cells of *S. cerevisiae*, *H. uvarum*, and *Starm. bacillaris* were distinguished clearly within the mixed yeast cultures by an epifluorescence microscope and flow cytometer (Figure 1). In the flow cytometry results, the difference between the non-stained cells (FITC less than 10^3) and the stained cells (FITC higher than 10^3) is clear.

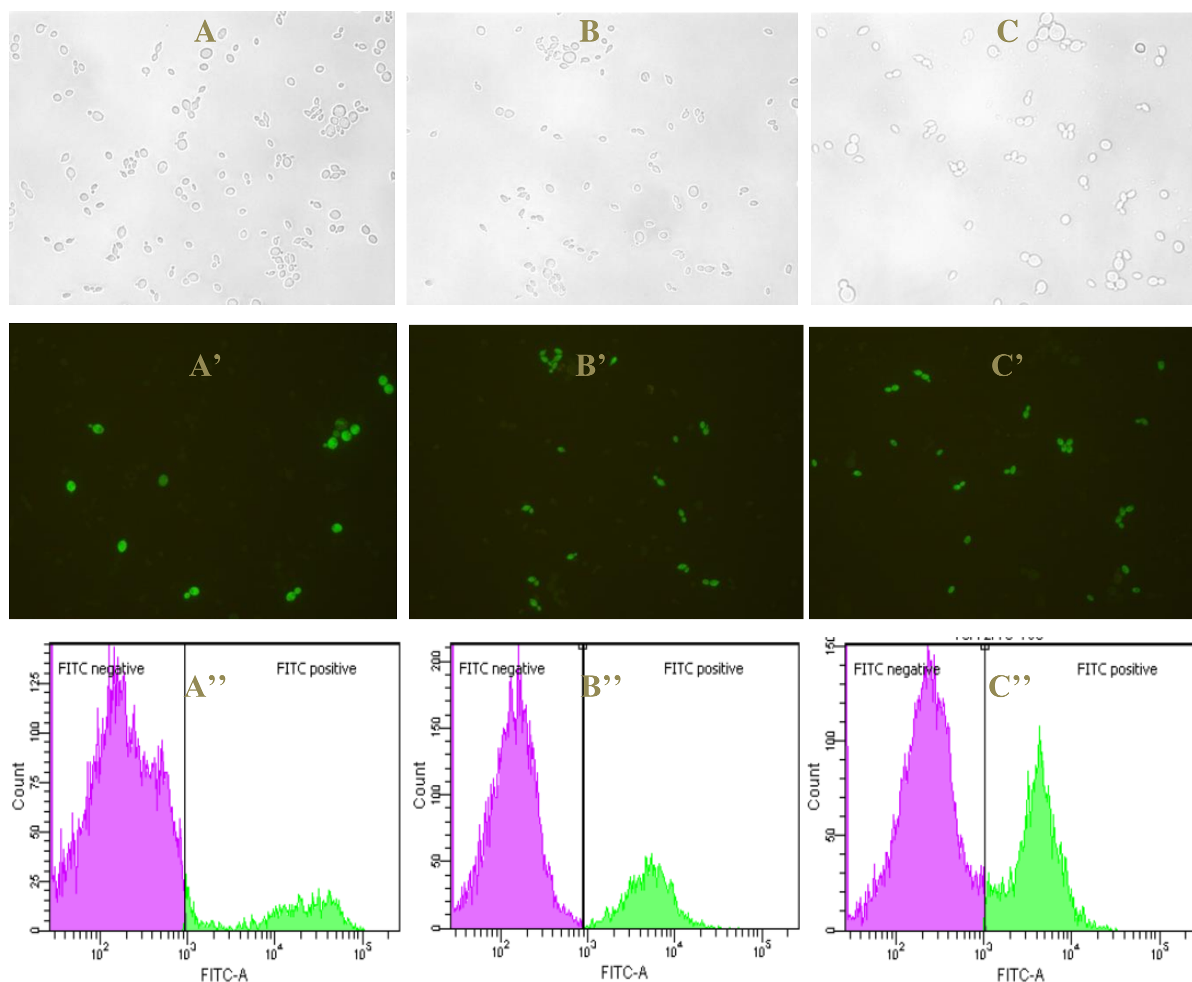
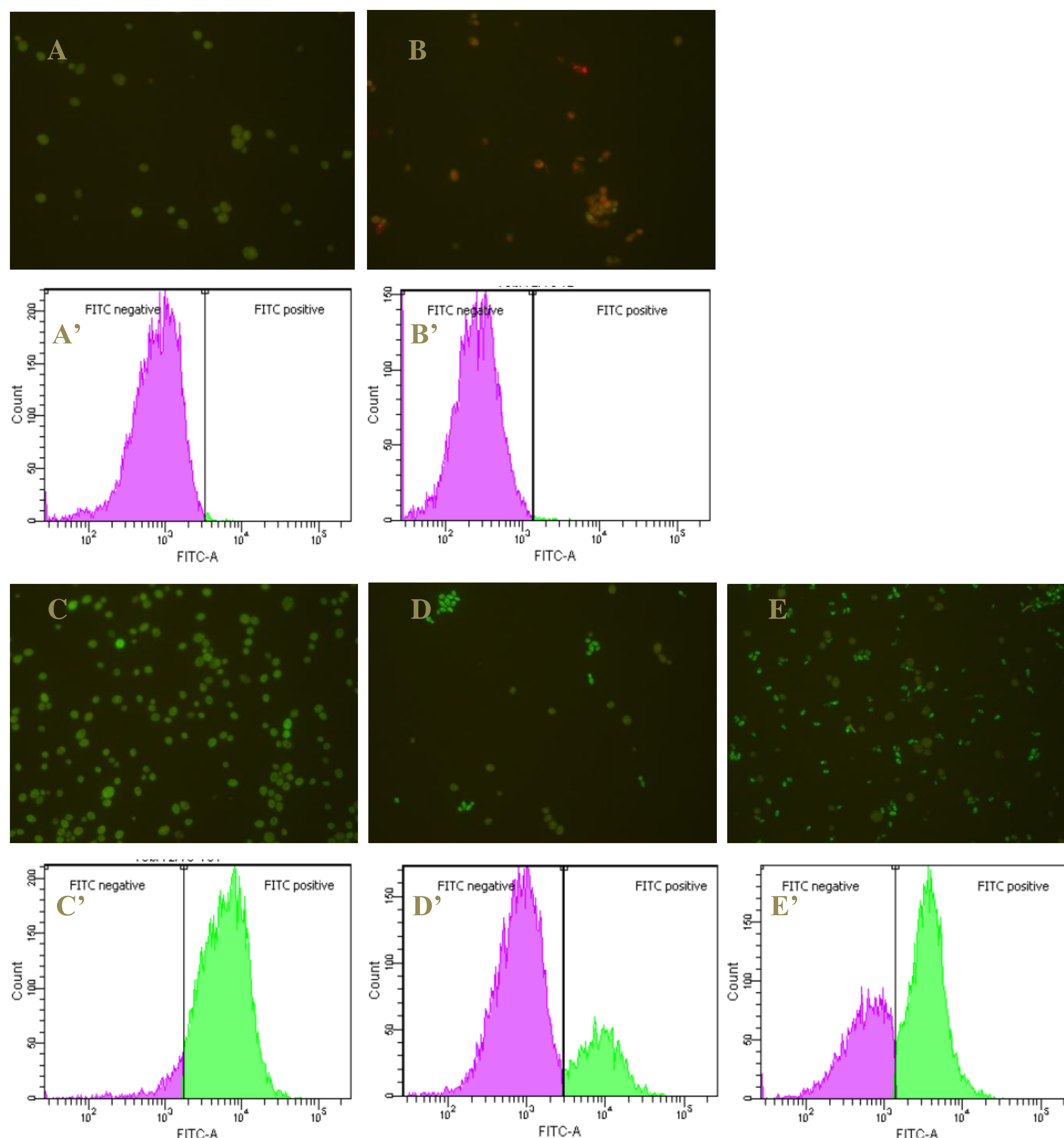


Figure 1. Fluorescent signal of target species in mixed yeast cultures visualized by microscopy and flow cytometry. Cells hybridized with probes Sce-FITC (A, A', A''), H8b-FITC coupled with H8b-Com (B, B', B''), and Sba-FITC (C, C', C'') and recorded with white light (A, B, C) and filter I3 (A', B', C') of the microscope and by flow cytometry (A'', B'', C'').

The non-hybridized wine samples W and R contained high background fluorescent signals because of the absorption of polyphenol pigments from the wine. However, for all three probes, the fluorescence intensities of the hybridized cells were much higher than the background signal (Figure 2). The hybridization results of the six wine samples demonstrated that the three target species, *S. cerevisiae*, *H. uvarum*, and *Starm. bacillaris*, can be differentiated in wine samples using both the

epifluorescence microscope and the flow cytometer (Figure 2). This capability was primarily achievable because the fluorescent color from the hybridized target cells differed from the background color and non-target cells in wine and because the target signal was more intense when using the filter for FITC. The H8b-FITC, when coupled with H8b-Com, Sce-FITC and Sba-FITC probes presented high specificity to the target species in the FISH tests performed on pure cultures, mixed cultures, and wine samples.



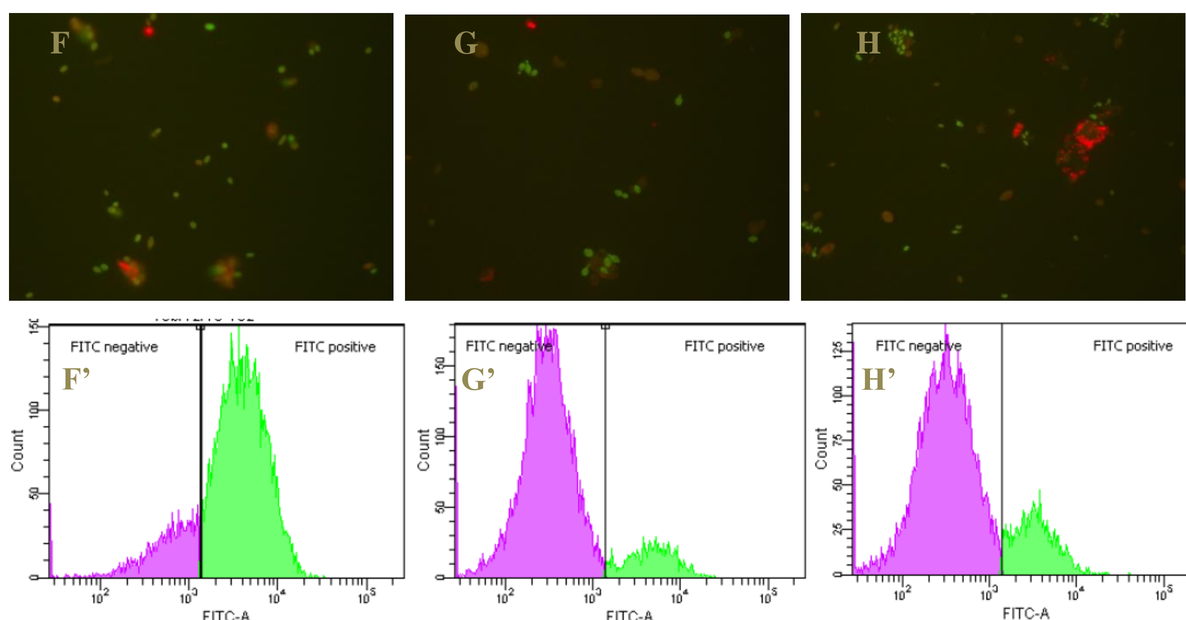


Figure 2. Fluorescent signals of background and target species in samples W, R, MW1, MW2, MR1, and MR2 visualized by microscopy (A, B, C, D, E, F, G, H) and flow cytometry (A', B', C', D', E', F', G', H'). Samples W (A, A') and R (B, B') without staining. Cells from sample W (C, C'), MW1 (D, D'), MW2 (E, E'), R (F, F'), MR1 (G, G'), and MR2 (H, H') hybridized with probes Sce-FITC (C, C', F, F'), H8b-FITC coupled with H8b-Com (D, D', G, G'), and Sba-FITC (E, E', H, H').

3.3. Yeast population analysis during alcoholic fermentations

At 25°C, all of the fermentations with *S. cerevisiae* (pure and mixed fermentations) were completed by 192 h, whereas the pure-culture fermentations with *Starm. bacillaris* (Sb) and *H. uvarum* (Hu) were slower and required 264 h and 336 h, respectively. All of the fermentations were relatively longer at 15°C: ScHu and ScHuSb terminated after 264 h; ScSb and Sc required 336 h; and Sb and Hu did not complete their fermentations until 336 h. For the pure-culture fermentations, Sb reached point 3 of the fermentation, whereas Hu only reached point 2 (25% CO_2 release). The FISH methodology was used to monitor the yeast population dynamics during the entire fermentation process and the population dynamics are shown in Figure 3. The population dynamics of *S. cerevisiae* were similar in all of the mixed fermentations, regardless of whether the results were obtained by epifluorescence microscopy or flow cytometry. In contrast, the populations of *H. uvarum* and *Starm. bacillaris* were slightly larger according to the microscopy results compared to those recorded by flow cytometry.

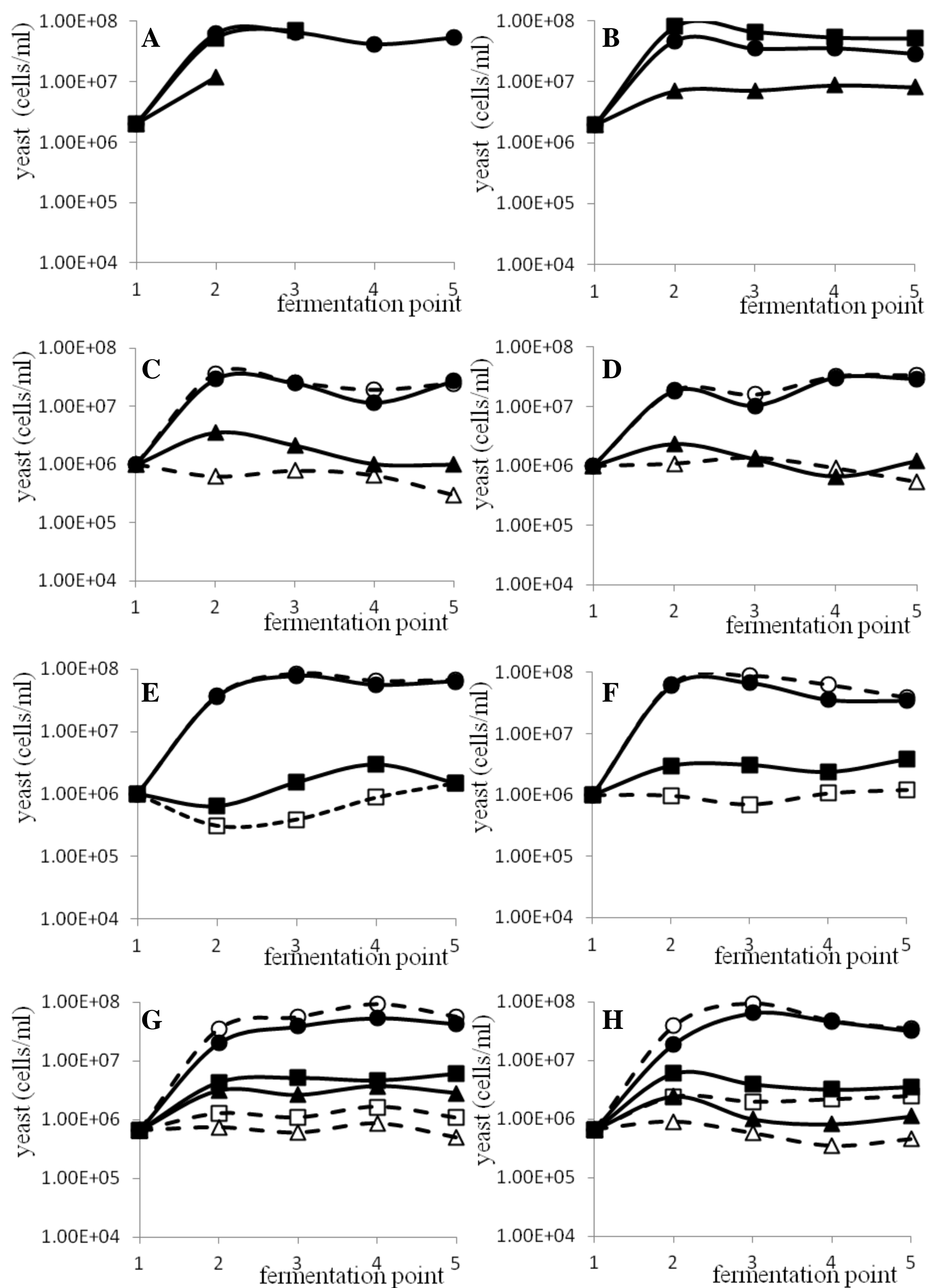


Figure 3. Yeast population dynamics during alcoholic fermentations at 15°C (A, C, E, G) and 25°C (B, D, F, H). Solid lines indicate populations measured by epifluorescence microscopy, and dotted lines indicate populations assessed by flow cytometry (the population quantity shown is the mean of triplicate values). Single fermentations of Sc, Hu, and Sb (A, B). Mixed fermentations of ScHu (C, D), ScSb (E, F), and ScHuSb (G, H). Symbols: (●, ○) *S. cerevisiae*; (▲, △) *H. uvarum*; (■, □) *Starm. bacillaris*.

The populations of *S. cerevisiae* and *Starm. bacillaris* in the single fermentations increased from their initial concentration of 10^6 cells/ml to 10^7–10^8 cells/ml, whereas *H. uvarum* grew more slowly with a population level of 10^6–10^7 cells/ml. In all of the mixed fermentations, the *S. cerevisiae* populations increased to 10^7–10^8 cells/ml after inoculation, whereas the populations of *H. uvarum* and *Starm. bacillaris* did not increase and remained at approximately 10^6 cells/ml. The temperature affected the fermentation process: the fermentations at 15°C were slower than those at 25°C, while mixed fermentations inoculated with *H. uvarum* were faster than the others at the low temperature. The population dynamics of *S. cerevisiae*, *H. uvarum*, and *Starm. bacillaris* at both 15°C and 25°C were similar for both the single and mixed fermentations and did not seem to be affected by the temperature.

While, the fluorescence intensity of the hybridized *S. cerevisiae* cells varied during the fermentation process, those of the *H. uvarum* and *Starm. bacillaris* cells did not. At point 1 of the fermentation, most of the *S. cerevisiae* cells had achieved the highest fluorescence intensity. This intensity declined in the later stages of the fermentations (decreasing by up to 10-fold from the initial fluorescence); however, the *S. cerevisiae* cells always exhibited a higher intensity than the background. These changes in intensity were always observed with the *S. cerevisiae* probes, but the cells within a sample yield did not always produce the same intensity. This result can be observed by the formation of two peaks in the flow cytometry data (supplementary data).

Finally, the validation of the probes was also tested in a natural fermentation of Macabeo must. As seen in Figure 4, we detected the presence of *H. uvarum* and *Starm. bacillaris* as the main non-*Saccharomyces* species in the must (approximately 10^6 cfu/ml) as well as traces of *T. delbrueckii* and *M. pulcherrima* (1 colony each at 0 and 12 h), which disappeared after the inoculation of *S. cerevisiae*. During the start of the alcoholic fermentation, the values obtained on plates and by FISH were very similar. However, at the end of the fermentation, these values differed greatly; *H. uvarum* was not detected by plating, while *Starm. bacillaris* differed by two log units and *S. cerevisiae* by 5-fold. For the total cell count, the values were greater on plates than under the microscope during the initial stages of fermentation and one order of magnitude lower at the end of the fermentation.

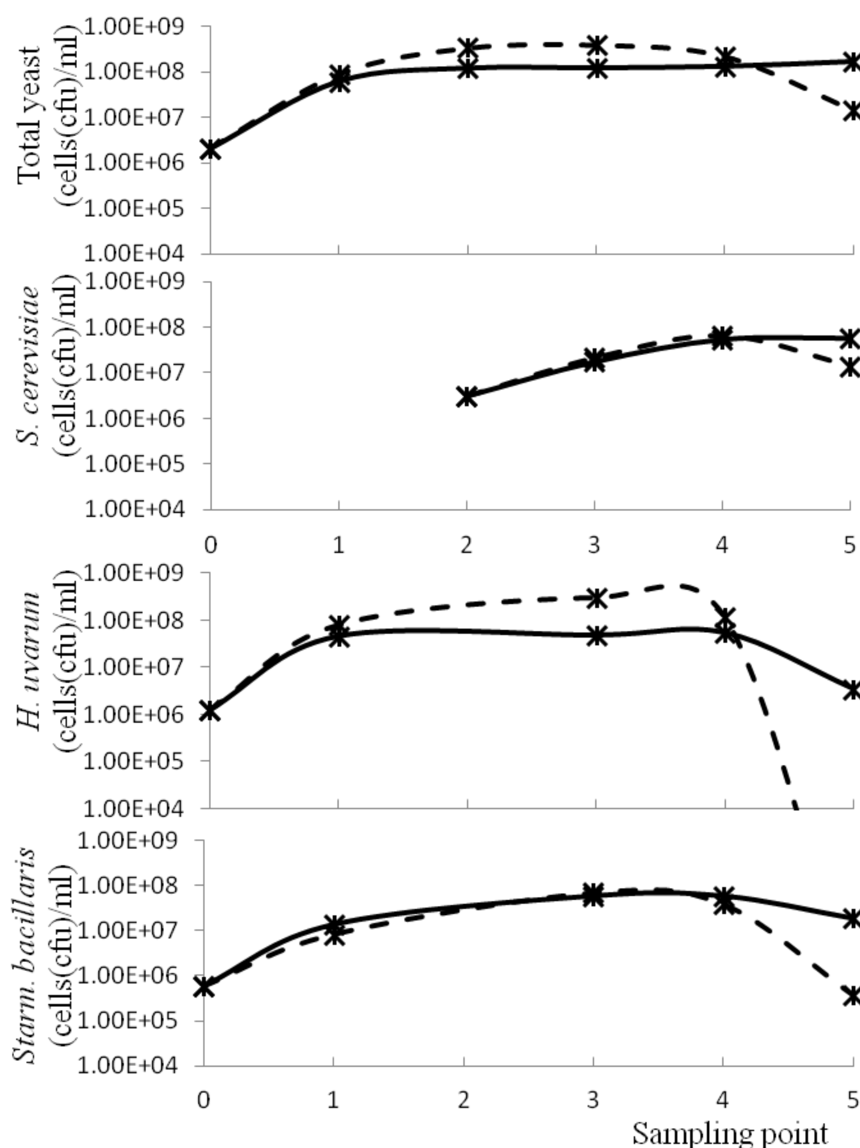


Figure 4. Yeast population dynamics during the natural fermentation of Macabeo must. Solid lines refer to populations assessed by flow cytometry and dashed lines to plates. Sampling points were: 0, must after settling (density 1100, 0h); 1, 12 h; 2, 48 h (time of *S. cerevisiae* inoculation); 3, 60 h; 4, mid-fermentation (density 1060, 84 h); and 5, final fermentation (density <1000, 312 h).

4. Discussion

Culture-independent techniques have been developed for the rapid enumeration of yeast populations during the wine-making process, with special emphasis on the determination of both the live and VBNC cells (Andorrà et al., 2010b; Andorrà et al., 2011; Branco et al., 2012; Cocolin and Mills, 2003; Hierro et al., 2006; Röder et al., 2007). Among them, FISH is a promising technique for

detecting target cells directly from wine samples because FISH probes are designed to hybridize with the rRNA inside of cells. In the present study, suitable probes and experimental conditions for detecting *S. cerevisiae*, *Hanseniaspora*, and *Starm. bacillaris* were selected and used for the quantification of each yeast species during the entire fermentation process. Thus, the evaluation of the potential for employing FISH in wine fermentation analyses was the goal of this study. Notably, the yeast cells detected by FISH should be strictly defined as cells with non-degraded rRNA. The stability of rRNA is species dependent (Andorrà et al., 2011); for example, 99% of the 26S rRNA of *S. cerevisiae* was found to be degraded within 24 h after cell death by heat-shock (Hierro et al., 2006).

Early reports have described FISH probes for several wine-related yeast species (Röder et al., 2007; Stender et al., 2001; Xufre et al., 2006); however, only probes for *D. bruxellensis* and *S. cerevisiae* have been successfully used for direct wine sample analyses (Andorrà et al., 2011; Röder et al., 2007). This is the first report of the use of oligonucleotide probes for *Hanseniaspora* and *Starm. bacillaris* in direct wine sample analysis. In addition, the design and evaluation of the oligonucleotide probes followed the important guidelines (regarding specific sequences, accessible sites, and high affinity) suggested by Inácio et al. (2003) and Yilmaz et al. (2011). For example, probes should be designed with −13 to −17 kcal/mol of $\Delta G^{o}_{overall}$ under typical FISH conditions according to principles of the mathFISH program (Yilmaz et al., 2011). However, the $\Delta G^{\circ}_{overall}$ values of the three probes (Hgu-FITC, Cst-FITC, and Huv-FITC) designed by Xufre et al. (2006) were outside of this recommended range, indicating a low affinity of those probes for target sites, which can most likely explain their low hybridization efficiencies. Unfortunately it could be difficult to achieve a good balance among these guidelines for some yeast species because the possible specific site for specific hybridization (for *Hanseniaspora* species, it was D441–D518) was located in an inaccessible region according to the accessibility map (Inácio et al., 2003). Subsequently, the experimental evaluation provided the final evidence. Furthermore, the probes should be subject to a multifactor trial to evaluate the conditions for the optimal hybridization efficiency. The percentage of formamide in the hybridization buffer is a relevant factor that should be tested for each probe, and mathFISH was demonstrated to be a useful tool with theoretical references for determining the appropriate formamide concentration (Yilmaz et al., 2011).

The high background of the fluorescent signal found in the white and red wine samples was the most likely reason for the limited use of FISH probes for the direct analysis of wine samples in past years. The polyphenols absorbed by yeast walls cause an intensive yellow fluorescence, while the anthocyanins that often form aggregates that include yeast cells cause intense red fluorescence. Thus, probes labeled with fluorochromes of these wavelengths or similar wavelengths (such as the FITC probe used in this study) should be tested for their clear discrimination between the background and the fluorescence of the target.

As proposed in Andorrà et al. (2011), FISH combined with epifluorescence microscopy and flow cytometry was used to monitor the yeast populations during the entire fermentation process. FISH combined with microscopy was useful for analyzing each cell in detail; however, for ecological studies, there is a need for the observation of a large number of cells and a highly experienced operator if the goal is an accurate quantification of the yeast populations present in small numbers. The analysis of a large number of cells under a microscope is tedious and time consuming, and the percentage of cells found in small populations can be biased by the subjectivity of the operator if they specifically look for those minor species. In contrast, the use of FISH combined with flow cytometry is fast, sensitive, and accurate because thousands of cells can be recorded in several seconds. Thus, it is suitable for the high throughput analysis of large numbers of wine samples. For our results, special attention should be paid to the aggregation of cells caused by the fixation step of FISH and this portion of cells should be excluded by gating when using flow cytometry. The aggregation of cells was a common phenomenon observed when using the FISH technique. Fortunately, the aggregation of yeast cells was not as serious as that of bacteria, making FISH combined with flow cytometry easier for yeast. Considering the complementary advantages of both methodologies, their combined use would be a better choice for the precise enumeration of yeast populations in wine samples.

Two interesting phenomena concerning yeast population dynamics during wine fermentations were observed in this study. On the one hand, the two non-*Saccharomyces* species maintained their inoculated population sizes in the mixed fermentations, while their populations increase in the single culture fermentations. This result could suggest that the capacity of the non-*Saccharomyces* population to grow was limited and that environmental pressure caused by *S. cerevisiae* might be

the primary factor. This finding was also observed in the natural fermentation. Meanwhile, the non-*Saccharomyces* populations did not decrease sharply in the late stages of mixed fermentations as normally observed by culture-dependent methods and as indicated in former ecological studies based on other culture-independent techniques (Andorrà et al., 2010b; Andorrà et al., 2011; Cocolin and Mills, 2003). Non-*Saccharomyces* yeasts can enter a viable but non-culturable state; under these conditions, they lose their cultivability but maintain their ribosomal RNA (without degradation), and a portion of these cells likely maintain their metabolic activities. However, we cannot completely rule out the possibility that the cells died and their rRNA persisted. In fact, at the end of the natural fermentation, we observed a meaningful difference between the number of cells observed under the microscope and the number of those able to grow on plates, which could have been attributed to either dead cells that maintained their cell structure (and thus their rRNA) or to cells that remained alive but were not able to be grown on plates (viable but unculturable). Furthermore, the variation in the fluorescence intensity of the stained *S. cerevisiae* cells during the fermentation, similar to the phenomenon observed by Andorrà et al. (2011), could be related to an increase in ribosome synthesis during the initial stages of alcoholic fermentation in *S. cerevisiae* cells (Novo et al., 2007).

The actual state of the cells detected by FISH was difficult to determine and most likely included VBNC cells, injured cells, and even dead cells with high rRNA stability. Herrero et al. (2006) and Regan et al. (2003) have suggested that the viability of the cells could be assessed using fluorescent dyes such as EMA and propidium iodide (PI) to stain cells with compromised membranes. Branco et al. (2012) reported that direct live/dead staining combined with FISH (LDS-FISH) can be used to effectively assess the viability of *S. cerevisiae* and *H. guilliermondii* during alcoholic fermentation. Thus, the combined use of such dyes and FISH could be evaluated for the detection of the true, viable target yeast population in future work.

In summary, the present study developed specific oligonucleotide probes for *Hanseniaspora* and *Starm. bacillaris*, which were applied in FISH combined with flow cytometry. We were able to directly identify *S. cerevisiae*, *H. uvarum*, and *Starm. bacillaris* in complex wine samples and thus demonstrated the potential for using FISH techniques in wine ecological studies. The design of new probes for other species will help to monitor the population dynamics of various yeast species (including minor populations) during industrial wine fermentations and to detect

spoilage yeasts during wine aging and storage. We also demonstrated the successful application of FISH in natural fermentations.

Acknowledgements

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Supplementary data

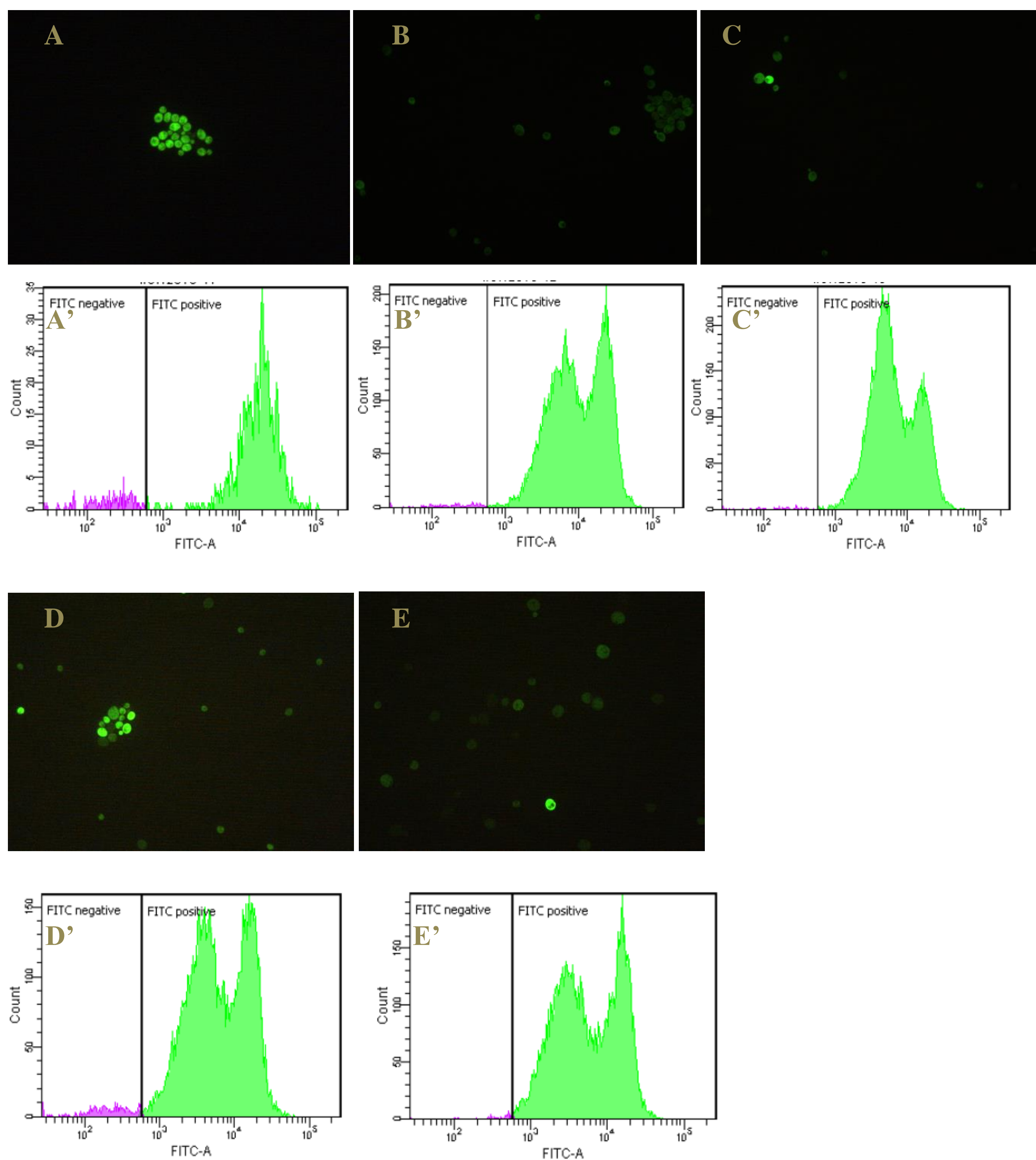

Figure 1. Fluorescent signal of stained *S. cerevisiae* cells at five fermentation points of ScSb. The five different fermentation points were marked with A (A'), B (B'), C (C'), D (D'), and E (E'), respectively.

Chapter 3

Viable and culturable populations of *Saccharomyces cerevisiae*, *Hanseniaspora uvarum* and *Starmerella bacillaris* (synonym *Candida zemplinina*) during Barbera must fermentation

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Abstract

The present study analyzed the viable and/or culturable populations of *Saccharomyces cerevisiae*, *Hanseniaspora uvarum* and *Starmerella bacillaris* (synonym *Candida zemplinina*) during laboratory grape must fermentation, in order to investigate the interaction between the three species considered. Firstly, population dynamics during wine fermentation were followed by culture-dependent techniques, and non-*Saccharomyces* yeast became non-culturable at late stages of fermentation when *S. cerevisiae* dominated. Four different culture-independent techniques were further applied to detect viable yeast cells at the late stage of fermentation. Both quantitative PCR techniques applied, namely ethidium monoazide bromide (EMA)-qPCR and Reverse Transcription (RT)-qPCR, detected *H. uvarum* and *Starm. bacillaris* at a concentration of 10^5 to 10^6 cells/mL. These non-culturable cells had membranes impermeable to EMA and stable rRNA. The background signals from dead cells did not interfere with the quantification of viable cells in wine samples by EMA-qPCR techniques. As a qualitative culture-independent technique, DGGE technique was coupled with EMA treatment (EMA-PCR-DGGE) or with RT (RT-PCR-DGGE). With EMA-PCR-DGGE non-*Saccharomyces* species during fermentation were detected although it was limited by the predominance of *S. cerevisiae*.

Keywords: Culture-independent technique, DGGE, Ethidium monoazide bromide (EMA), Quantitative PCR (qPCR), Wine, Non-*Saccharomyces*.

1. Introduction

Wine fermentations are complex ecological processes with a succession of variable yeast species that could provide different characteristics to the fermentation and final wine (Fleet, 2003). Considerable efforts have been devoted to investigate and monitor population dynamics during mixed fermentations with *Saccharomyces* and non-*Saccharomyces* species. According to culture-dependent analysis, the non-*Saccharomyces* species are only isolated from early stages and the fermentations are dominated by *Saccharomyces* at late stages (Fleet, 2008). Recently, using culture-independent techniques, it was highlighted that viable non-*Saccharomcyes* populations could be quantified at late stages of fermentation (Andorrà et al., 2008; Wang et al., 2014) supporting their possible role also at the end of the transformation process. For these reasons, a thorough study is required to understand the states (culturable, live, injured or dead) of non-*Saccharomyces* during fermentation.

The application of culture-independent techniques in wine ecology studies is considered a valid approach to investigate the presence of viable but non-culturable (VBNC) cells (Cocolin et al., 2013). For the quantitative techniques, qPCR was firstly used to directly quantify yeast DNA from wine samples (Hierro et al., 2007; Tofalo et al., 2012), but because of the stability of DNA, dead cells were also quantified resulting in an overestimation of yeast populations. To solve this problem, RT-qPCR, fluorescence in situ hybridization (FISH) and qPCR using ethidium monoazide bromide treatment or propidium monoazide bromide (EMA-qPCR or PMA-qPCR) were explored (Hierro et al., 2006; Rawsthorne and Phister, 2009; Andorrà et al., 2010a; Shi et al., 2012; Vendrame et al., 2014; Wang et al., 2014). In RT-qPCR and FISH the cells with rRNA are considered viable, because the rRNA is less stable than DNA and, thus, is not quantified in dead cells (Hierro et al., 2006, Andorrà et al., 2011, Wang et al., 2014). EMA-qPCR excludes cells with compromised membranes; EMA enters these cells and covalently combines with DNA which is not amplified by subsequent PCR reactions (Rudi et al., 2005). All of these quantitative techniques require specific primers, which increase the sensitivity for detection, avoiding the detection of non-targeted yeast species. As qualitative techniques, PCR-DGGE and RT-PCR-DGGE have been developed with universal primers to detect all probable yeast species without the need to know their sequences (Cocolin et al., 2000; Mills et al., 2002). However, detection sensitivity of DGGE depended on the disparity of orders of magnitude among different populations (Mills et al., 2002; Andorrà et al.,

2008; Cocolin et al., 2011). Therefore, to analyze the yeast species in complicated must samples during fermentations, it is better to use both qualitative and quantitative techniques.

This study focused on three main species in must fermentations: *Saccharomyces cerevisiae*, *Hanseniaspora uvarum* and *Starmerella bacillaris* (synonym *Candida zemplinina*), with the aim to analyze the vitality state of the cells during fermentation, especially at late stages. These two non-*Saccharomyces* species were studied here because of their common appearance on Barbera grape in Piedmont region of Italy (Alessandria et al., 2015). The population dynamics during the whole fermentation was monitored by culture-dependent techniques. When the cell culturability was lost for the non-*Saccharomyces* species, EMA-qPCR and EMA-PCR-DGGE, as well as RT-qPCR and RT-PCR-DGGE were used to determine the cells' status in wine samples. Dead cells, after 75% ethanol treatment, and culturable cells were used as negative and positive controls, respectively, to raise the standard curves for qPCR techniques and markers for DGGE.

2. Materials and methods

2.1 Yeast strains

H. uvarum Y1 (Mills et al., 2002) and *Starm. bacillaris* CBE4 (Englezos et al., 2015) were obtained from yeast culture collection of the DISAFA (Dipartimento di Scienze Agrarie, Forestali e Alimentari, University of Torino, Italy). Uvaferm BC (*S. cerevisiae*) was obtained from Lallemand (Montreal, Canada). The yeast cultures from DISAFA were grown on YPD agar (1% yeast extract, 2% peptone, 2% dextrose, all from Biogenetics, Italy), and then were inoculated into 10 mL of sterile Barbera must for a preadaptation. The Uvaferm BC was activated following the suppliers' instructions and subsequently preadapted in the same must.

2.2 Wine fermentation and sampling

Red Barbera grape berries were harvested in 2013 and after crushing grape must was stored at -20 °C. Before use, the grape must was defrosted at 4 °C, and then pasteurized at 65 °C for 1 h. Flasks of 250 ml containing 100 mL of Barbera must (Glucose+Fructose 234.00 g/L, malic acid 3.8 g/L, citric acid 0.3 g/L, tartaric acid 5.8 g/L, pH 2.95, and YAN 179.60 mg N/L) were inoculated with 1 x 10^5 cells/mL of preadapted Uvaferm BC, *Starm. bacillaris* CBE4 and *H. uvarum* Y1.

Fermentations were performed in duplicate at 25 °C, statically in presence of air (with screw cap not totally tight), and the whole process was monitored by weight every 24 h until no further weight loss. Samples (3 mL) were taken at day 0, 1, 4, 6, 8, 11 and 14. One mL sample was used for microbiological analysis and another two tubes with 1 mL sample were centrifuged at 14,000 rpm for 10 min. The supernatants were collected for analysis of main parameters using an HPLC (Agilent Technologies 1260 Infinity, USA) according to Giordano et al. (2009), and the content of YAN was measured by L-arginine / urea / ammonia assay kit (Megazyme, Ireland) and primary amino nitrogen assay kit (Megazyme, Ireland). The pellet in one tube was passed to EMA treatment and further DNA extraction, and the pellet in the other tube was suspended in 100 µL of RNA*later*® solution (Ambion, USA), and then kept at -20 °C for further RNA extraction.

2.3 Microbiological analysis

Appropriate dilutions in ten-fold series by Ringers solution (Oxoid, Italy) from 1 mL of must were spread onto WL nutrient agar (Biogenetics) and Lysine medium (Oxoid). Counting was done after five days' growth at 28°C. Colonies of the three yeast strains were discriminated by different morphologies and colors on WL nutrient agar (Cavazza et al., 1992). Lysine medium was used for quantification of non-*Saccharomyces* population when *S. cerevisiae* dominated the fermentation.

2.4 EMA treatment and DNA extraction

The cells' pellet from 1 mL of sample was resuspended in 1 mL of YPD broth and kept at 13 °C for 2 h to recover cells' membrane from the ethanol interference (Andorrà et al., 2010a). Then cells were collected, suspended in 1 mL of sterile water and treated with EMA (Sigma-Aldrich, Italy) solution (5 g/L) using the same device and procedure as described by Andorrà et al. (2010a). Briefly, EMA was dissolved in sterile Milli-Q water to prepare the solution of 5 g/L. The box with a 650-W halogen lamp was constructed, and the distance between the lamp and the tube was approx. 20 cm. Two microliters of EMA solution were added to the cell suspension (final concentration of EMA was 24 µM), and incubated in dark for 10 min. The samples were exposed to light for 30 s, kept on ice for 1 min with light off, and exposed again to light for 30 s. Cells were collected by centrifugation and washed with 1 mL of sterile water to remove the unstained EMA. Masterpure™ Complete DNA & RNA

Purification kit (Epicentre, USA) was used to extract DNA according to the manufacturer's instruction. The DNA extracted was conserved at -20 ºC and used for both EMA-qPCR and EMA-PCR-DGGE analysis.

2.5 RNA extraction and reverse transcription

The cell suspension preserved in RNA*later*® solution was centrifuged, and RNA from cell pellet was extracted by Masterpure™ Complete DNA & RNA Purification kit (Epicentre, USA) following manufacturer's instructions. RNA was treated with TURBO™ DNase (Ambion, USA) at 37 °C for 3 h or overnight (if needed) to completely remove DNA. Complete DNA removal was confirmed by qPCR. Reverse transcription of the extracted RNA was performed with the following procedure: 0.5 µL of RNA was mixed in 4.5 µL of DNase and RNase-free water containing 0.5 µL of Random Primers (500 µg/mL, Promega, Italy), and incubated at 72 °C for 5 min. The reaction sample was kept on ice for 5 min, and then added with 7.5 µL of mixture containing 2.5 µL of M-MLV RT 5× Buffer, 2.5 µL of 10mM dNTPs, 20 U of RNase inhibitor (all from Promega, Italy), 100 U of M-MLV Reverse transcriptase (Promega), and 1.5 µL of DNase and RNase-free water. The reaction continued with incubation at 42 °C for 1 h and stopped with a step of 72 °C for 10 min. The cDNA synthesized was conserved at -20 ºC and used for further RT-qPCR and RT-PCR-DGGE analysis.

2.6 Standard curves and qPCR analysis

The qPCR was performed with the primers (all from Sigma-Aldrich, Italy) YEASTF/YEASTR for total yeast (Hierro et al. 2006), CESPF/SCERR for *S. cerevisiae* (Hierro et al. 2007), CESPF/HUVR for *H. uvarum* (Hierro et al. 2007), and AF/200R for *Starm. bacillaris* (Andorrà et al. 2010b). Each reaction was carried out by the MiniOpticon™ Real-Time PCR System (Bio-Rad, Italy) in a total volume of 13 µL of reaction mixture, which contained 6 µL of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), 0.4 µM of the primers, and 1 µL of DNA or cDNA template. PCR conditions were as in Andorrà et al. (2010b). Standard curves of EMA-qPCR and RT-qPCR were constructed for each yeast species in triplicate using 10-fold serial dilutions of preadapted cells. These cells were obtained from one day's yeast cultures in 10 mL of sterile Barbera juice as mentioned in 2.1. The serial dilutions were performed using 9 mL of sterile must to which 1 mL of cell suspension

was added. The final cell suspensions were counted by hemocytometer under microscope to determine the real concentrations of serial cell dilutions.

Dead cells were prepared from the same cultures, and then treated with 75% ethanol for 24 h. The lysis process of cells was confirmed by the absence of growth in YPD broth after 24 h and on WL nutrient agar after 5 days.

2.7 PCR and DGGE analysis

Primers $NL1^{GC}$ and LS2 were used to amplify the ribosomal region of extracted DNA or synthesized cDNA for further DGGE analysis (Mills et al., 2002). Five μL of the PCR products were firstly checked for the sizes by agarose gel electrophoresis, then, the same volume was further separated by DGGE gel electrophoresis in a DCode universal mutation detection system (Bio-Rad). Both kinds of electrophoresis were operated and the gels were stained and photographed according to the descriptions of Mills et al. (2002) with minor modifications: DGGE gel used a denaturing gradient from 30% to 60% of urea and formamide, and was run at 120 V for 4 h. PCR products from DNA or cDNA of pure yeast cultures were used as markers in DGGE gel. Different cell mixtures were prepared from pure yeast cultures, and subjected to EMA-PCR-DGGE analysis to determine the detection limits of the three species.

2.8 Data analysis

Statistical analyses of variations were performed by One-Way ANOVA to calculate the value of F and significance, with post-hoc Tukey test when needed, using IBM SPSS Statistics 23. The Ct values from live and dead cells were used directly for variation analysis, and the yeast population numbers analyzed by different techniques were converted to logarithm value for further variation analysis.

3. Results

3.1 Culturable yeast populations during must fermentation

The fermentations terminated after 11 days, although culturability analysis was extended up to 14 days. During the whole fermentation process, culturable populations of the three species showed different trends, especially at late stages of fermentations (Fig. 1). The three species grew to population of 10^7 colony forming units (cfu)/mL during the first days of fermentation. *S. cerevisiae* (Uvaferm BC)

maintained the maximum population level during fourteen days. *Starm. bacillaris* CBE4 kept similar population level as *S. cerevisiae* during 8 days, decreasing sharply to undetectable levels by plating on day 11. The other non-*Saccharomyces* species, *H. uvarum* Y1, grew faster to reach a population of 10^7 cfu/mL, but decreased to undetectable level earlier (day 6) than the *Starm. bacillaris* CBE4 strain.

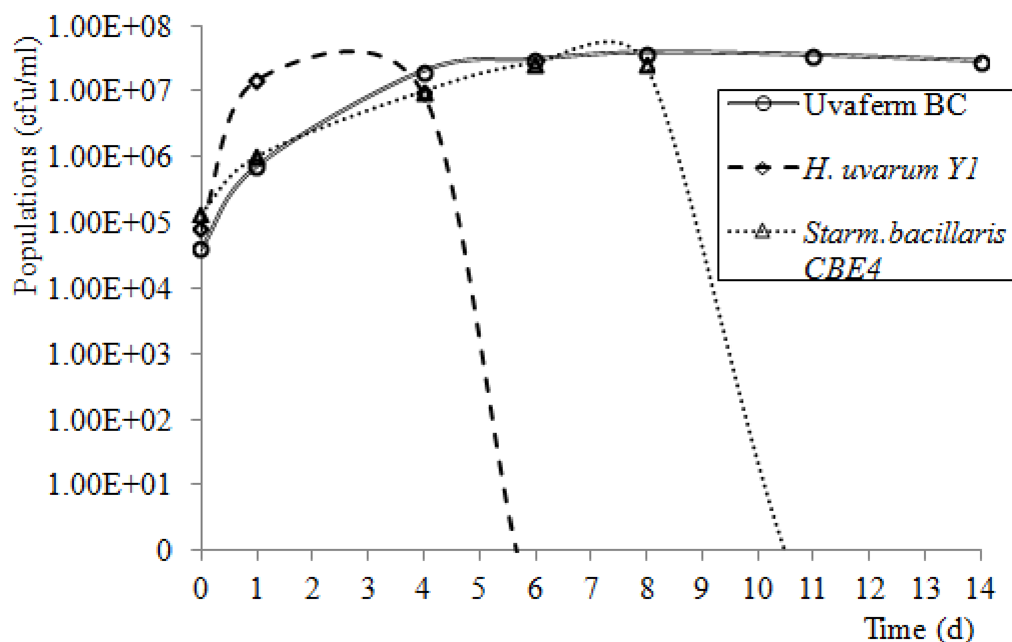

Figure 1. Culturable *S. cerevisiae* (Uvaferm BC), *H. uvarum*, and *Starm. bacillaris* populations during grape must fermentation as determined by plating on WL nutrient agar and Lysine medium. The values are the mean of duplicates.

3.2 qPCR analysis of wine samples

First, standard curves for each species and both techniques (EMA and RT-qPCR) were separately constructed. Background signal from dead cells at different concentrations were also quantified. Finally, the populations of each species in the selected samples were quantified and compared among the two culture-independent techniques and the culture-dependent techniques.

3.2.1 Standard curves for EMA-qPCR

Cells adapted in sterile Barbera must were serially diluted in the same must and quantified by microscope to associate the logarithm values of cells' concentration and Ct values. Good correlations were obtained for populations between 10^3 and 10^7 cells/mL by EMA-qPCR analysis (Table 1).

Table 1. The slope, intersection, correlation coefficient (R^2) and efficiency of standard curves of total yeast, *S. cerevisiae*, *H. uvarum*, and *Starm. bacillaris* by EMA-qPCR analysis and RT-qPCR analysis. The efficiency was calculated by the formula $10^{-1/slope}$ -1. Mean and standard deviation of triplicate qPCR amplifications are shown.

Technique	Yeast	Slope	Intersection	R^2	Efficiency (%)
EMA-qPCR	Total yeast	-2.8250±0.2490	36.924±0.813	0.9943±0.0061	125.93±23.17
	S. cerevisiae	-3.4097±0.0807	42.319±0.484	0.9909±0.0005	96.46±3.21
	H. uvarum	-3.2230±0.0198	38.599±0.103	0.9976±0.0037	104.30±0.89
	Starm. bacillaris	-3.8530±0.3224	47.819±2.024	0.9912±0.0152	81.78±9.06
RT-qPCR	Total yeast	-2.4045±0.0770	30.095±0.3734	0.9796±0.0021	160.55±8.00
	S. cerevisiae	-2.9293±0.0741	40.454±0.479	0.9870±0.0009	119.47±4.39
	H. uvarum	-3.1147±0.1020	41.340±0.4815	0.9964±0.0030	109.44±5.21
	Starm. bacillaris	-3.3408±0.0364	37.036±0.3539	0.9923±0.0006	99.22±1.48

The preadapted cells were also used to prepare dead cells, and the dead cells of three species were analyzed by EMA-qPCR technique to quantify the background. The Ct values from dead cells were much higher when compared to those generated by live cells at the same cell concentration (Table 2). The ANOVA analysis demonstrated the significant difference of Ct values between live and dead cells due to the lower significance value than 0.05, despite that no difference was observed for *Starm. bacillaris* at the concentration of 10^3 cells/mL (the significance value > 0.05), most likely due to the high difference of Ct values within live and dead cells. Interference from background signal appeared only in the presence of large populations of dead cells (10^7 cells/mL), which were detected by the method as approx. 10^4 live cells/mL according to the standard curves.

3.2.2 Standard curves of RT-qPCR

Good correlations were also obtained for culturable populations between 10^2 and 10^7 cells/mL by RT-qPCR analysis (Table 1). Ct values of *S. cerevisiae*, *H. uvarum*, and *Starm. bacillaris* were also checked before and after the killing treatment (75% ethanol for 24h) and approx. 10^4 to 10^5 cells/mL background was produced from 10^6 to 10^7 dead cells/mL of each species according to the standard curve in Table 1 (data not shown).

Table 2. Ct values for a dilution series of live or dead cells by EMA-QPCR. Cell concentration is expressed as log units, and Ct values were shown as mean ± standard deviation of triplicate qPCR amplifications. The significance level for One-Way ANOVA calculation was 0.05.

Species	Cell concentration	Live	Dead	Variation between live & dead cells	Variation within live & dead cells	F	Significance
S. cerevisiae	7	17.89±0.04	28.76±0.38	117.61	0.07	1599.65	0.001
	6	22.70±0.00	29.23±0.21	42.60	0.02	1893.26	0.001
	5	25.12±0.16	31.65±0.04	44.09	0.01	3391.51	<0.001
	4	28.70±0.17	31.81±0.01	14.87	0.01	1020.81	0.001
	3	31.94±0.35	34.19±0.01	4.00	0.06	63.92	0.015
H. uvarum	7	16.25±0.10	24.39±0.09	66.34	0.01	7270.25	<0.001
	6	18.98±0.08	30.87±0.19	141.25	0.02	6472.08	<0.001
	5	22.33±0.16	31.53±0.59	84.82	0.19	457.40	0.002
	4	26.01±0.34	32.98±0.18	48.58	0.07	652.09	0.002
	3	28.85±0.21	32.95±0.82	16.77	0.36	45.98	0.021
Starm. bacillaris	7	20.83±0.09	30.63±0.16	97.42	0.02	5582.63	<0.001
	6	24.14±0.01	33.95±0.74	96.33	0.27	356.23	0.003
	5	29.15±0.25	35.21±0.46	31.08	0.14	225.18	0.004
	4	32.97±0.01	37.49±0.50	20.43	0.13	162.08	0.006
	3	35.68±0.71	38.00±0.01	5.41	1.46	3.69	0.195

3.2.3 Analysis of fermentation samples

The DNA and cDNA extracted from fermentation samples were subjected to amplification and specific yeast populations were quantified using the previously generated standard curves. More specifically, samples at day 6 and 11, in which the *H. uvarum* and *Starm. bacillaris* respectively became non-culturable, were analyzed. The chemical composition of the fermented must samples is shown in Table 3.

Table 3. Chemical composition of grape must main components at different fermentation stages. The values are means of duplicate analysis.

Day	Glucose g/L	Fructose g/L	Ethanol % vol	Glycerol g/L	Acetic acid g/L
0	116.60	117.40	0.00	0.10	0.00
6	24.44	34.22	10.00	6.94	0.12
11	0.27	1.09	13.99	8.07	0.26

The EMA-qPCR and RT-qPCR quantification results were compared to those obtained by culture-dependent techniques (Table 4). The ANOVA analysis did not

differentiate among three techniques for the total yeast and *S. cerevisiae* quantification on day 6, but some differences existed for the other samples. The post-hoc Tukey test was further performed to find out the culture-independent technique, by which the quantification result was significantly different from the result by culture-dependent techniques, as shown in Table 4. Therefore, three main conclusions could be obtained. First of all, the total yeast populations from all samples were kept at similar level (10^7 cells/mL) by culture-dependent and culture-independent techniques because of the existence of large culturable *S. cerevisiae* population. Secondly, when the culturable species were quantified on plates, the population size was similar to the one detected by EMA-qPCR, both for *S. cerevisiae* and *Starm. bacillaris*. However, quantification by RT-qPCR yielded counts that were one log unit lower for *S. cerevisiae* and even two log units lower for *Starm. bacillaris* comparing to culture-dependent methods. Then, when no colonies of non-*Saccharomyces* were recovered on plates, still populations of about 10^5 cells/mL (*H. uvarum*) or 10^6 cells/mL (*Starm. bacillaris*) were quantified by EMA-qPCR. The counts of the non-culturable non-*Saccharomyces* by RT-qPCR were similar to those obtained by EMA-qPCR.

Table 4. Yeast quantification on day 6 and 11 by culture-dependent (microbiological analysis) and culture-independent techniques (EMA-qPCR and RT-qPCR). The values of populations in the table are the average from duplicate fermentation and expressed as cfu/mL (plate counting) or cells/mL (qPCR methods), nd means not detectable. The significance level for One-Way ANOVA calculation was 0.05.

Day	Yeast	WL and LM plates	EMA-qPCR	RT-qPCR	Variation between techniques	Variation within techniques	F	Significance
6	Total yeast	5.71×10^7	2.08×10^7	1.08×10^8	0.28	0.04	6.45	0.082
	S. cerevisiae	3.03×10^7	2.79×10^7	6.03×10^6	0.30	0.03	9.20	0.053
	H. uvarum	nd	3.16×10^5 *	9.10×10^5 *	21.67	0.06	392.29	<0.001
	Starm.bacillaris	2.68×10^7	1.52×10^7	5.40×10^5 *	1.72	0.03	60.81	0.004
11	Total yeast	3.50×10^7	1.41×10^7	1.08×10^8	0.40	0.01	66.69	0.004
	S. cerevisiae	3.50×10^7	2.60×10^7	4.33×10^6 *	0.48	0.02	36.61	0.008
	H. uvarum	nd	6.50×10^5 *	4.28×10^5 *	21.84	<0.01	28285.08	<0.001
	Starm.bacillaris	nd	1.88×10^6 *	4.42×10^5 *	23.39	0.07	338.84	<0.001

** The mean difference was significant from culture-dependent technique by post-hoc Tukey test.*

3.3 DGGE electrophoretic profiles of wine samples

In order to study the effect of the EMA treatment on the detection of the three species by DGGE, eight different cell mixtures were prepared from pure yeast cultures in sterile Barbera juice (Fig. 2). The differences in population sizes affected the detection and when *S. cerevisiae* was predominant in the mixture with 10^7 cells/mL, *Starm. bacillaris* could be detected at 10^5 cells/mL but not at 10^3 cells/mL, whereas *H. uvarum* was not detected in any of those tested concentrations. When low populations of *S. cerevisiae* were present in the mixture (10^3 cells/mL), *Starm. bacillaris* at 10^5 and 10^7 cells/mL could be detected, while *H. uvarum* could only be seen at concentrations of 10^7cells/mL or when *Starm. bacillaris* was at the same concentration (10^5 cells/mL).

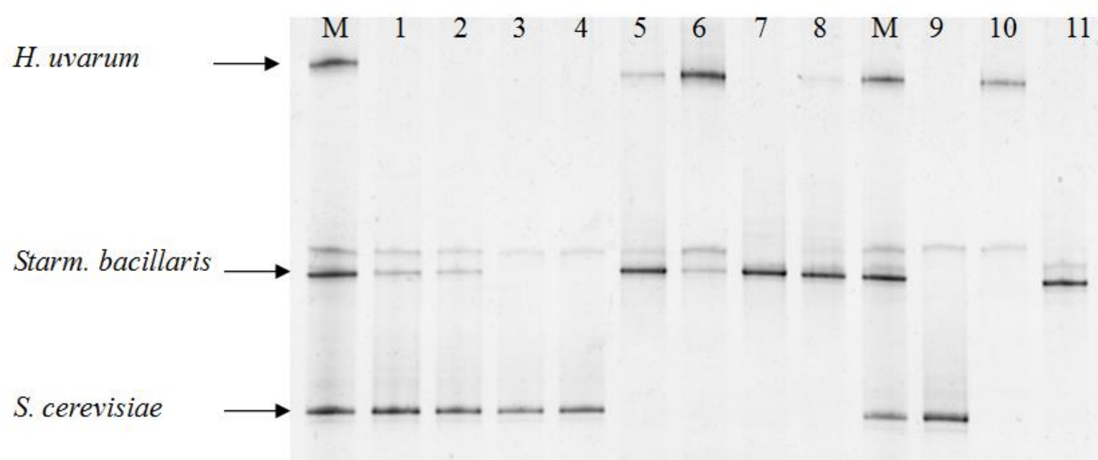

Figure 2. Detection of viable cells from eight different mixtures of *S. cerevisiae* (sacc), *H. uvarum* (huv) and *Starm. bacillaris* (star) by EMA-PCR-DGGE. M, DNA marker made with the three pure species; Lanes 1-11 represent samples of different cell and population mixtures: 1, 10^7sacc+10^3huv+10^5star; 2, 10^7sacc+10^5huv+10^5star; 3, 10^7sacc+10^3huv+10^3star; 4, 10^7sacc+10^5huv+10^3star; 5, 10^3sacc+10^7huv+10^7star; 6, 10^3sacc+10^7huv+10^5star; 7, 10^3sacc+10^5huv+10^7star; 8, 10^3sacc+10^5huv+10^5 star; 9, 10^7sacc; 10, 10^7huv; 11, 10^7star.

The fermentation samples (day 6 and 11) were also analyzed by the EMA-PCR-DGGE and RT-PCR-DGGE techniques. The results are shown in Fig. 3. With EMA-PCR-DGGE, *S. cerevisiae* was detected in both tested days (6 and 11), *Starm. bacillaris* was only found on day 6 while *H. uvarum* was not detected. Although these results were similar to plating, the differences in population sizes between all the species affected clearly the detection of the minority species (*H. uvarum* and *Starm. bacillaris*).

Analysis by RT-PCR-DGGE yielded very different results. *S. cerevisiae*, *H. uvarum* and *Starm. bacillaris* could be detected in all samples although *H. uvarum* presented very strong bands.

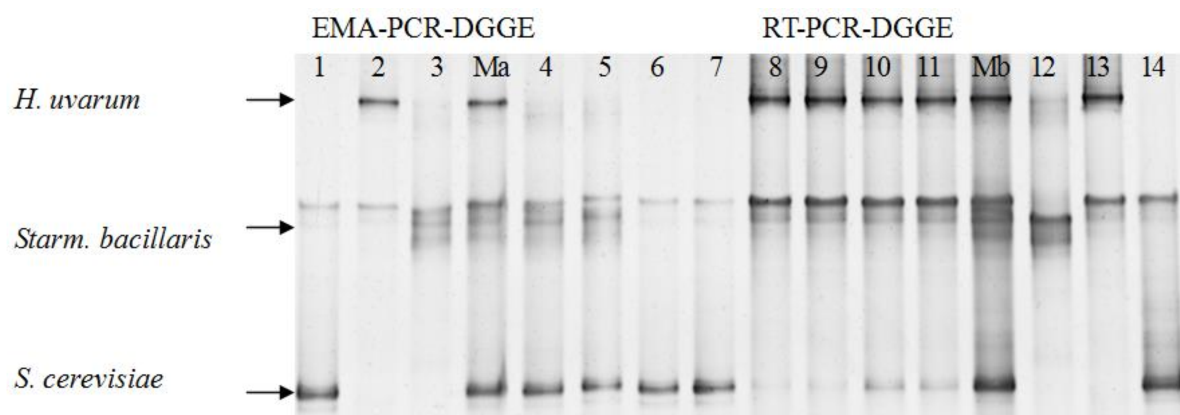

Figure 3. Detection of viable cells of samples from grape must fermentation by EMA-PCR-DGGE and RT-PCR-DGGE analysis. Ma, marker containing mixed DNA of three pure species; Mb, marker containing mixed cDNA of three pure species; Lanes 1-14 were obtained with DNA (1-7) or cDNA (8-14) templates from different samples: 1 and 14, 10^7 *S. cerevisiae*; 2 and 13, 10^7 *H. uvarum*; 3 and 12, 10^7 *Starm. bacillaris*; 4, 5, 8 and 9, samples of day 6 from duplicate fermentations; and 6, 7, 10 and 11, samples of day 11 from duplicate fermentations.

4. Discussion

The definition of live cells in wine ecology has changed with the development of detection techniques. Starting from traditional methods (i.e. use of culture media), analysis has moved to the application of culture-independent molecular techniques represented by the use of DNA (qPCR or PCR-DGGE), determination of cell membrane integrity (EMA-qPCR or PMA-qPCR) or use of RNA (RT-qPCR, RT-PCR-DGGE and FISH) (Cocolin and Mills, 2003; Hierro et al., 2006; Andorrà et al., 2008; Andorrà et al., 2010a; Andorrà et al., 2010b; Shi et al., 2012; Vendrame et al., 2014; Wang et al., 2014). The combined use of culture-dependent and culture-independent techniques was considered in this paper, and the aim was to study how the interactions between *S. cerevisiae*, *H. uvarum* and *Starm. bacillaris* during alcoholic fermentation of a natural must could be reflected at population level. A need for truly dead cells was necessary for comparison and background estimation. Heat shock (65ºC) and ethanol toxicity (75%) were tested by reactivation in both rich medium (YPD broth) and differential media (WL nutrient agar). Heat shock and ethanol toxicity yielded the same results (data not shown). Ethanol toxicity was chosen for

the similarity to the increased concentrations of ethanol during alcoholic fermentations, which could finally produce cell death.

The agreement between culture-dependent techniques and EMA-qPCR for culturable *S. cerevisiae* and *Starm. bacillaris* cells was considered as a proof that culturable cells had fully functional cell membrane and EMA-qPCR could quantify them accurately, as previously described by Andorrà et al (2010a). EMA-qPCR presented good linearity with culturable populations between 10^3 to 10^7 cells/mL, but a low background signal was produced from the dead cells at high concentrations. This background signal was also observed by Andorrà et al. (2010a) and Nkuipou-Kenfack et al. (2013), and it could not be removed by regulation of EMA treatment conditions (Nkuipou-Kenfack et al., 2013). Nevertheless, from the view of application, this background signal did not actually interfere with the quantification of live cells from wine fermentations, because the signal (10^5 to 10^6 cells/mL) was always higher than background signals (10^4 cells/mL). This point was also verified by Andorrà et al. (2010a), by addition of a constant population of dead cells to serial dilutions of viable cells obtaining a standard curve that was not influenced by the dead cells.

The analysis of rRNA integrity through the RT-qPCR analysis resulted in an underestimation of the culturable population by one or two log units. Considering the good linearity of the standard curves, this result is probably related with a decreased ribosome level inside the culturable cells facing environmental stress (ethanol production, nutrient depletion) and initiating survival strategies. Although there is no evidence in the present work, other studies based on FISH (Andorrà et al., 2011; Wang et al., 2014) also observed the variation in the fluorescence intensity of the stained *S. cerevisiae* cells during fermentation. The variation of rRNA concentration to some extent questioned the quantification accuracy of RT-qPCR, especially as reference for live cells. Furthermore, the rRNA of dead cells might be degraded at different rates depending on the lytic process. By ethanol treatment (75% ethanol for 24h), the reduction of rRNA was obvious, although some stable rRNA still existed after 48h (data not shown) and probably interfered with the quantification of live cells. Previous data from dead cells originated by heat shock (60 °C 20 min, Hierro et al., 2006) also showed the relative stability of rRNA, which takes at least 24h for significant degradation in *S. cerevisiae* cells. The death of non-*Saccharomyces* species during wine fermentation is probably dependent on a variety of factors (Wang et al., 2015) and their effect on the relative stability of rRNA in these dying

cells is still far from being understood. More information is needed to understand the process, and the use of rRNA-dependent techniques to evaluate it.

Previous studies based on culture-independent techniques have reported the existence of non-*Saccharomyces* populations during late stages of fermentation, when *Saccharomyces* dominated the process (Andorrà et al., 2010a; Wang et al., 2014). Our results were consistent with these reports because of the detection of 10^5 to 10^6 cell/mL of *H. uvarum* and *Starm. bacillaris* after no culturable cells were obtained from these species. Based on our results, we can conclude that a subpopulation of non-culturable cells had an injured membrane (therefore were not detected by EMA-qPCR) and are considered dead while a quantifiable number of non-culturable cells were still alive with functional membranes (detected by EMA-qPCR) and non-degraded RNA (detected by RT-qPCR).

DGGE was firstly used in this study with combination of EMA treatment, and the approximate detection limits for *Starm. bacillaris* and *H. uvarum* were also tested. It could be used as a basic qualitative culture-independent technique for monitoring wine fermentation. The limitation of EMA-PCR-DGGE was the uncertain detection of minor populations when some predominant populations existed at one or two log units higher concentrations. RT-PCR- DGGE in this study detected all of the three species at late stages of fermentation, especially from *H. uvarum*, which was the species undetected on plates. The high intensity of rRNA signal from *H. uvarum* could be interpreted as a high concentration of rRNA, as seen before (Andorrà et al., 2011) on a strain of *Hanseniaspora guilliermondii*. However, the application of this technique to follow live cells during wine fermentation needs the support of further data due to the unclear rRNA relative stability in dead cells which is also probably species-dependent.

In conclusion, the present work detected viable but non-culturable *H. uvarum* and *Starm. bacillaris* cells by culture-independent techniques. These cells presented functional membranes and non-degraded rRNA. Also both *S. cerevisiae* and *Starm. bacillaris* presented cell membrane integrity, relatively stable rRNA and culturability during late stages of grape must fermentation. The comparative analysis among different techniques demonstrated the potential of EMA-qPCR and EMA-PCR-DGGE for wine ecological studies. This work also indicates some underlying obstacles for the application of RT-qPCR and RT-PCR-DGGE on the estimation of viable populations of different species during alcoholic fermentation. The relative stability of

rRNA during the process of cell lysis needs to be determined with precision before being applied systematically for routine analysis of viable populations in alcoholic fermentations.

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Chapter 4

Interaction between *Hanseniaspora uvarum* and *Saccharomyces cerevisiae* during alcoholic fermentation

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Abstract

During wine fermentation, *Saccharomyces* clearly dominate over non-*Saccharomyces* wine yeasts, and several factors could be related to this dominance. However, the main factor causing the reduction of cultivable non-*Saccharomyces* populations has not yet been fully established. In the present study, various single and mixed fermentations were performed to evaluate some of the factors likely responsible for the interaction between *Saccharomyces cerevisiae* and *Hanseniaspora uvarum*. Alcoholic fermentation was performed in compartmented experimental set ups with ratios of 1:1 and 1:9 and the cultivable population of both species was followed. The cultivable *H. uvarum* population decreased sharply at late stages when *S. cerevisiae* was present in the other compartment, similarly to alcoholic fermentations in non-compartmented vessels. Thus, cell-to-cell contact did not seem to be the main cause for the lack of cultivability of *H. uvarum*. Other compounds related to fermentation performance (such as sugar and ethanol) and/or certain metabolites secreted by *S. cerevisiae* could be related to the sharp decrease in *H. uvarum* cultivability. When these factors were analyzed, it was confirmed that metabolites from *S. cerevisiae* induced lack of cultivability in *H. uvarum*, however ethanol and other possible compounds did not seem to induce this effect but played some role during the process. This study contributes to a new understanding of the lack of cultivability of *H. uvarum* populations during the late stages of wine fermentation.

Keywords: Wine, Ethanol, Nitrogen, Killer, Cell-to-cell contact.

1. Introduction

Wine fermentation is an ecologically complex process that involves diverse yeast species at different stages. These yeast species interact with each other and show a population succession, which is mostly characterized by large populations of non-*Saccharomyces* species at early stages and the dominance of *Saccharomyces* at late stages (Fleet, 2008). Research efforts to monitor this population succession have provided many hypotheses for this phenomenon. This process will be understood by testing these hypotheses and identifying the factors responsible for the dominance relations between yeast species.

Until now, investigations of yeast interactions have primarily emphasized four aspects during wine fermentations. First is the nutrient level, with carbon and nitrogen as the main limiting factors (Andorrà et al., 2012). The lack of equilibrium in natural grape musts between carbon and nitrogen is well known, and it is a limiting factor. *Saccharomyces* consumes both of them faster than non-*Saccharomyces*, causing nutrient depletion at the beginning of fermentation (Monteiro and Bisson, 1991; Albergaria et al., 2003). Furthermore, *Saccharomyces* is more efficient at producing biomass than non-*Saccharomyces* under fermentation conditions, i.e., non-*Saccharomyces* need higher nutrient concentrations to produce the same biomass (Andorrà et al., 2012). Thus, nutrient concentrations are regarded as an important factor for the dominance of *Saccharomyces*. Certain fermentation factors such as oxygen and temperature are a second factor correlated to yeast interactions related to the Crabtree effect and heat production, which is described as a fitness advantage of *Saccharomyces* in the niche construction theory (Goddard, 2008; Salvadó et al., 2011). SO_2 was also regarded as a selective fermentation factor because of different sensitivities from different yeast species (Ribéreau-Gayon et al. 2006). Thirdly, yeast metabolites such as ethanol, medium-chain fatty acids and killer toxins have been considered as well (Thomson et al., 2005; Piškur et al., 2006; Albergaria et al., 2010). Ethanol and medium-chain fatty acids are known to decrease the growth rate and even cause growth arrest of non-*Saccharomyces* yeasts due to their toxicity and the relatively inefficient regulation caused by these metabolites (Fleet, 2003; Thomson et al., 2005; Piškur et al., 2006). Killer toxins secreted by *Saccharomyces cerevisiae* are found to be death inducing factors for non-*Saccharomyces*, such as enzymes with glucanase activity (Magliani et al., 1997), proteinaceous compounds (Pérez-Nevado et al., 2006), and antimicrobial

peptides (AMPs) derived from fragments of *S. cerevisiae* glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein (Albergaria et al., 2010; Branco et al., 2014). Finally, some direct factors such as quorum sensing phenomena (Granchi et al., 1998; Smid and Lacroix, 2013) and cell-to-cell contact (Nissen et al., 2003; Arneborg et al., 2005) might also explain the interaction between *Saccharomyces* and non-*Saccharomyces* yeasts.

Although evidence as noted above is increasingly provided to elucidate the displacement of non-*Saccharomyces* by *Saccharomyces* in wine fermentations, the mechanisms have still not been completely unraveled. For example, the role of ethanol is considered to be overestimated, because the lack of cultivability of non-*Saccharomyces* was found to be related to the presence of *Saccharomyces* cells instead of ethanol (Granchi et al., 1998; Pérez-Nevado et al., 2006), and several reports have indicated that some non-*Saccharomyces* yeasts have higher tolerances to ethanol than previously thought (Pina et al., 2004; Pérez-Nevado et al., 2006). Additionally, the role of cell-to-cell contact reported by Nissen et al. (2003) seems to be contradictory with the findings from Pérez-Nevado et al. (2006), which indicated that one or more toxic compounds from *S. cerevisiae* induce the death of *Hanseniaspora* cells. A recent study of Branco et al. (2014) strongly supports the lethal role of AMPs secreted by *S. cerevisiae* against several non-*Saccharomyces* species by using mutant *S. cerevisiae* with the AMP encoding genes deleted.

In this study, we determine the interactions by performing a series of alcoholic fermentations with *S. cerevisiae* and *Hanseniaspora uvarum.* The evolution of the yeast population was followed by plating on solid media and counting under a microscope. To analyze the influence of cell-to-cell contact, cells of different species were mixed or kept separated by dialysis tubes. To analyze the effects of different metabolites, adapted cells of *H. uvarum* were cultured in different media to follow the influence of *S. cerevisiae* metabolites. The ethanol effect and nutrient factors such as sugar availability were tested by simulating the fermentation conditions.

2. Materials and methods

2.1. Yeasts strains, culture conditions and fermentation trials

The yeast strains of *S. cerevisiae* NSa and *H. uvarum* NSb were natural isolates from wines and maintained in our group collection (Wang et al. 2014). They were identified by 5.8S-ITS-RFLP analysis (Esteve-Zarzoso et al., 1999) and

sequence analysis of D1/D2 domain of 26S rDNA (Kurtzman and Robnett, 1998). Yeasts were grown overnight in YPD (1% yeast extract, 2% peptone and 2% glucose, w/v, pH 6.2) medium at 28 ºC before use. All the fermentations were done in the presence of air (with screw cap not totally tight) at 25 °C with 120 rpm of shaking speed and inoculated with $1×10^6$ cells/mL of each yeast species.

2.2. Analytical methods and population quantification methods

Concentrations of acetic acid, glycerol and total sugar in the samples were tested by Miura One Multianalyzer (TDI, Barcelona, Spain) using the enzymatic kit from Biosystems S. A. (Barcelona, Spain); and those of ethanol, fructose and glucose by enzymatic kit from Roche Diagnostics (Darmstadt, Germany). Samples were diluted and analyzed according to the manufacturer's instructions.

Culture methods and microscopy counting were used to quantify the yeast populations of all samples in this study. The samples were taken aseptically and spread onto solid YPD plates after appropriate dilution in sterile water. Solid lysine medium (Oxoid LTD., England) was used for *H. uvarum* quantification in samples from mixed cultures or mixed fermentation, because *S. cerevisiae* does not grow well on it. Sample spreading and colony counting were performed using an automated spiral spreader and a colony counter (Biomérieux, France).The cells were stained using the LIVE/DEAD®BactLight™ Bacterial Viability kit (Molecular Probes Inc., USA) and counted using a fluorescence microscope (LeicaDM 4000B) as stated in Andorrà et al., 2010a.

2.3. Analysis of interaction through fermentations with separation between species

2.3.1. Experimental set up with or without dialysis tube

Dialysis tubes (Sigma-Aldrich, MWCO 12.4 kDa, diameter 49 mm, St. Louis, United States) were soaked in sterile water for 1 h before use. The dialysis tubes were cut into suitable lengths: one end was knotted; another tube with a smaller diameter was inserted inside the dialysis tube, and this end of the dialysis tube was tightly tied with cotton thread to seal the gap between the smaller tube and the dialysis tube. The inner tube then provided a means to introduce samples into the dialysis tube and remove samples from the dialysis tube. This dialysis tube unit was then placed into a vessel to give two culture compartments: the compartment within

the dialysis tube (inner compartment) and the compartment within the vessel but outside of the dialysis tube (outer compartment). Using the dialysis tube unit, three series of fermentations were conducted. In one experiment (A) the outer vessel was a 250 ml screw cap bottle in which the volume of culture inside the dialysis tube was 20 ml and the volume of culture inside the vessel but outside the dialysis tube was 180 ml. In a second experiment (B), the outer vessel was a 50 ml conical tube (Falcon®, Corning, USA) in which the volume of culture in both of the inner and outer compartments was 10 ml. The last experimental set up (C), the vessel was a 250 ml screw cap bottle into which no dialysis tube unit was introduced. These experimental set ups are summarized in Figure 1. For experimental set ups A and B, similar levels of culture media were formed between two compartments, and thus no obvious volume changes caused by different metabolite diffusion were observed. All of the operations above were performed in sterile conditions, and each vessel prepared with medium was autoclaved before use.

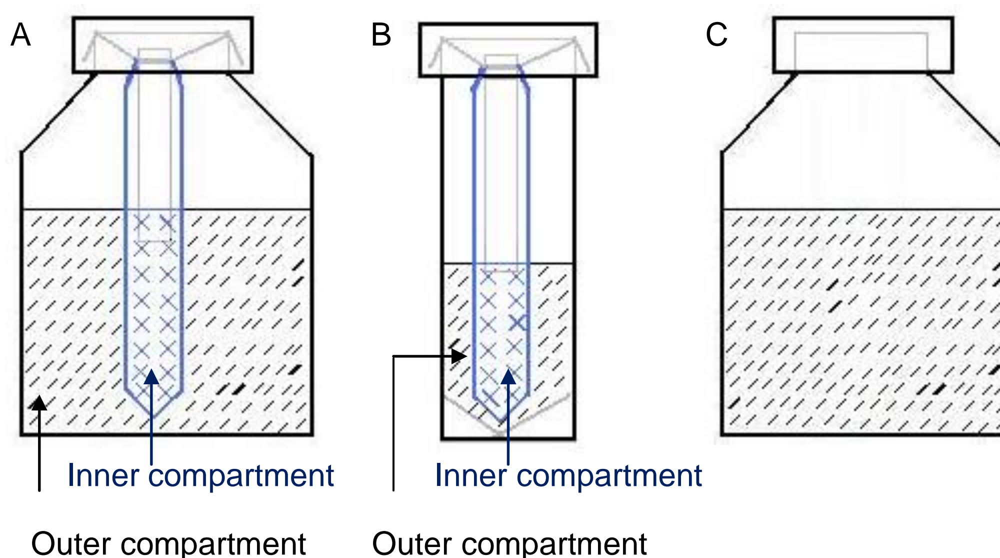

Figure 1. Diagrams of the different experimental set ups (A, B, C) with or without dialysis tube inside. The size of each part is not strictly in the real proportion.

In order to check the conditions of metabolite circulation between two separate compartments, YPD medium was introduced into both experimental set ups (A and B), and *S. cerevisiae* were inoculated into either inner or outer compartments. Contents of acetic acid, ethanol, glycerol and sugar in both compartments were measured after 3 d of growth. Cell quantification of *S. cerevisiae* by culture methods

and microscopy were done at the same time to check the possibility of cell exchange through the dialysis tube unit. Furthermore, considering the exchange speed of water might be higher than that of other molecular such as glucose, contrast trials were done to examine the possible volume changes between two compartments: natural concentrated must diluted to 20 g/L and 200 g/L sugar that were separately added into different compartments of the experimental set up B, then it was autoclaved, put onto a shaker with a constant speed of 120 rpm, and 3 d later, the volume and sugar content of both compartments were checked.

2.3.2. Fermentations

To examine the interactions between the two yeast species, fermentations with either YPD or natural musts were done in different experimental set ups (Figure 1). YPD was used as a negative control, since the sugar concentration, the ethanol produced and the availability of nutrients do not allow yeast interactions. In contrast, in natural musts the sugar and other nutrient conditions allow inhibitions of growth between different species.

- Fermentations in experimental set up A using natural must (Concentrats Pallejà Tarragona Spain, pH 3.7, without sulfite treatment, sugar 200 g/L by dilution from concentrated must) as the culture medium. Four different fermentations were conducted: (i) *S. cerevisiae* inoculated into and cultured in the outer compartment and *H. uvarum* inoculated into and cultured in the inner compartment (within the dialysis tube), abbreviation A-S(H); (ii) *S. cerevisiae* inoculated into and cultured in the inner compartment and *H. uvarum* inoculated into and cultured in the outer compartment, abbreviation A-H(S); (iii) *S. cerevisiae* inoculated into and cultured in both compartments, abbreviation A-S(S); (iv) *H. uvarum* inoculated into and cultured in both compartments, abbreviation A-H(H). In this experimental set up the volume ratio between inner and outer compartments was 1:9.

- Fermentations in experimental set up B used the same natural must as in experimental set up A, but a different volume ratio between compartments (1:1). Two kinds of fermentations were performed: (i) *S. cerevisiae* inoculated into and cultured in the outer compartment and *H. uvarum* inoculated into and cultured in the inner compartment, abbreviation B-S(H); (ii) *S. cerevisiae* inoculated into and cultured in the inner compartment and *H. uvarum* inoculated into and cultured in the outer compartment, abbreviation B-H(S).

- In fermentation C, either natural must (as used in fermentations A and B) or YPD was used as culture media. Both of *S. cerevisiae* and *H. uvarum* were inoculated together, abbreviation C-S+H.

Fermentations were done in duplicate, and thus 16 fermentations in total were performed. All of the fermentations were monitored by weight every 24 h until no weight loss, and samples were daily taken to quantify the yeast cells. For further validating the metabolite circulation between two compartments, the concentrations of acetic acid, glycerol and sugar from both compartments of experimental set up A were measured at middle and end stages. As a control, the same indexes were also checked in experimental set up C.

2.4. Production of supernatants and yeast cells to test yeast interactions

To test the possible effect of culture metabolites from *S. cerevisiae* upon *H. uvarum* culturability, cell-free supernatants from *S. cerevisiae* fermentation (ScSN) were used. First, a mixed fermentation was performed in synthetic must with 200 g/L of sugar (pH 3.3, as in Andorrà et al. 2012) to set up the fermentation stages when the interaction was analyzed. Three stages were set: 1. When the cultured population of *H. uvarum* was the highest; 2. When the plate recovery of *H. uvarum* was below the initial population and thus, a decline phase was evident; and 3. When no *H. uvarum* colonies could be seen on plates. Once these fermentation stages were set, single species fermentations were performed to provide the ScSN (from the same fermentation stages set up previously) and *H. uvarum* cells. The need of having *H. uvarum* cells originated in fermentation is due to the changing conditions during alcoholic fermentation and the adaptation of the cells to these changes. In the wine industry it is well known that to restart a stuck fermentation is easier from another ongoing fermentation than with fresh inoculum. Finally, adapted *H. uvarum* cells were inoculated in ScSN to observe the changes in cultivability during 48 h.

As controls, adapted *H. uvarum* cells were incubated not only in YPD but also in synthetic media mimicking the different fermentation stages. The use of YPD was aimed at checking that the *H. uvarum* cells were fully viable and cultivable. The synthetic musts mimicking stages 1, 2 and 3 were used to analyze the effect of the changes on the main substrates (alcohol and sugars) on the viability and cultivability of the *H. uvarum* cells, but not the presence of putative *S. cerevisiae*-produced compounds. Thus, synthetic media 1, 2 and 3 had the same ethanol and sugar

concentrations as the fermentation media (see Table 1 “Mixed fermentation”), and 30 mg N/L of nitrogen as most of the nitrogen is taken up during the first 24 h (Beltran et al. 2005). All the experiments were performed in triplicate; 30 experiments were included in this analysis.

Table 1. Concentrations of ethanol, fructose and glucose at each fermentation stage from three fermentations: mixed fermentation (providing sampling references as indicated in Figure 5), *S. cerevisiae* fermentation (providing supernatants) and *H. uvarum* fermentation (providing adapted cells).

Fermentation	Fermentation stages	Ethanol (%)	Fructose (g/L)	Glucose (g/L)
Mixed fermentation	1	3.4	84.5	62.2
	2	10.3	28.5	5.5
	3	11.5	8.6	0.4
S. cerevisiae fermentation	1	4.0	90.8	66.8
	2	10.8	13.6	0.8
	3	12.4	0.5	0.0
H. uvarum fermentation	1	5.1	59.5	60.6
	2	6.0	48.5	49.7
	3	6.8	48.2	48.7

2.5. Analysis of ethanol toxicity

Experiments were also conducted to analyze the possible effect of ethanol on *H. uvarum*’s cultivability. For these experiments “adapted” *H. uvarum* cells from fermentation stage 2 were inoculated into and cultured in synthetic media with different concentrations of ethanol. Fermentations were started in 30 mL of synthetic media with 12.5 g/L of glucose, 12.5 g/L of fructose and 30 mg N/L of nitrogen (pH 3.3), while ethanol concentrations were set form 6 to 12% (v/v). All fermentations were done in duplicates. After the inoculation, *H. uvarum* cells were sampled, counted and plated to monitor their culturable populations at 24h and 48h, and the contents of sugar and ethanol of each medium were examined after 72 h.

3. Results

3.1. Population changes during alcoholic fermentation in separated compartments

3.1.1. Experimental set up characterization

A first experiment was set to analyze the barrier of the dialysis tube to the free diffusion of cells and metabolites. The use of either natural must with different sugar concentrations or YPD did not result in any changes in the final composition of the media in the inner or outer compartment. Similarly, *S. cerevisiae* added in one compartment was not recovered in the other compartment, although acetic acid, glycerol, ethanol and sugar were the same in both compartments. Thus, the experimental set up allowed the diffusion of these molecules but prevented cell transfer.

A small volume change was observed between the two compartments of experimental set up B, when a 10-fold difference of initial sugar concentration was set for both compartments. This volume change indicated a possibility of different circulation speeds of different molecules when there were different concentrations. Due to the different fermentation rates between *S. cerevisiae* and *H. uvarum*, different sugar concentrations appeared in the experimental set ups with separated cells (S(H) and H(S)). The lower sugar concentrations in the *S. cerevisiae* compartment resulted in less volume, whereas *H. uvarum* compartments presented more volume. To test the possible effect of the volume differences, experimental set ups with different volume ratios (A, 1:9; B, 1:1) were used.

3.1.2. Yeast population changes

Mixed alcoholic fermentations performed in YPD versus natural must showed very different results (Figure 2): In YPD no relevant interaction between the two species was observed, the sugar consumption was very fast, and a high population size was attained. The same population level was observed for both species. By contrast, in natural must, a strong interaction in cultivability was observed: the cells of *H. uvarum* were visible under the microscope and maintained the same population size during the fermentation; however, the cultivable population of *H. uvarum* cells decreased to less than 10^6 cfu/mL (the inoculation level) after 3 days of fermentation, and this trend continued until no *H. uvarum* cells were recovered (<200 cfu/mL, the detection limit) on plates after 5 days of fermentation. The use of the LIVE/DEAD viability kit confirmed the counting under the microscope without staining: yeast

populations remained stable and *H. uvarum* remained viable at populations of 10^6 cells/mL during all the fermentation.

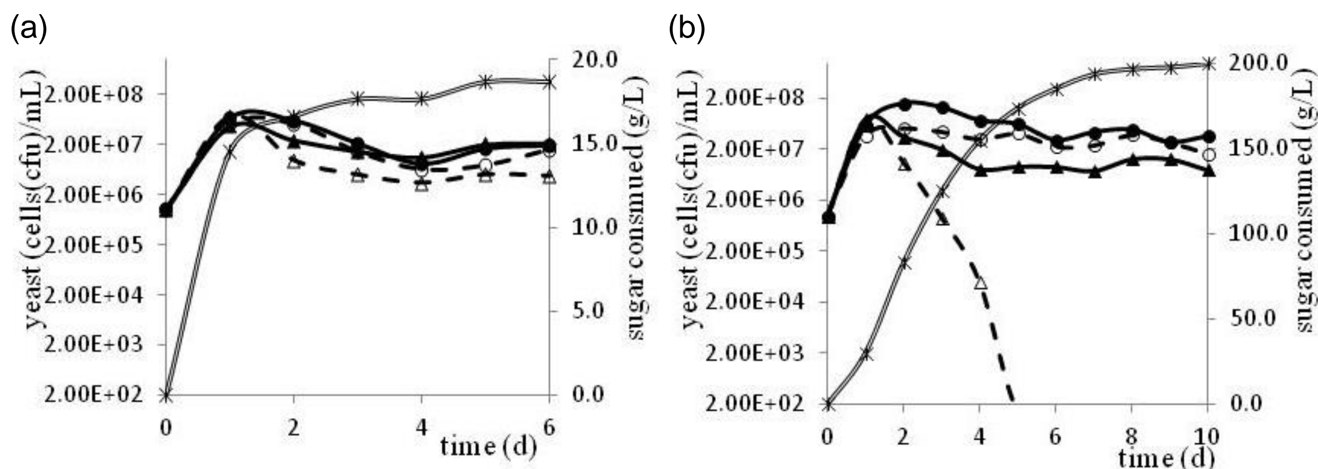

Figure 2. Changes of yeast population in mixed fermentations performed in experimental set up C (C-S+H) with YPD (a) and natural must (b). Solid lines show population assessed by microscopy and dotted lines show population assessed by culturing (values are the mean of duplicates). Symbols: (●,⊖) *S. cerevisiae*; (▲, △) *H. uvarum*; (✱) sugar consumed.

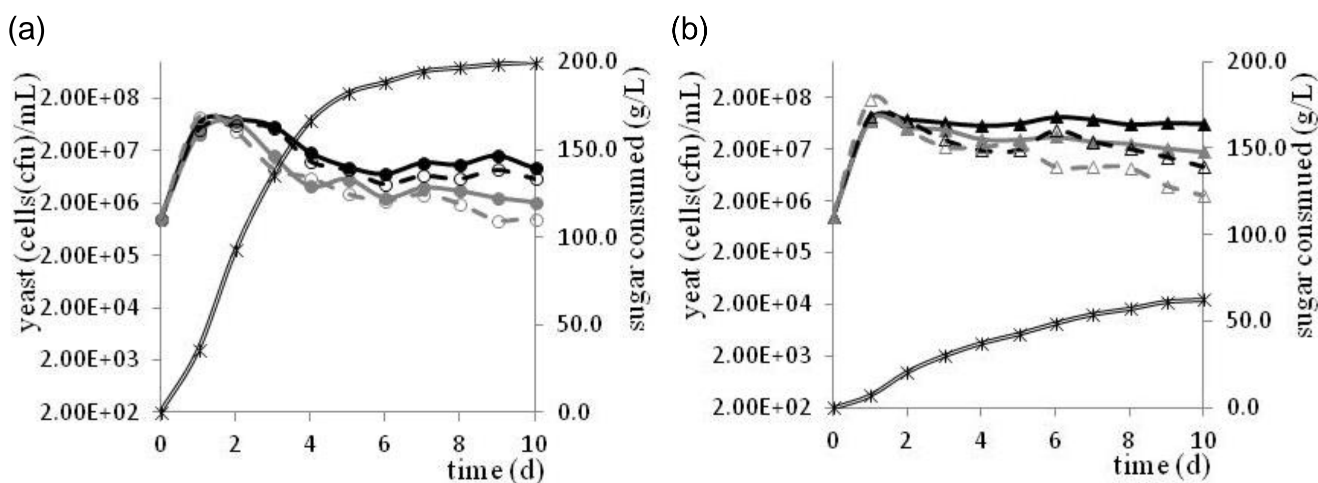

Figure 3. Development of the fermentation and yeast population in experimental set up A with the same species inoculated in both compartments of the fermenter: A-S(S) (a) and A-H(H) (b). Solid lines show population assessed by microscopy, dotted lines show population assessed by culturing, black lines show yeast in outer compartment and gray lines show yeast in inner compartment (values are the mean of duplicates). Symbols: (●,⊖) *S. cerevisiae*; (▲, △) *H. uvarum*; (✱) sugar consumed.

Alcoholic fermentations in separated compartments showed different fermentation kinetics and *H. uvarum*'s cultivability compared with the mixed

fermentation using natural must. To test if the presence of the cells in both compartments affected the cultivability and fermentation process, the same species were tested in both compartments in experimental set up A (Figure 3). It can be seen that the recoveries were similar in both compartments, no matter if it was assessed by microscopy or by plating. The only observable difference was the different sugar consumption between *S. cerevisiae* (Figure 3a) and *H. uvarum* (Figure 3 b).

Once the effect of the experimental set up was discarded, we performed the fermentations with different species inoculated in the two separate compartments, and in the two different experimental set ups with the same or different volume ratios (Figure 4). The fermentation performed in experimental set up B yielded the same results: the fermentation proceeded in 10 days with a sharp decrease on the cultivability of *H. uvarum* after six days of fermentation and no recovery on day 8. Only a small difference was observed by microscopy when *H. uvarum* was in the outer compartment (Figure 4b), because the counts were decreasing with time, while when it was in the inner compartment the counts were similar to *S. cerevisiae* population along the fermentation (figure 4a). However, when the volume differential were greater the effects could not be easily determined (in experimental set up A): When *S. cerevisiae* was in the outer compartment (Figure 4c), the fermentation proceeded similarly, yet the effect upon the cultivability of *H. uvarum* appeared earlier (decrease on day 3 and without recovery on day 5), probably due to the changes induced by a larger population of *S. cerevisiae*. The opposite experimental set up did not proceed properly because the inner compartment dried out due to faster fermentation and the flow of water to the outer compartment (Figure 4d).

Thus, three primary conclusions could be drawn. First, the volume ratio between the *S. cerevisiae* and *H. uvarum* compartments affected the sharp decrease of the cultivable *H. uvarum* population. When the volume of *S. cerevisiae* compartment was equal to or larger than *H. uvarum* compartment, the cultivability of *H. uvarum* was easily affected by *S. cerevisiae* at late stages of the fermentation. Second, the sharp decrease of *H. uvarum*'s cultivability always started at late stages of fermentation. Third, no obvious effects of cell-to-cell contact were observed on the interaction between *H. uvarum* and *S. cerevisiae* if we compare the results of mixed fermentation and compartmented fermentation with *S. cerevisiae* in the large compartment. The sharp decrease happened regardless of separation between the populations, indicating that certain compounds in the medium caused this decrease.

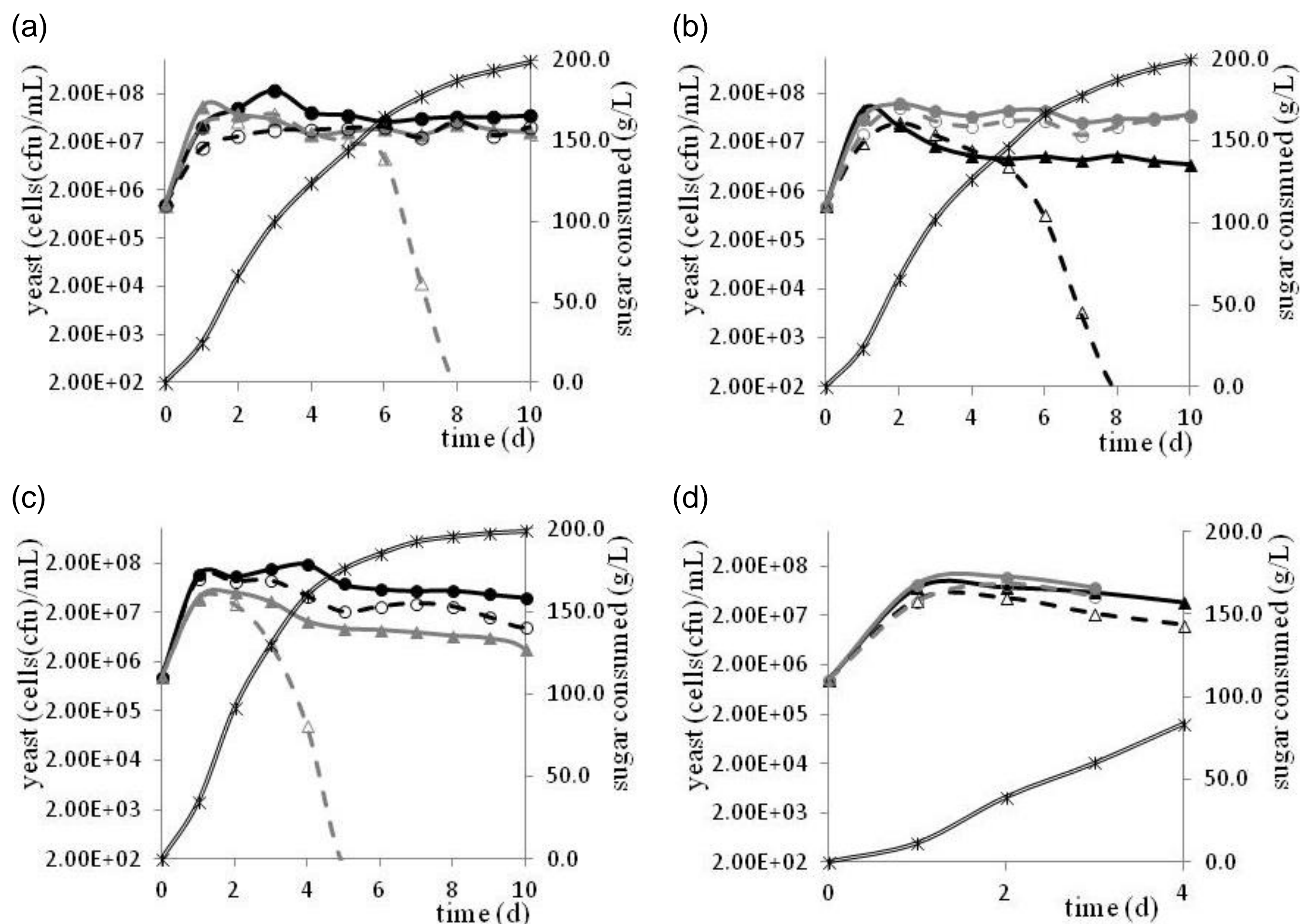

Figure 4. Development of the fermentation and yeast population in experimental set ups A and B with different species inoculated in inner and outer compartments: B-S(H) (a), B-H(S) (b), A-S(H) (c), and A-H(S) (d). Solid lines show population assessed by microscopy, dotted lines show population assessed by culturing, black lines show yeast in outer compartment and gray lines show yeast in inner compartment (values are the mean of duplicates). Symbols: (●,○) *S. cerevisiae*; (▲, △) *H. uvarum*; (✳) sugar consumed.

3.2 The influence of fermentation-produced compounds on the cultivable population of *H. uvarum*.

To further elucidate the probable factors influencing the interaction between *S. cerevisiae* and *H. uvarum*, we performed a new set of experiments. We wanted to differentiate the effect of the changes in the media composition due to the alcoholic fermentation and the effect of compounds produced by *S. cerevisiae* that could induce the observed effects. With this objective, a precisely defined medium (synthetic must) was used to perform an initial mixed fermentation (figure 5). From this fermentation, we could define three different stages regarding the cultivability of *H. uvarum*: stage 1, when the cultivable *H. uvarum* population reached the

maximum; stage 2 when that population declined below the initial population; and stage 3 when no *H. uvarum* colonies could be seen on plates. These points were analyzed to set the changes in the medium composition (table 1) and to provide the values of ethanol and sugar that will be used to define the synthetic media mimicking these stages. Furthermore, single species fermentations were performed to provide cell-free supernatants from *S. cerevisiae* fermentations (ScSN) and adapted *H. uvarum* cells at the same stages of fermentation. The fermentation performed with single culture of *S. cerevisiae* to provide ScSN yielded similar results as the mixed fermentation, while in the fermentation to obtain *H. uvarum* cells 6.8% ethanol was reached (Table 1).

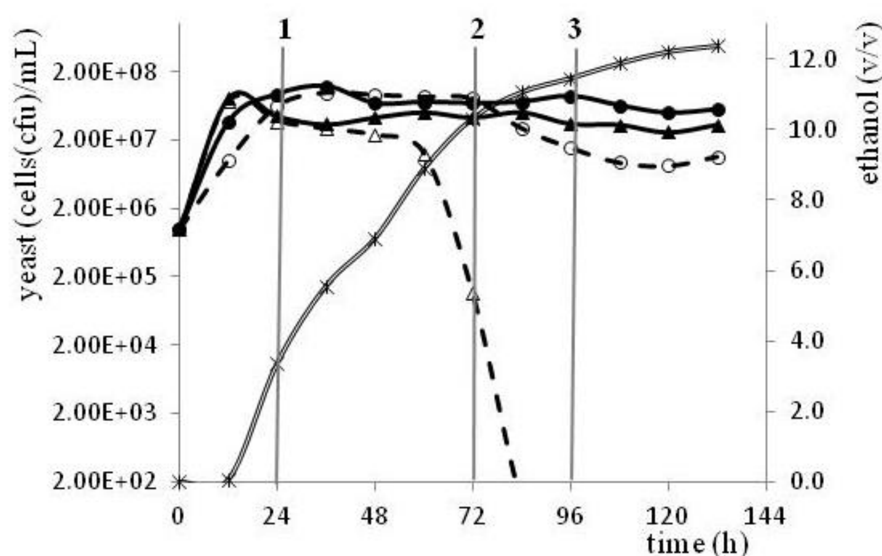

Figure 5. Mixed fermentation of *S. cerevisiae* and *H. uvarum* in synthetic must to determine the three fermentation stages (1, 2, 3) according the cultivable *H. uvarum* population. The populations were analyzed by microscopy (solid lines) and plate culture (dotted lines). Vertical lines indicate the three fermentation stages. Symbols: (●,○) *S. cerevisiae*; (▲, △) *H. uvarum*; (✱) ethanol.

The *H. uvarum* population quantified by microscopy maintained always a similar level, however those cells were not always cultivable (Figure 6). When *H. uvarum* was cultured in either ScSN of stage 1 or YPD, practically no effects upon cultivability were seen (Figure 6a1). Only a small decrease in cultivability was seen at 24 h in the synthetic medium mimicking fermentation stage 1 (Figure 6b1). Incubation of *H. uvarum* cells with ScSN from fermentation stages 2 and 3 produced a sharp decrease in *H. uvarum* cultivability to negligible amounts (Figures 6a2 and 6a3). The synthetic medium mimicking fermentation stages 2 and 3 produced similar

effects, although the cells exposed to synthetic medium of stage 2 were less affected and the decrease in cultivability proceeded slowly (Figure 6b2), whereas after 48 h no cells were recovered in plates (Figure 6b3).

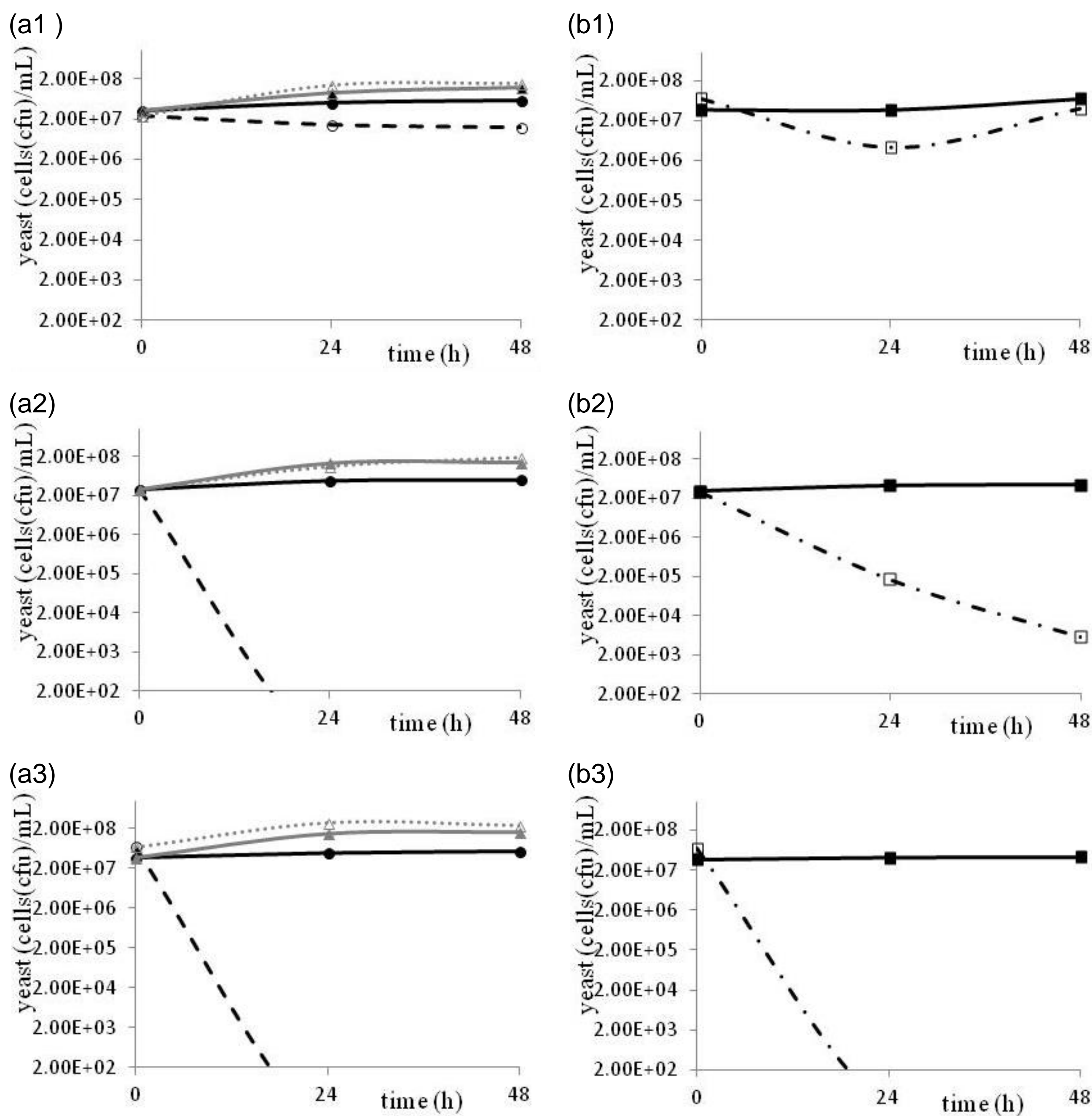

Figure 6. Quantification of adapted cells of *H. uvarum* after being inoculated in YPD, *S. cerevisiae* supernatant (ScSN) and synthetic media mimicking fermentation stages 1, 2 and 3. Cells were quantified by microscopy (solid lines) and plate culture (dotted lines). Adapted *H. uvarum* cells were inoculated into YPD (—▲—, ···△···) and ScSN at fermentation stages 1 (a1), 2 (a2) and 3 (a3) (—●—, – ⊖ –). Adapted *H. uvarum* cells were also inoculated in synthetic juice mimicking the three fermentation stages (b1, b2 and b3, —■—, – □–). The population values are the mean of triplicates.

By comparing the population of cultivable cells in ScSN and synthetic media from the same fermentation stages, metabolites secreted by *S. cerevisiae* seemed to play a main role in the cultivability of *H. uvarum*, while changes of other compounds related to the fermentation process (ethanol and sugar) also seemed to play some role. Furthermore, *H. uvarum* cells in YPD medium had their typical apiculate morphology and size. However, a portion of the cells in synthetic media of stage 1 changed their morphology to an elongated type as observed by microscopy, and in the other media some or all of the cells were reduced in size (data not shown).

3.3. The influence of ethanol and sugar on the cultivability of *H. uvarum*

Experiments with different ethanol concentrations were performed to determine the influence of ethanol on the cultivability of *H. uvarum*. Adapted *H. uvarum* cells from fermentation stage 2 were inoculated in synthetic media with concentrations of ethanol from 6% to 12% and 25 g/L of sugar. In these conditions ethanol affected cultivability to some extent (Table 2). The cultivability decreased and correlated well with the increase of ethanol concentration, although this decrease was only of one log unit during the first 48 h and, thus, not comparable to the decrease in the previous sections or in the mixed fermentations.

Table 2. Cultivability and fermentation ability of *H. uvarum* cells in synthetic media with different ethanol concentrations. The initial cell concentration of cultivable *H. uvarum* in all media was 3.80×10^{7} cfu/ml, the cultivable population size at 24h and 48h is the mean of duplicate cultures ± SD (standard deviation). The initial sugar concentration of all media was 25.0 g/L with a 1:1 ratio of glucose and fructose.

Initial ethanol concentration (%)	**Cultivable population size (cfu/mL)**		**Concentration of sugar (g/L) or ethanol (%) after 72h**			
	24h	**48h**	**Ethanol**	**Fructose**	**Glucose**	**Sugar**
6.0	$2.74\pm1.39\times10^{7}$	$1.67\pm0.28\times10^{7}$	7.0	7.4	5.8	13.2
7.0	$1.85\pm0.04\times10^{7}$	$8.07\pm0.85\times10^{6}$	7.9	8.1	6.5	14.5
8.0	$1.21\pm0.33\times10^{7}$	$7.88\pm1.47\times10^{6}$	9.1	8.9	7.4	16.4
9.0	$9.62\pm0.96\times10^{6}$	$5.20\pm1.26\times10^{6}$	10.0	8.8	8.4	17.2
10.0	$8.80\pm0.31\times10^{6}$	$3.84\pm0.03\times10^{6}$	10.1	9.6	9.2	18.8
11.0	$5.78\pm0.32\times10^{6}$	$1.13\pm0.07\times10^{6}$	10.6	9.7	10.2	19.9
12.0	$1.27\pm0.12\times10^{6}$	$8.27\pm1.80\times10^{4}$	11.9	10.2	11.0	21.2

The concentrations of sugar and ethanol were examined at 72 h (Table 2). In all the cases, *H. uvarum* consumed sugar from the medium. The cells were able to further ferment 35-50% of the sugar when the initial ethanol concentration was between 6% and 9%, whereas at higher ethanol concentrations some sugar was consumed but no further production of ethanol was observed. Low sugar concentrations maintained *H. uvarum* cultivability above 10^5 cfu/mL after 48 h. Thus, the concentration of sugar and ethanol at late stages of fermentation could not be the main reason for the sharp decrease of *H. uvarum*'s cultivability observed in the mixed fermentations or the reconstitution experiments with cells and ScSN.

4. Discussion

The interaction between *Saccharomyces* and non-*Saccharomyces* wine yeasts during wine making is well established. In this study, we analyzed this interaction further in experimental set ups that separate the populations of the different species and prevent cell-to-cell contact, and we also tried to evaluate the roles of primary compounds in the must such as sugar, ethanol and the metabolites secreted by *S. cerevisiae* by reconstitution fermentations.

Although cell-to-cell contact was first proposed by Nissen et al. (2003) and confirmed by Renault et al. (2013), this process was challenged by Pérez-Nevado et al. (2006) who found a lack of cultivability of *H. guilliermondii* cells after exposing them to cell-free supernatants from mixed cultures of *H. guilliermondii* and *S. cerevisiae*. In our study, compartmented set ups with the same or different volumes in the two compartments were used to keep the cells of the different species separated. Furthermore, cell-free supernatants from different fermentation stages of the *S. cerevisiae* fermentation were used to evaluate the cultivability of *H. uvarum* cells. Our results in both aspects (the compartmented fermentation and culture trials in cell-free supernatants) demonstrated that without cell-to-cell contact, the interaction between *H. uvarum* and *S. cerevisiae* still exists, and even in a similar fashion as in mixed fermentation when the same fermentation conditions and a given volume proportion were present. Thus, we believe that certain metabolites have a role in the lack of cultivability of *H. uvarum* rather than cell-to-cell contact. However, the different results between the observations of Nissen et al (2003) and Renault et al (2013) and those from Pérez-Nevado et al (2006) could be due to the differences of strains used in the different experiments.

When seeking for the probable explanation for the interaction between *H. uvarum* and *S. cerevisiae*, we found some possible hints correlated with the fermentation process: firstly, strong interaction was observed in mixed fermentation using natural must with 200g/L sugar while no obvious interaction appeared in mixed culture using YPD medium with 20g/L sugar. Secondly, late stages of fermentation seemed to be a key stage for *H. uvarum* cells, because sharp decrease of the cultivability always begun at late stages in this study, and a similar phenomenon has been observed in other reports (Granchi et al. 1998; Pérez-Nevado et al. 2006). Thus, factors correlated with the fermentation process are probable promoting this lack of cultivability such as nutrition depletion, ethanol and other metabolites.

Our results indicated that the low sugar concentration affected *H. uvarum*'s cultivability to some extent. Similarly, Albergaria et al. (2003) and Andorrà et al. (2012) noted that sugar and nitrogen depletion, which are caused by fast and efficient consumption by *S. cerevisiae*, most likely limit the growth rate of *H. uvarum* and eventually induce the population decline. However, nutrient depletion does not seem to be the only possible answer according to other recent studies: Pérez-Nevado et al. (2006) reported that the loss of cultivability of *H. guilliermondii* was slower in fermentations with 100 g/L initial sugar compared to those with 200 g/L initial sugar, indicating that the inducing agent could be more related to the total amount of sugar metabolized than the residual sugar level. This fact could be related to the influence that small variations in the concentrations of nitrogen and carbon sources have on the amount, nature and diversity of secreted proteins (Buerth et al., 2011, Mattanovich et al., 2009). Therefore, the role of nutrient levels in the yeast interaction might be more complicated than previously thought, and it is likely correlated with the metabolic pathways of sugar and nitrogen during alcoholic fermentation (Salvadó et al., 2011; Branco et al., 2014). *H. uvarum* showed preferences for different sugars at different stages of fermentation in this study, and different preferences for nitrogen sources have previously been reported for *S. cerevisiae*, *H. uvarum* and *Candida zemplinina* (Andorrà et al., 2012), which could be helpful in further research on the correlation of sugar and nitrogen metabolic pathways with yeast interactions.

As the main wine fermentation metabolite, ethanol was confirmed in this study not to be the critical factor behind the sharp decrease of cultivable *H. uvarum*, although high concentrations of ethanol (6% to 12%) reduced the growth rate of *H.*

uvarum to some extent. Our results also demonstrated the tolerance of *H. uvarum* cells to high ethanol concentrations as indicated by Pina et al. (2004).

We tested the possibility of a killer effect of *S. cerevisiae* on the *H. uvarum* strains used in this study by MB plate analysis (Pérez-Nevado et al. 2006) without any success (data not shown). However, our results indicated that metabolites secreted by *S. cerevisiae* are likely one of the main causes of the lack of cultivability of *H. uvarum*. This can be deduced from the obvious effect that supernatants from *S. cerevisiae* fermentations at early and late stages had on the growth of *H. uvarum* cells together with the limited effect when cultured in synthetic media with similar sugar and ethanol concentration. Albergaria et al. (2010) and Branco et al. (2014) proposed that antimicrobial peptides (AMPs) derived from fragments of *S. cerevisiae* glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein show fungistatic and/or fungicidal effects on a wide variety of wine related microorganisms. These AMPs with molecular weight of 4, 4.5 and 6 kDa probably crossed the dialysis tubes with MWCO of 12.4 kDa used in the compartmented experimental set ups of this study. Moreover, Mostert and Divol (2014) reported that some proteins observed in pure cultures are absent in mixed fermentations, indicating that the presence of two or more different yeast species may modify the exoproteome. Nevertheless, further analysis on these metabolites is still needed, and the mechanisms underlying the production and accumulation of metabolites during wine fermentations also need further elucidation.

Two more aspects that should be tested in the future are the interactions with other non-*Saccharomyces* species and also the interaction between *Saccharomyces* strains. Regarding the first aspect, Mostert and Divol (2014) showed that a precursor of a killer toxin can be detected when *Saccharomyces*-led fermentations are performed in the presence of *Metschnikowia pulcherrima*, but when the fermentation is together with *Lachancea thermotolerans*, this precursor is not found. Interactions between populations can also be seen among strains of the same species (*S. cerevisiae*), and such phenomena might be even more complex. The interaction between two types of *S. cerevisiae* strains previously characterized as dominant and non-dominant in alcoholic fermentations was found to be different when they were placed in a set up with separate compartments but sharing the same must. In this case the dominance was not present, revealing that this phenomenon is related to competition for the same space (Perrone et al., 2013).

Finally, an underlying factor relevant to these experiments is the relation between lack of cultivability and cell death. In fact, when we compared the total yeast cells as quantified by microscopy with cultivable yeast by plating, we were considering this fact together with the fact that a viable but not cultivable (VBNC) status has been reported in wine microorganisms during alcoholic fermentation (Millet and Lonvaud-Funel, 2000). We observed population changes similar to those of previous reports that used both microscopy and molecular techniques involving DNA or RNA detection (Andorrà et al., 2010b, 2011) that demonstrate the probable existence of viable but non-culturable cells. Although VBNC status could be understood as a first step leading to cell death, it is evident that our study was not aimed at elucidating this aspect. Although we observed changes in size and morphology in the *H. uvarum* cells, these cannot be directly related to cell death. Finally, it is relevant to emphasize that cell morphology cannot be used as a criterion to follow and identify different species during alcoholic fermentations.

In summary, we analyzed the interaction between *S. cerevisiae* and *H. uvarum* by integrating the effects of ethanol and sugar concentrations during the alcoholic fermentation and the metabolites excreted by *S. cerevisiae* that decrease the cultivability of *H. uvarum*. In our hands, metabolites rather than cell-to-cell contact were most likely the main factor causing this limitation. Further research on these metabolites and their correlation with nutrient metabolic pathways is needed to fully understand this yeast interaction.

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Supplementary data

Table 1 Concentrations of acetic acid, glycerol and sugar in different stages of fermentations using natural must in fermenter A and C (values are the mean of duplicates).

Fermentation	Compartment	Inoculated species	Fermentation stages	Acetic acid (g/L)	Glycerol (g/L)	Sugar (g/L)
A-S(H)	outer	*S. cerevisiae*	middle	0.73	4.39	95.91
	inner	*H. uvarum*	middle	0.70	4.48	101.91
	outer	*S. cerevisiae*	end	0.63	6.56	0.19
	inner	*H. uvarum*	end	0.60	6.56	0.20
A-H(S)	outer	*H. uvarum*	middle	1.00	4.24	100.25
	inner	*S. cerevisiae*	middle	0.97	4.05	87.55
A-S(S)	outer	*S. cerevisiae*	middle	0.75	4.64	95.58
	inner	*S. cerevisiae*	middle	0.73	5.05	98.62
	outer	*S. cerevisiae*	end	0.86	6.93	0.06
	inner	*S. cerevisiae*	end	0.87	6.72	0.05
A-H(H)	outer	*H. uvarum*	end	1.11	1.46	126.43
	inner	*H. uvarum*	end	1.14	1.66	122.33
C-S+H	—	*S. cerevisiae* + *H. uvarum*	middle	0.80	3.90	101.23
	—	*S. cerevisiae* + *H. uvarum*	end	0.66	5.30	0.06

Chapter 5

The Interaction between *Saccharomyces cerevisiae* and non-*Saccharomyces* yeast during alcoholic fermentation is species and strain specific

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Abstract

The present study analyzes the lack of culturability of different non-*Saccharomyces* strains due to interaction with *Saccharomyces cerevisiae* during alcoholic fermentation. Interaction was followed in mixed fermentations with 1:1 inoculation of *S. cerevisiae* and ten non-*Saccharomyces* strains. *Starmerella bacillaris* and *Torulaspora delbrueckii* indicated longer coexistence in mixed fermentations compared with *Hanseniaspora uvarum* and *Metschnikowia pulcherrima*. Strain difference in culturability and nutrient consumption (glucose, alanine, ammonium, arginine or glutamine) were found within *each species in mixed fermentation with S. cerevisiae*. The interaction was further analyzed using cell-free supernatant from *S. cerevisiae* and synthetic media mimicking both single fermentations with *S. cerevisiae* and using mixed fermentations with the corresponding non-*Saccharomyces* species. Cell-free *S. cerevisiae* supernatants induced faster culturability loss than synthetic media corresponding to the same fermentation stage. This demonstrated that some metabolites produced by *S. cerevisiae* played the main role in the decreased culturability of the other non-*Saccharomyces* yeasts. However, changes in the concentrations of main metabolites had also an effect. Culturability differences were observed among species and strains in culture assays and thus showed distinct tolerance to *S. cerevisiae* metabolites and fermentation environment. Viability kit and recovery analyses on non-culturable cells verified the existence of viable but not-culturable status. These findings are discussed in the context of interaction between non-*Saccharomyces* and *S. cerevisiae*.

Keywords: Contact-dependent interaction, Culturability loss, Excreted compounds, Viable but not-culturable (VBNC), Wine.

1. Introduction

Spontaneous wine fermentation is driven by a succession of different yeast species. A great variety of non-*Saccharomyces* yeast species originate from grape berries and survive during the early stages of fermentation, such as species from the genera *Candida, Hanseniaspora, Lachancea*, *Metschnikowia*, *Pichia* and *Torulaspora* (Fleet, 2003). Some species such as *Starmerella bacillaris* and *Hanseniaspora uvarum* grow to a high density (10^5-10^7 cells/mL) and dominate other non-*Saccharomyces* species (Wang *et al.*, 2015b). There is a growing interest regarding the impact of non-*Saccharomyces* yeasts on the final wines, and some species are now used as fermentation starters (Jolly *et al.*, 2014). However, fermentative *Saccharomyces cerevisiae* soon replaces non-*Saccharomyces* species to become the main or the only species present in the late stages of fermentation. Non-*Saccharomyces* strains are assumed to "die off" because these cells gradually lose their ability to form colonies on growth media, i.e. they lose the capacity to grow. The culturability loss of non-*Saccharomyces* strains has drawn widespread attention in recent years due to new findings that mention the role of excreted compounds in the interaction between *Saccharomyces* and non-*Saccharomyces* yeasts (Ciani and Comitini, 2015; Liu *et al.*, 2015; Albergaria and Arneborg, 2016). Moreover, in a work by Branco *et al.* (2015), it was shown that viable but not-culturable (VBNC) status was related to interaction through excreted compounds. Therefore, as more non-conventional wine yeasts have been explored as wine starters in mixed fermentation with *S. cerevisiae* (Masneuf-Pomarede *et al.*, 2016), studies on culturability loss of different non-*Saccharomyces* strains will help in understanding their final impact on wine quality.

The culturability loss of non-*Saccharomyces* strains at the late stages of alcoholic fermentation is a complicated phenomenon due to the multitude of factors involved. It is conventionally regarded to be related to their insufficient adaptability to environmental changes in fermentations, such as nitrogen limitation (Monteiro and Bisson, 1991), low oxygen availability (Holm Hansen *et al.*, 2001) and inhibition of increased ethanol (Fleet, 2003), as well as extrinsic factors such as SO_2 (Ribéreau-Gayon *et al.*, 2006). However, Nissen *et al.* (2003) proposed that *S. cerevisiae* S101 adopted a contact-dependent mechanism to induce the culturability loss of some non-*Saccharomyces* strains (*Lachancea thermotolerans* and *Torulaspora delbrueckii*). Subsequently, the contact-dependent mechanism was confirmed by

studies using the same *S. cerevisiae* strain (Nissen *et al.*, 2004; Renault *et al.*, 2013; Kemsawasd *et al.*, 2015a). However, it was found that *S. cerevisiae* CCMI 885 excreted toxic compounds, which inhibited the growth of *Hanseniaspora guilliermondii* and *H. uvarum*, demonstrating the interaction of these species through excreted antimicrobial compounds (Pérez-Nevado *et al.*, 2006). Recent studies further elucidated that *S. cerevisiae* CCMI 885 produced antimicrobial peptides, which altered intracellular pH, membrane permeability and culturability of non-*Saccharomyces* strains (Albergaria *et al.*, 2010; Branco *et al.*, 2014; Branco *et al.*, 2015). Interestingly, in the work of Wang *et al.* (2015c), not only the excreted products from *S. cerevisiae* NSa but also the synthetic media, induce a lack of culturability of *H. uvarum*. However, the synthetic must was weaker at inducing a lack of culturability of *H. uvarum* than *S. cerevisiae* supernatant, which included the same media plus the yeast metabolites. Thus, the role of environmental changes should be taken into consideration when studying the interaction between different yeasts.

Until now, studies on culturability loss of non-*Saccharomyces* yeasts have mainly focused on several potential wine starters: *H. guilliermondii*, *H. uvarum*, *Kluyveromyces marxianus*, *L. thermotolerans* and *T. delbrueckii* (reviewed in Albergaria and Arneborg, 2016). However, few studies have focused on the culturability differences among strains. According to Branco *et al.* (2014), different *D. bruxellensis* strains showed strain-specific sensitivity towards antimicrobial peptides excreted by *S. cerevisiae*. The differences between contact-dependent mechanisms and interactions through extracellular compounds were ascribed to the *S. cerevisiae* strains used (Kemsawasd *et al.*, 2015a). Therefore, more yeast species and strains should be considered to gain a better understanding of the interaction between *S. cerevisiae* and non-*Saccharomyces* yeasts.

This study was aimed at (i) investigating the strain and species differences in culturability loss, (ii) analyzing the interaction mechanisms that exist in different strains, and (iii) determining the viable status of non-culturable cells. We investigated the interaction between *S. cerevisiae* NSa (the same strain used in our former work, Wang *et al.*, 2015c) and ten non-*Saccharomyces* strains from different sources belonging to *H. uvarum*, *S. bacillaris*, *M. pulcherrima* and *T. delbrueckii* to analyze the interactions in mixed fermentation between *S. cerevisiae* and each individual strain. Through the use of three types of media (supernatants from *S. cerevisiae*

fermentation, synthetic media mimicking *S. cerevisiae* fermentation and mixed fermentation), the performance of each non-*Saccharomyces* strain was compared and studied. Synthetic must was used to rule out other effects and to define the media to mimic the must at different stages of fermentation. Recovery analysis and viability assays were also conducted to evaluate the status of non-culturable cells.

2. Material and methods

2.1. Yeasts strains and culture conditions

Eleven yeast strains were used in this study, containing *H. uvarum* CECT13130, NSb and CECT1444^T, *S. bacillaris* NSc, NSd and CECT11046, *M. pulcherrima* Mp com and Mp 51, *T. delbrueckii* Td com and CECT13135, and *S. cerevisiae* NSa. These strains were obtained from different collections: CECT13130, NSa, NSb, NSc, NSd, Mp 51, CECT13135 and NSa were natural isolates from our collection (Wang et al., 2014). CECT1444 and CECT11046 were from Spanish Type Culture Collection. Mp com (Flavia) and Td com (Biodiva) were commercial strains from Lallemand Inc. (Canada).

The species identity of all strains was determined by 5.8S-ITS-RFLP analysis (Esteve-Zarzoso *et al*., 1999) and sequence analysis of the D1/D2 domain of 26S rDNA (Kurtzman and Robnett, 1998). Yeasts were grown overnight in YPD medium (1 % yeast extract, 2 % peptone and 2 % glucose, w/v, pH 6.2) at 28 ºC before use.

2.2. Alcoholic fermentations, sampling and setting culturability

Synthetic must (100 g/L fructose, 100 g/L glucose, 290 mg N/L amino nitrogen, and 120 mg N/L ammonium nitrogen, pH 3.3) was prepared according to Andorrà *et al.* (2012). 350 mL of synthetic must was added to a 500 mL screw cap bottle, inoculated with 10^6 cells/mL of each yeast strain and kept at 25 °C in a shaker at a speed of 120 rpm. Fermentations were performed in the presence of air because the caps were not screwed tightly on the bottles. Each of the mixed fermentations was inoculated with one non-*Saccharomyces* strain and *S. cerevisiae* NSa. As a comparison, a single *S. cerevisiae* fermentation was carried out with the NSa strain. Fermentations were conducted in duplicate; when an interaction was observed, the fermentations were repeated in another duplicate and thus four replicas were used to set the interaction analysis.

Samples were taken every day to follow sugar and nitrogen consumption, ethanol production and yeast population dynamics until the end of fermentation. Concentrations of ethanol, fructose and glucose were tested using an enzymatic kit from Roche Diagnostics (Germany). The level of individual amino acids and ammonium was analyzed by HPLC according to Andorrà *et al.* 2012. Yeast populations in all samples were quantified using a microscope and plating after appropriate dilution in sterile water. YPD agar medium was used to calculate the total number of yeast cells present, and lysine agar medium (Oxoid LTD., England) was used for quantification of non-*Saccharomyces* strains.

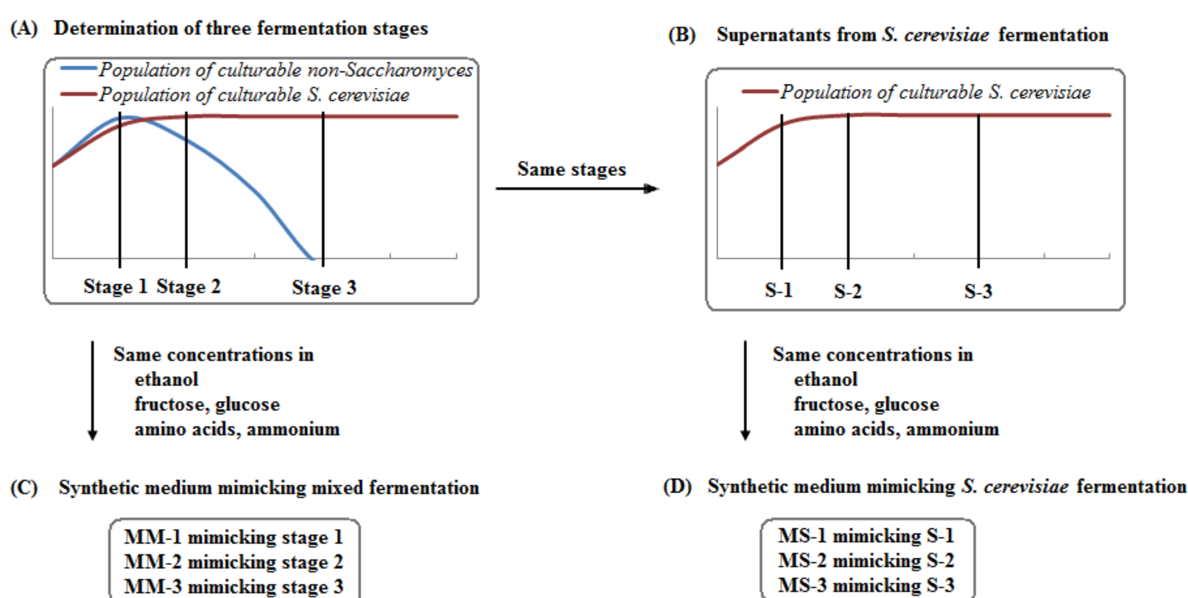

Figure 1 Experimental design for setting up three fermentation stages (**A**) and preparing three types of synthetic media (**B, C, D**).

Three stages were set up for each species depending on the culturability of the non-*Saccharomyces* species in mixed fermentations (Figure 1A): 1. When culturable populations reached the highest level; 2. When culturable populations started to decrease; 3. When no colonies grew on plates, or at the end of fermentation for some strains if colonies were still seen on plates. The concentration of main chemical components (ethanol, fructose, glucose, individual amino acids and ammonium), fermentation time and non-*Saccharomyces* strain culturability at these stages were listed to mimic the conditions of each fermentation stage where the interaction between non-*Saccharomyces* strains and *S. cerevisiae* was set (Figure 1

and Table 1). Data from these stages at mixed and single fermentations were used for the interaction assays in next step.

Table 1. Fermentation stages, population size and chemical characteristics of the media at different single and mixed fermentation stages.

Species	Fermentation time (h)	Culturable non-*Saccharomyces* (cfu/mL)	Ethanol (v/v)	Fructose (g/L)	Glucose (g/L)	Total assimilable nitrogen (mg N/L)	Names of synthetic media
H. uvarum	24	$2.6±1.3×10^7$	1.6±0.2	74.6±4.3	59.0±4.1	66.8±26.7	MM-1
	24	—	1.8±0.0	76.7±0.0	52.3±0.0	5.0±0.0	MS-1
	48	$2.0±1.1×10^7$	3.1±0.6	49.8±2.1	25.1±6.1	6.6±3.3	MM-2
	48	—	4.2±0.1	45.2±1.6	13.7±1.7	1.2±0.0	MS-2
	96	nd	9.4±0.5	12.7±5.0	0.9±0.7	nd	MM-3
	96	—	10.2±0.4	4.1±2.9	nd	nd	MS-3
M. pulcherrima	24	$8.1±3.3×10^6$	2.2±0.6	89.6±4.1	53.1±7.2	87.4±54.1	MM-1
	24	—	1.8±0.0	76.7±0.0	52.3±0.0	5.0±0.0	MS-1
	48	$3.2±6.8×10^6$	6.7±0.9	56.1±4.9	23.6±5.7	2.5±1.2	MM-2
	48	—	4.2±0.1	45.2±1.6	13.7±1.7	1.2±0.0	MS-2
	96	nd	10.2±0.5	15.9±6.0	0.7±0.8	nd	MM-3
	96	—	10.2±0.4	4.1±2.9	nd	nd	MS-3
S. bacillaris	24	$4.2±3.7×10^7$	1.5±0.2	68.6±3.0	50.7±2.2	18.3±4.7	MM-1
	24	—	1.8±0.0	76.7±0.0	52.3±0.0	5.0±0.0	MS-1
	96	$8.8±8.3×10^6$	9.9±0.5	5.1±1.9	nd	nd	MM-2
	96	—	10.2±0.4	4.1±2.9	nd	nd	MS-2
	120	$4.7±8.2×10^1$	11.6±0.1	0.1±0.1	nd	nd	MM-3
	120	—	11.5±0.4	0.1±0.1	nd	nd	MS-3
T. delbrueckii	24	$1.9±0.7×10^7$	3.1±0.1	90.8±3.4	48.4±0.9	20.1±13.9	MM-1
	24	—	1.8±0.0	76.7±0.0	52.3±0.0	5.0±0.0	MS-1
	96	$1.1±1.9×10^6$	10.4±0.4	19.8±5.1	1.1±1.9	nd	MM-2
	96	—	10.2±0.4	4.1±2.9	nd	nd	MS-2
	144	$1.8±2.4×10^6$	11.7±0.6	1.5±1.9	nd	nd	MM-3
	120	—	11.5±0.4	0.1±0.1	nd	nd	MS-3

This chemical composition was used to define the media mimicking the three selected fermentation stages. All values are the average of different strains within the same species. "MM" means synthetic media with main metabolites (ethanol, fructose, glucose and nitrogen) mimicking mixed fermentations, whereas "MS" is named after synthetic media with main metabolites mimicking S. cerevisiae fermentation. The Arabic numbers 1, 2 and 3 refer to the three stages selected in fermentations. "—" refers to the absence of a non-Saccharomyces population as derived from single S. cerevisiae fermentations, whereas "nd" means not detected. The total assimilable nitrogen is the sum of nitrogen from assimilable amino acids and ammonium. Only amino acid concentrations higher than 0.9 mg N/L are considered, and the concentrations are shown in Supplementary Table 1.

2.3. Culture assays using different synthetic media

To further understand the culturability of non-*Saccharomyces* strains in mixed fermentation, three types of synthetic media were prepared (Figure 1): supernatant from *S. cerevisiae* fermentation (S), synthetic medium mimicking *S. cerevisiae* fermentation (MS) and synthetic medium mimicking mixed fermentation (MM). S was collected from *S. cerevisiae* fermentation (Figure 1B), centrifuged and filtered using a 0.22 µm Whatman syringe filter (GE Healthcare Life Science, Germany). S was spread onto YPD agar plates to confirm the absence of *S. cerevisiae* cells. As a comparison, MS was prepared with metabolites (ethanol, fructose, glucose, individual amino acid and ammonium) mimicking S, with the absence of *S. cerevisiae* excreted compounds (Figure 1D). By performing culture assays using S and MS, the effect of main fermentation metabolites (the same for S and MS) and other putative *S. cerevisiae* metabolites (only in S) could be observed. Considering the possible differences of the main metabolites produced by *S. cerevisiae* fermentation and mixed fermentation, MM was prepared with corresponding components mimicking the mixed fermentation (Figure 1C). Moreover, no micronutrients or vitamins were added to MS and MM due to fast consumption at the beginning of alcoholic fermentation. All of the synthetic media were prepared for the three fermentation stages selected in 2.2 and were named with Arabic numbers to differentiate these stages (Figure 1 and Table 1).

Single fermentations of each non-*Saccharomyces* strain were then performed to provide adapted cells as described in Wang *et al.* (2015c). These adapted cells were incubated in YPD to ensure viability and incubated in synthetic media to check their culturability on plates. Culture assays were conducted at 25 °C in duplicate with a shaking speed of 120 rpm; when a culturability decrease was observed, the culture assays were repeated in another duplicate and thus four replicas were used to follow the cultuability change of non-*Saccharomyces* yeasts. Samples were taken at 24 h, 48 h and 120 h to quantify yeast cells using a microscope and YPD plating after appropriate dilution in sterile water. Cells losing culturability in synthetic media were collected for the following recovery analysis and viability assay.

2.4. Recovery analysis and viability assay of non-culturable cells from synthetic media

To test the viability of non-culturable cells from synthetic media, two approaches were used. Membrane integrity was analyzed by using the LIVE/DEAD® BactLight™ Bacterial Viability kit (Molecular Probes Inc., USA). In this assay, yeast cells were stained and observed using a fluorescence microscope equipped with filter system I3 and N2.1 (Leica DM 4000B) as in Hierro *et al.* (2006). The capacity to grow in rich liquid media was analyzed by incubating the cells in fresh YPD medium. Cells that could be recovered were considered to be viable but not culturable in synthetic media. Cells that could not be recovered after two consecutive 48 h incubations in fresh YPD medium were analyzed again by the LIVE/DEAD® BactLight™ Bacterial Viability kit.

2.5. Statistical analysis

One-way ANOVA by IBM SPSS Statistics 23 was used to calculate the value of significance for the variation analysis, and included a post-hoc Tukey test when needed. The consumption ratio (% of the total) of nutrients was used directly for the analysis of variation.

3. Results

3.1. Culturable population and metabolic characteristics of non-Saccharomyces strains during alcoholic fermentation

Overall, both *S. cerevisiae* and non-*Saccharomyces* strains reached the maximum population number of 10^7- 10^8 cells/mL 24 h after inoculation, and this size was maintained during mixed and single fermentations. Culturability of non-*Saccharomyces* strains decreased in all mixed fermentations. This decrease varied not only among different yeast species but also among some strains within the same species (Figure 2). Culturable *H. uvarum* increased to 10^7 - 10^8 cfu/mL at 24 h and began to decrease at 48 h. No colonies were formed on lysine plates for CECT1444 after 72 h and for CECT13130 and NSb after 96 h. Similar to *H. uvarum*, *M. pulcherrima* grew to 10^7 cfu/mL and quickly started to decrease. At 96 h, no colonies of Mp com were recovered on lysine plates, and for Mp 51, no colonies were recovered after 48 h. Culturable *S. bacillaris* maintained a population size of 10^7 - 10^8 cfu/mL until 96 h at which time the population started to decline. After 120 h, 10

to 100 cfu/mL of NSd and CECT11046 were recovered, however no colonies of NSc grew from lysine plates. Finally, *T. delbrueckii* strains reached approximately 10^7 cfu/mL and were maintained up to 48 h. After 48 h, CECT13135 started to decline, and no colonies were recovered after 144 h. The other *T. delbrueckii* strain, Td com, showed a slow decrease in culturable population, and at 144 h, 10^6 cfu/mL of Td com were still culturable. Based on the culturability of non-*Saccharomyces* strains in mixed fermentations, three stages were set up for each species. The fermentation times and main metabolites of these three fermentation stages are shown in Table 1. As a comparison, the same stage in fermentation of *S. cerevisiae* is also listed.

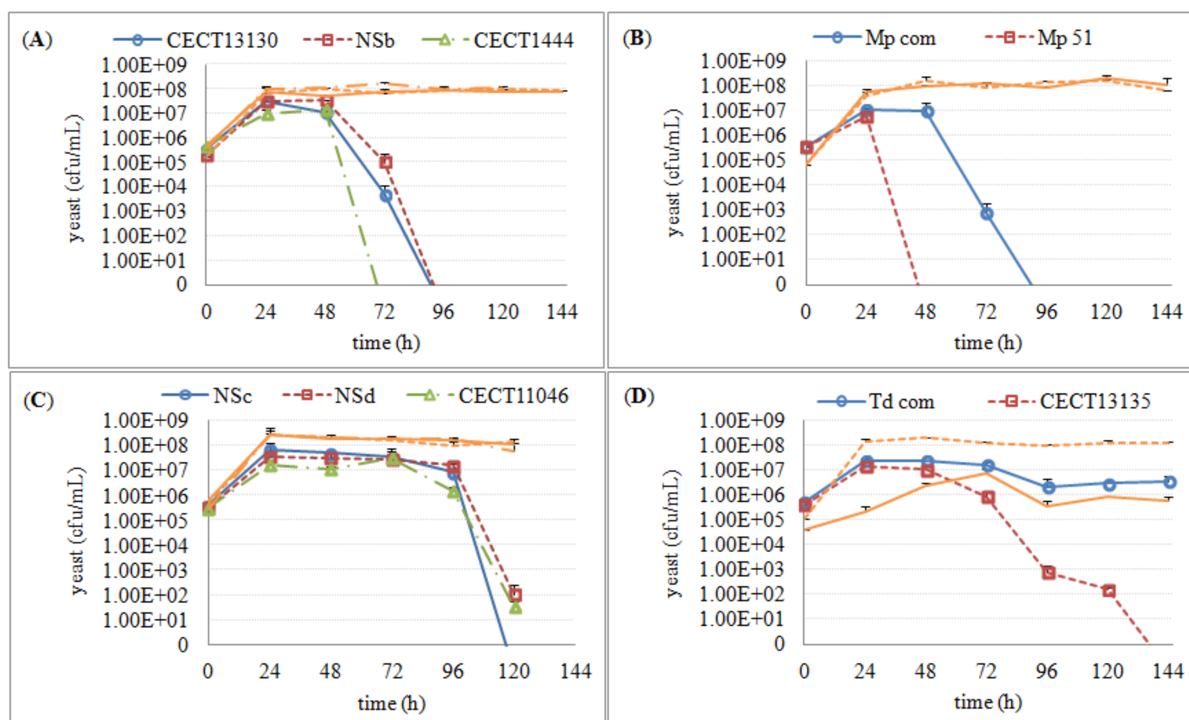

Figure 2 Culturable population of non-*Saccharomyces* in mixed fermentations with *S. cerevisiae*. Culturable *S. cerevisiae* populations were shown in orange line using the same line type as the non-*Saccharomyces* co-inoculated. (**A**) *H. uvarum* (**B**) *M. pulcherrima* (**C**) *S. bacillaris* (**D**) *T. delbrueckii.*

Despite the different culturability of non-*Saccharomyces* strains in mixed fermentations, no obvious variations in fermentation length were observed, and all fermentations finished after 120 or 144 h. Similar to *S. cerevisiae* fermentation, all mixed fermentations consumed glucose faster than fructose, and the final ethanol concentration reached 11 % vol to 12 % vol (Table 1). However, analysis of the consumption of these main metabolites after 24 h revealed strain-dependent differences. As shown in Table 2, the strains that lost culturability faster were those

that consumed some metabolites faster in the mixed fermentations: (i) Within *H. uvarum*, mixed fermentations inoculated with CECT1444 consumed glucose, ammonium and arginine faster than the other two strains; (ii) For *M. pulcherrima*, mixed fermentations with Mp 51 metabolized fructose, glucose, alanine, ammonium, arginine and glutamine faster than mixed fermentations with Mp com; (iii) Arginine was consumed faster during mixed fermentations with *S. bacillaris* NSc than the other two strains; (iv) More arginine was consumed during mixed fermentations with *T. delbrueckii* CECT13135 than Td com.

Table 2. Consumption ratio of glucose, alanine, ammonium, arginine and glutamine at 24 h of fermentation.

	Non-*Saccharomyces* strains	% of the total						Time of culturability loss
		Fructose	Glucose	Alanine	Ammonium	Arginine	Glutamine	
sc		23.3	47.7	100.0	100.0	100.0	100.0	nd
sc+hu	CECT13130	27.2	37.6*	90.4*	68.1*	65.1*	100.0	96 h
	NSb	23.3	39.2*	93.1*	63.4*	66.7*	100.0	96 h
	CECT1444	25.7	46.2#	91.6*	100.0#	71.9*#	100.0	72 h
sc+mp	Mp com	6.9*#	40.7*#	13.4*#	69.1*#	53.7*#	88.4*#	96 h
	Mp 51	13.9*	53.2*	96.7	91.2*	67.8*	100.0	48 h
sc+sb	NSc	35.1*	51.6	100.0	100.0	91.4*#	100.0	120 h
	NSd	30.4*	48.5	96.9	100.0	82.7*	100.0	nd
	CECT11046	28.8*	47.7	98.3	100.0	84.1*	100.0	nd
sc+td	Td com	10.8*	51.6	99.4	100.0	75.9*#	100.0	nd
	CECT13135	7.7*	51.6	99.9	100.0	93.5*	100.0	144 h

*sc means S. cerevisiae single fermentation, and mixed fermentations are presented as sc+hu (S. cerevisiae + H. uvarum), sc+mp (S. cerevisiae + M. pulcherrima), sc+sb (S. cerevisiae + S. bacillaris), sc+td (S. cerevisiae + T. delbrueckii). nd means not detected. * significance ≤0.05 with respect control (sc) by one-way ANOVA. # significantly different from the other strains of the same species as determined by a post-hoc Tukey test.*

3.2. The influence of excreted compounds from *S. cerevisiae* and media composition on the culturability of non-*Saccharomyces* strains

To further elucidate the culturability of non-*Saccharomyces* strains and the interaction with *S. cerevisiae* during mixed fermentation, we performed culture assays using S (supernatant from *S. cerevisiae* fermentation), MS (synthetic media mimicking *S. cerevisiae* fermentation) and MM (synthetic media mimicking mixed fermentation) based on the three stages of fermentation (Table 1). Although the non-*Saccharomyces* strains maintained a population size of 10^7 - 10^8 cells/mL for 120 h,

as determined by cell counting under a microscope, not all strains were culturable during the five-day period. The culturability was dependent on the media used, as well as the yeast species and strain.

No effect on culturability was seen in any media in fermentation stage 1 (S-1, MS-1, MM-1), which corresponded to the fermentation stage where culturable populations of non-*Saccharomyces* strains were the highest (generally between 10^7 and 10^8 cfu/ml). However, in the media from fermentation stage 2 (only S-2), and in the media from fermentation stage 3 (all the media), a decrease in culturable populations was observed (Figures 3-6), although the extent of the decrease was species- and strain-dependent.

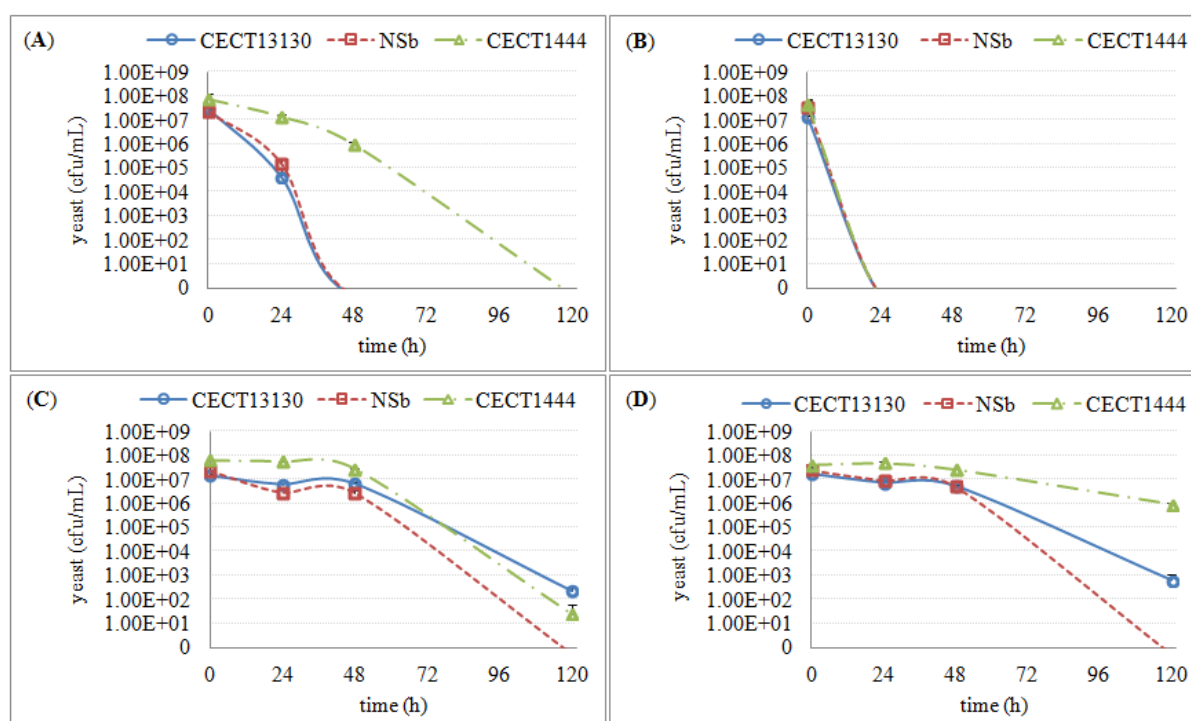

Figure 3 The culturable population of three *H. uvarum* strains (CECT13130, NSb and CECT1444) grown in different synthetic media for 120 h. (**A**) the growth in supernatant from the second stage of *S. cerevisiae* fermentation S-2 (**B**) the growth in supernatant from the third stage of *S. cerevisiae* fermentation S-3 (**C**) the growth in synthetic media MS-3 (**D**) the growth in synthetic media MM-3.

Within *H. uvarum* strains, the decrease of culturability was seen at 24 h in S-2 and S-3 and at 120 h in MS-3 and MM-3 (Figure 3). *H. uvarum* strains lost culturability in both S-2 and S-3; however, the decrease in culturability in S-2 occurred more slowly than in S-3. Further, among the three strains, CECT1444 showed a much slower decrease in culturability in S-2. The media (MS-3 and MM-3)

also affected the culturability, but to a lesser extent, and they were evident only at 120 h. There was also a strain difference, such that NSb was more affected than the other two strains.

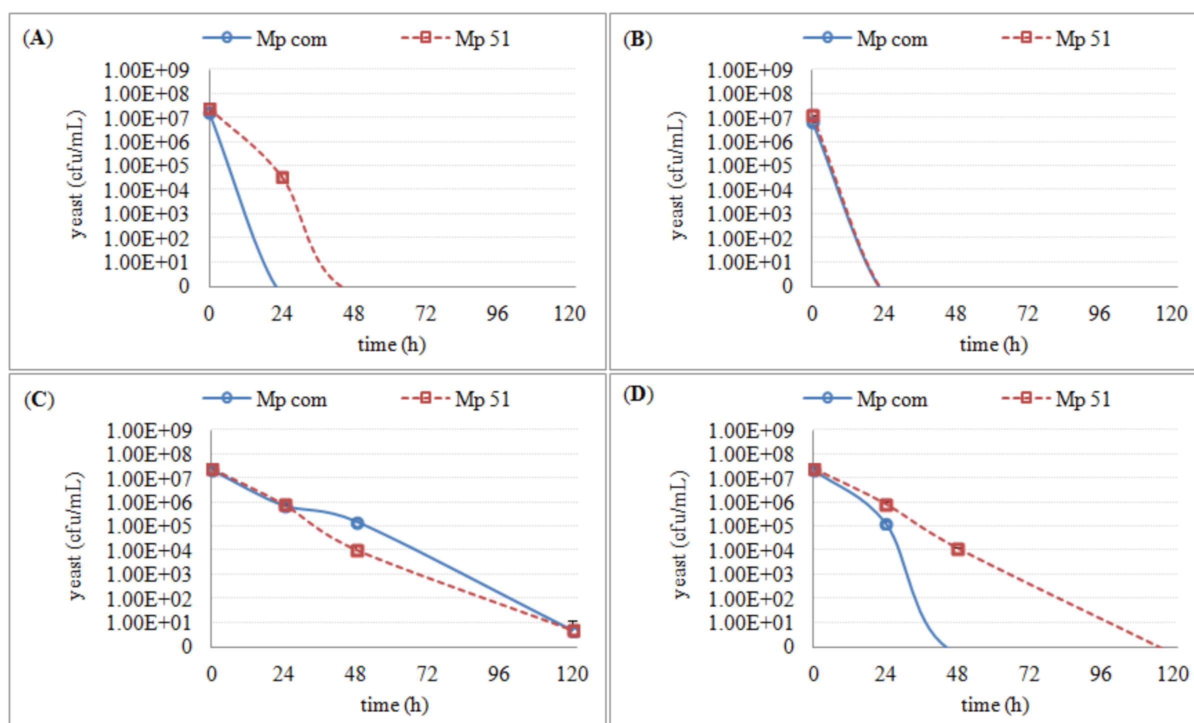

Figure 4 The culturable population of two *M. pulcherrima* strains (Mp com and Mp 51) grown in different synthetic media for 120 h. (**A**) the growth in supernatant from the second stage of *S. cerevisiae* fermentation S-2 (**B**) the growth in supernatant from the third stage of *S. cerevisiae* fermentation S-3 (**C**) the growth in synthetic media MS-3 (**D**) the growth in synthetic media MM-3.

Regarding the *M. pulcherrima* strains, a slow decrease in culturability was observed in all media mimicking the fermentation stages (MS-2, MM-2, MS-3 and MM-3), whereas a sharp decrease was seen in the *S. cerevisiae* supernatants (S-2 and S-3). Mp com and Mp51 showed different culturability in both S-2 and MS-3 (Figure 4), with Mp com exhibiting higher sensitivity.

The culturability of three *S. bacillaris* strains was less affected by different synthetic media, as some colonies were recovered (Figure 5). All of the strains showed a decrease in culturability during all studied periods (120 h), with no relevant differences between strains. Only in S-3 was a difference in sensitivity observed with strain NSc, which showed much lower culturability than the other two strains.

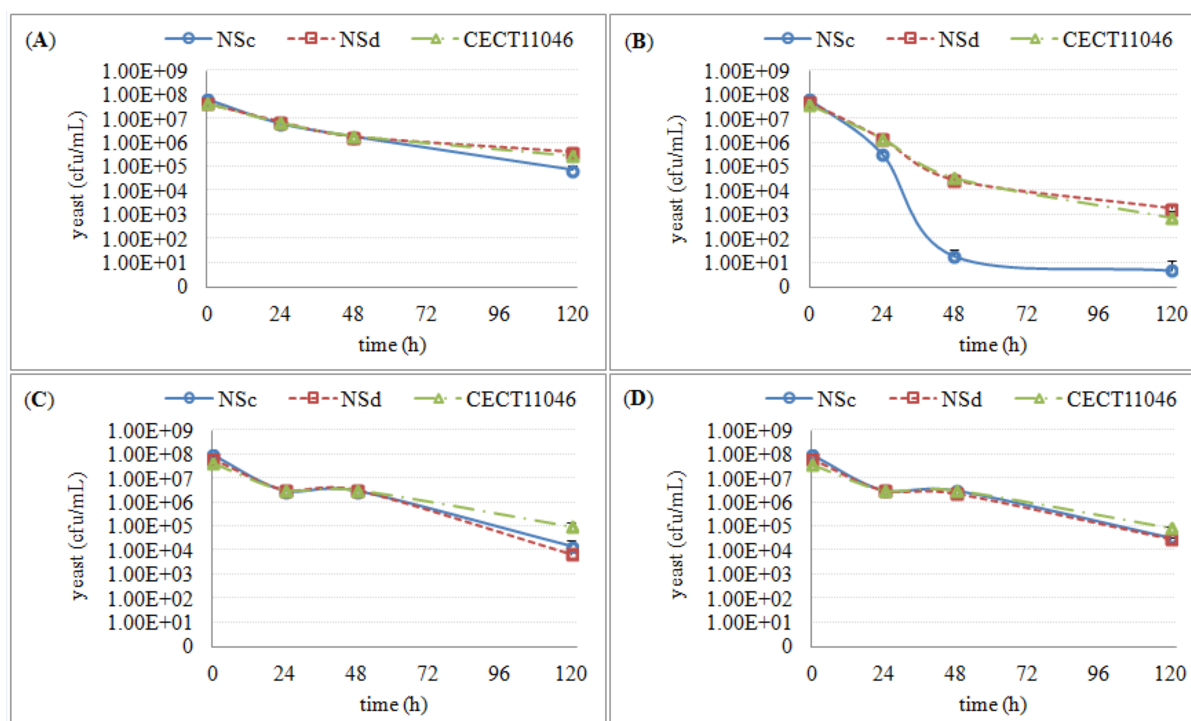

Figure 5 The culturable population of three *S. bacillaris* strains (NSc, NSd and CECT11046) grown in different synthetic media for 120 h. (**A**) the growth in supernatant from the second stage of *S. cerevisiae* fermentation S-2 (**B**) the growth in supernatant from the third stage of *S. cerevisiae* fermentation S-3 (**C**) the growth in synthetic media MS-3 (**D**) the growth in synthetic media MM-3.

Similar to *S. bacillaris*, the effect of synthetic media on the culturability of the two *T. delbrueckii* strains was limited (Figure 6). However, when the cells were cultured in S-3, no colonies were recovered after 120 h. Instead, only a small decrease of culturability was observed in MS-3 and S-2.

Although the decrease of culturability varied among different species, S-3 consistently showed the most obvious effect compared with other synthetic media. For the two species more affected (*H. uvarum* and *M. pulcherrima*), a more obvious effect was shown in S-2 than in MS-3 or MM-3. Thus, it is likely that some substances secreted from *S. cerevisiae* played a principal role in the interaction between *S. cerevisiae* and non-*Saccharomyces* strains, and that changes in the media (ethanol, nitrogen and sugar) also mediated the interaction.

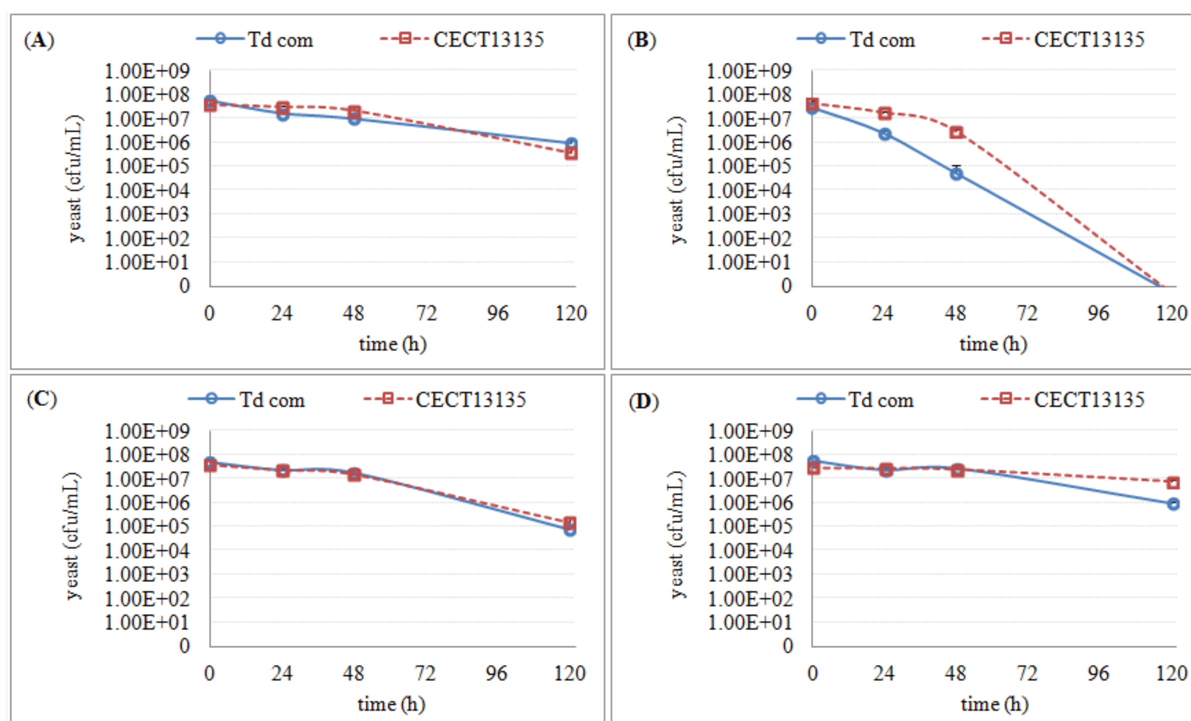

Figure 6 The culturable population of two *T. delbrueckii* strains (Td com and CECT13135) grown in different synthetic media for 120 h. (**A**) the growth in supernatant from the second stage of *S. cerevisiae* fermentation S-2 (**B**) the growth in supernatant from the third stage of *S. cerevisiae* fermentation S-3 (**C**) the growth in synthetic media MS-3 (**D**) the growth in synthetic media MM-3.

3.3. The viability of the non-culturable cells

To improve the understanding of the lack of culturability of the non-*Saccharomyces* strains, all of the samples (34 cases in total) that did not grow on plates were tested using two different methods: membrane integrity using the LIVE/DEAD viability kit and recovery by suspension in liquid YPD with agitation.

On one hand, these non-culturable cells were immediately analyzed using the LIVE/DEAD viability kit (Supplementary Figure 1). The results showed that live fluorescent cells were only found three times: non-culturable cells of the Mp com strain at 24 h in S-2 yielded 0.13% of live fluorescent cells, at 48 h in MM-3 8.17% and the Mp 51 strain at 120 h in MM-2 2.84 %. All other non-culturable cells yielded dead fluorescent cells.

On the other hand, all non-culturable cells were evaluated by recovery analysis. The non-culturable cells with live fluorescent were recovered when incubated in liquid YPD medium, whereas some of the non-culturable cells with dead fluorescent could also be recovered. The latter cases were found seven times

involving three non-*Saccharomyces* species. For example, after 120 h exposure to mimicking media MS-3 and MM-3, *H. uvarum* NSb, as well as *H. uvarum* CECT1444 after S-2 and the two *T. delbrueckii* strains after S-3, appeared dead in the fluorescence analysis but could be recovered. In the case of *H. uvarum* CECT13130, this observation was seen only in the early stages of exposure (48 h) to the media. Likewise, Mp com could be recovered after 120 h exposure to mimicking media MM-2 but could not be recovered from MM-3. Both cases indicate the existence of an intermediate, transient step before the cells are completely dead.

The LIVE/DEAD viability kit was again used to check the cells that could not be recovered by consecutive incubation in liquid YPD medium. All cells that could not be recovered yielded only dead fluorescent.

4. Discussion

The culturability loss of non-*Saccharomyces* strains during late stages of alcoholic fermentation has been well documented (Fleet, 2003). However, despite recent advances, the cellular mechanism underlying culturability loss is still a matter of discussion (Ciani and Comitini, 2015; Liu *et al.*, 2015; Albergaria and Arneborg, 2016). In a previous study (Wang *et al.*, 2015c), we investigated how *S. cerevisiae* NSa interacted with *H. uvarum* NSb by the use of a compartmented dialysis system, cell-free supernatant and mimicking synthetic media. Due to the absence of a contact-dependent mechanism in *S. cerevisiae* NSa, in the present study we decided to focus on the effects of compounds secreted by *S. cerevisiae* NSa, and the changes in main metabolites (ethanol, glucose, fructose, amino nitrogen and ammonium nitrogen).

Our results indicated that cell-free supernatant from *S. cerevisiae* fermentation influenced cellular culturability much more than mimicking synthetic media at the same fermentation stage (same chemical composition for major metabolites). Therefore, as mentioned in Wang *et al.* (2015c), some putative *S. cerevisiae* metabolites played a main role in the interaction between *S. cerevisiae* NSa and other non-*Saccharomyces* strains. A faster culturability loss was induced by *S. cerevisiae* supernatant at stage 3 than the initial two stages, which demonstrated the possible accumulation, or higher effect, of the *S. cerevisiae* secreted compounds as fermentation proceeded. Studies from Pérez-Nevado *et al.* (2006) and Williams *et al.* (2015) further related the accumulation to the amount of sugar consumed by *S.*

cerevisiae. Likewise, antimicrobial peptides identified by Branco *et al.* (2014) were derived from a glycolytic enzyme, showing a probable link with sugar metabolism of *S. cerevisiae*. More research is still required to illustrate how sugar consumption regulates the secretion of antimicrobial peptides or other putative metabolites.

Moreover, according to our previous report (Wang *et al.*, 2015c), as fermentation proceeds, the changes of the main metabolites also decreased the culturability of the cells, and the present results indeed showed that synthetic media at stage 3 caused a decrease in culturability. However, this effect occurs more slowly in the sensitive species and strains, and in our culture assays, complete culturability loss was mostly found after 48 h or 120 h. This indicates that the changes in the main metabolites play a role in the interaction between *S. cerevisiae* NSa and other non-*Saccharomyces* strains, and vice versa. Because not all of the species were equally affected, it also showed the capacity of some non-*Saccharomyces* strains to withstand a harsh environment (ethanol higher than 10% vol, glucose lower than 1 g/L, fructose lower than 16 g/L and no available nitrogen).

The interaction between *S. cerevisiae* and non-*Saccharomyces* strains also relied on the participating yeast species. In our mixed fermentations, cells of *S. bacillaris* and *T. delbrueckii* could coexist longer with *S. cerevisiae* than *H. uvarum* and *M. pulcherrima*. Other studies proposed that oxygen availability, glucose uptake rate and nitrogen source might contribute to the longer co-existence (Holm Hansen *et al.*, 2001; Nissen *et al.*, 2004; Andorrà *et al.*, 2012; Taillandier *et al.*, 2014). We indeed found that mixed fermentation inoculated with *S. bacillaris* or *T. delbrueckii* present a consumption rate of glucose, alanine, ammonium and arginine more similar to single fermentations with *S. cerevisiae* than those mixed fermentations inoculated with *H. uvarum* and *M. pulcherrima*. However, the relation between species tolerance and consumption of some nutrients still needs further investigation.

As expected, strain differences within each species were observed in mixed fermentation, culture assays and recovery analyses. First, strains decrease their culturability to a different extent during mixed fermentation. Second, when incubated in the same synthetic media in culture assays, strains showed different culturability or tolerance to a harsh environment. Third, non-culturable cells from the same synthetic media showed different recovery abilities depending on the strain. The strain difference, to some extent, increased the complexity of interaction analysis

between *S. cerevisiae* and non-*Saccharomyces* strains. In our case, the hypothesis of “strain tolerance to hard environment” cannot simply explain the strain differences in mixed fermentations. *H. uvarum* CECT1444 exhibited a slow culturability decrease in S-2, MM-2 and MM-3 as compared to the other two strains and thus was regarded as a strain that is highly tolerant to harsh environments. However, despite being a highly tolerant strain, in mixed fermentation, the culturability decreased even earlier than the other two *H. uvarum* strains. When we analyzed the metabolites at 24 h of mixed fermentation inoculated with *H. uvarum* CECT1444, a faster consumption of glucose, ammonium and arginine was detected. Andorrà *et al.* (2012) and Kemsawasd *et al.* (2015b) reported the influence of nitrogen consumption on yeast growth and fermentation performance. Nevertheless, further research should be undertaken to elucidate this effect, which was also observed in strains Mp 51, *S. bacillaris* NSc and *T. delbrueckii* CECT13135 compared with other strains within the same species.

Another important finding was the appearance of non-culturable cells when incubated with synthetic media, yielding more than 90 % of cells with “dead” fluorescence by viability analysis but that could be recovered by incubation in YPD medium. However, when these cells were incubated longer in the synthetic media (24 h more), all showed dead fluorescence and could no longer be recovered in YPD medium. This phenomenon demonstrated the existence of VBNC status of at least *H. uvarum*, *M. pulcherrima* and *T. delbrueckii* during alcoholic fermentation. As hypothesized by Branco et al. (2015), VBNC status could be understood as a transition status of yeast from culturable cells to dead cells, involving sub-lethally and severely injured cells. During this transition process, the ability to form colonies is the first lost vital activity and progressive changes in the permeability of cell membrane occur as found in this study, however the DNA or RNA remains stable (Andorrà et al., 2010; Wang et al., 2014; Wang et al., 2015a). Branco et al. (2015) measured how antimicrobial peptides secreted by *S. cerevisiae* affected cell viability and reported that injured cells had a similar pH as the external pH, whereas cells without compromised membranes (impermeable to propidium iodide) maintained a higher pH. Further research is still required to determine how the interactions between *S. cerevisiae* and non-*Saccharomyces* impacts physiological status and metabolic capacity of cells in different status.

In conclusion, we investigated the interaction between one *S. cerevisiae* strain and ten non-*Saccharomyces* strains during alcoholic fermentation. We demonstrated that the decrease of culturability was mainly caused by metabolites secreted by *S. cerevisiae*, although the change of the main metabolites in the media also played a role. We also found that culturability loss of non-*Saccharomyces* yeasts was not only species-dependent but also strain-dependent. The finding of VBNC status and strain differences in culturability is meaningful to the exploration of *Saccharomyces* - non-*Saccharomyces* interactions. The understanding of these interactions is relevant for the development of non-*Saccharomyces* strains as starters in wine production.

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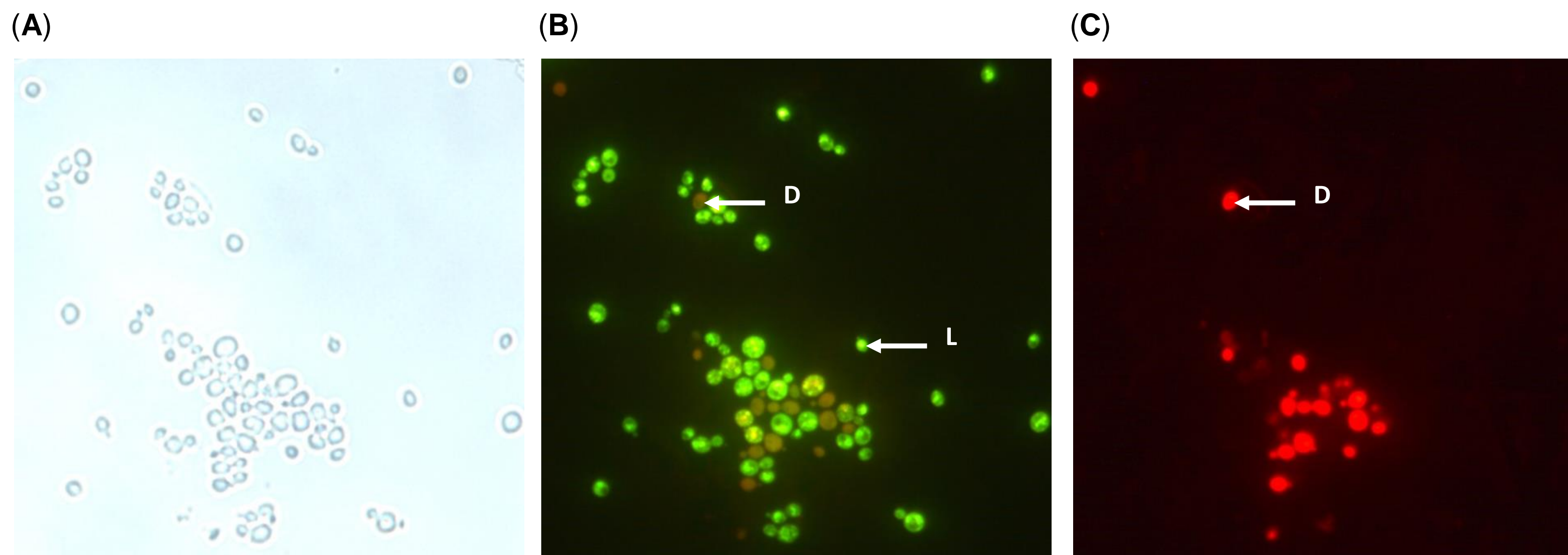
Supplementary Table 1. The concentration of amino acids and ammonium in three selected stages of mixed fermentations inoculated with *S. cerevisiae* and one non-*Saccharomyces* species, and single fermentation with *S. cerevisiae*. All values are the average of different strains within the same species. Synthetic medium mimicking mixed fermentations is named MM, and synthetic medium mimicking *S. cerevisiae* fermentation is named MS. The Arabic numbers 1, 2 and 3 used in the name of synthetic media stand for the three stages selected in the fermentations. “nd” means not detected or the concentration is lower than 0.9 mg N/L. Synthetic media with only proline (30 mg N/L) is not listed.

Species	Synthetic media	Alanine	Ammonium	Arginine	Aspartic	Cysteine	Glycine	Glutamic	Glutamine	Leucine	Proline	Tryptophane
		(mg N/L)										
H. uvarum	MM-1	1.9	27.5	28.7	1.7	1.2	1.5	nd	nd	1.5	30.0	2.8
	MS-1	nd	nd	nd	1.3	1.1	nd	nd	nd	1.7	30.0	0.9
	MM-2	nd	nd	3.9	1.5	nd	nd	nd	nd	nd	30.0	1.2
	MS-2	nd	nd	nd	nd	nd	nd	nd	nd	nd	30.0	1.2
M. pulcherrima	MM-1	10.4	23.9	35.1	2.1	1.0	2.2	1.6	5.6	0.5	30.0	5.1
	MS-1	nd	nd	nd	1.3	1.1	nd	nd	nd	1.7	30.0	0.9
	MM-2	nd	nd	nd	1.6	nd	nd	nd	nd	0.9	30.0	nd
	MS-2	nd	nd	nd	nd	nd	nd	nd	nd	nd	30.0	1.2
S. bacillaris	MM-1	nd	nd	12.5	1.6	2.0	nd	nd	nd	1.0	30.0	1.4
	MS-1	nd	nd	nd	1.3	1.1	nd	nd	nd	1.7	30.0	0.9
T. delbrueckii	MM-1	nd	nd	13.7	1.5	1.1	nd	nd	nd	nd	30.0	3.8
	MS-1	nd	nd	nd	1.3	1.1	nd	nd	nd	1.7	30.0	0.9



Supplementary Figure 1. The cells with "L" (live) and "D" (dead) fluorescence in the viability assay. Cells were observed with white light (**A**), filter system I3 (**B**) and filter system N2.1 (**C**).

GENERAL DISCUSSION

GENERAL DISCUSSION

The alcoholic fermentation for wine production involves great diversity of yeast species, which drive the development of the process and influence the final flavor characteristics of wine. Those diverse yeast species participate in alcoholic fermentation at different stages with specific yeast dynamics and succession during the process. A good assessment of yeast dynamics is desirable, to better control the metabolites appearing in the final product (Bisson and Joseph 2009). Especially in recent years mixed fermentation using selected *S. cerevisiae* and non-*Saccharomyces* yeasts has been proposed for creating wine complexity (Jolly et al. 2014, Lleixà et al. 2016, Masneuf-Pomarede et al. 2016) and is a trend in some wineries. Thus, the basic estimation and understanding of yeast dynamics is a requirement for the successful exploitation of those mixed cultures. At the same time, the emergence of culture-independent techniques indeed improves our assessment capacity after the finding of viable but non-culturable status of yeast in winemaking process (Divol and Lonvaud-Funel 2005). Further development of those culture-independent techniques means a more rapid and accurate assessment of yeast dynamics. Furthermore, the role of yeast interaction in the dynamics has also been highlighted in recent studies (Albergaria and Arneborg 2016). The correlation between yeast interaction and yeast dynamics needs more studies to fully understand the process.

In that framework this thesis has aimed to apply new culture-independent techniques to investigate yeast diversity, to analyze yeast status and to understand the yeast interaction during the alcoholic fermentation.

When it comes to the reality of alcoholic fermentation during wine making, it is often considered that alcoholic fermentation is a complex microbiological process and that a great diversity of yeast participates in the process. Although the dominant species have been usually paid more attention, wine is never a product from alcoholic fermentation of single pure strains or species. Rather, a high diversity and species richness could be observed in the process ranging from dozens of to hundreds of them (Chapter 1), which mainly roots in microbiota exist on grapes and subsequently enter grape must when berries break (Barata et al. 2012). It would be interesting to follow these non-dominant species during alcoholic fermentation in the

future, because their existence not only contributes to ecological complexity but also probably correlates with evolution principles based on microbial interaction and even the complexity of final product.

In addition to the great microbial diversity, another new finding that recently caused increasing interests is that microbial diversity distributes differently in each region (Pinto et al. 2015) or even vineyard (Chapter 1). The regional microbial community could be related with regional wine style, a factor neglected in the past and recently recovered (Bokulich et al. 2014, Setati et al. 2015). The European project WILDWINE has been performed to determine the “microbial fingerprint” of different wine regions and to establish artificial “natural winemaking” by applying selected multi-strain and multi-species starters (Mas et al. 2016). The final recognition of “microbial terroir” needs more experimental tests to demonstrate that this regional differential microbiota actually modulate wine styles (Barata et al. 2012). From our perspective, a necessary step is to know how they develop grape must by their participation in the alcoholic fermentation.

Although the diverse yeast community is involved in alcoholic fermentation, their participation in alcoholic fermentation basically follows a pattern of early dominance of fungi, fast giving way to non-*Saccharomyces* yeasts and the final dominance of *S. cerevisiae*. This dynamic pattern can be varied due to artificial strategies including inoculation design, temperature control and nutrition regulation (Medina et al. 2012, Taillandier et al. 2014, Maturano et al. 2015). However, a full control of the alcoholic fermentation with great diversity has not yet been achieved, mainly due to incomplete understanding of yeast dynamics. To our knowledge, it is likely that yeast population dynamic correlates with interaction between *S. cerevisiae* and non-*Saccharomyces* yeasts. Yeast interaction and environmental changes impose yeast cells on a stressful environment, which cause that non-*Saccharomyces* gradually lose their culturability especially at late stage of fermentation. Therefore, systematic studies on the interaction between *S. cerevisiae* and non-*Saccharomyces* have been carried out in this thesis to know the nature of the interaction and its correlation with yeast dynamics during alcoholic fermentation.

To restore and better explain yeast interaction during alcoholic fermentation, we established a simulation system. On one hand, to estimate the influence of environmental changes we used synthetic musts mimicking different stages of mixed fermentations. On the other hand, both cell-free supernatants from *S. cerevisiae*

fermentation and synthetic must mimicking these cell-free supernatants were designed to determine the toxic effect of *S. cerevisiae* exudates. In addition, cell contact was analyzed by using compartmented vessels and cell-free supernatants in all our trials. Based on this system, we mainly analyzed four factors: availability of nutrients and toxicity of ethanol, presence of toxin in *S. cerevisiae* exudates, cell contact, and the specificity of yeast species and strains. The methodology of this system could also be reference for the evaluation of other factors (such as pH, SO_2 and temperature) or other microbial interaction analysis in the future.

The two environmental factors — availability of nutrients and toxicity of ethanol were evaluated together since nutrient depletion and ethanol production are simultaneous common stresses induced by alcoholic fermentations. Non-*Saccharomyces* yeast species were conventionally regarded to be poorly adapted to such stresses and thus this could be a reason for the dominance of *Saccharomyces*. Marsit and Dequin (2015) even proposed that this is a strategy used by *Saccharomyces* yeast to establish competitive dominance in the ecological niche: they rapidly consume nutrients, transform carbohydrates into ethanol and finally they could catabolize ethanol for energy. Non-*Saccharomyces* yeast in our simulation system did present a tendency to decrease their population but at a much slow rate than in presence of *S. cerevisiae*. The slow decrease in population of non-*Saccharomyces* yeasts in harsh environment with both limited availability of nutrients and high ethanol concentration shakes the former ideas, which over-emphasize the importance of these environmental factors.

Although we know that nutrition addition can increase fermentation capacity of *Saccharomyces* and non-*Saccharomyces* yeast (Albergaria 2007, Taillandier et al. 2014), more research is required to know the single factor effect of nutrition depletion on vitality of non-*Saccharomyces* yeast. Considering that *Saccharomyces* consume nutrients faster and produce biomass more efficiently than non-*Saccharomyces* (Andorrà et al. 2012), we can try to add some specific nutrients (Alanine, Ammonium, Arginine or Glutamine depending on the species, Chapter 5) or complex nutrition (yeast extract or peptone) into mixed fermentation to postpone the culturability loss of non-*Saccharomyces* yeast. It would be also interesting to try to establish the specific nitrogen requirements of different non-*Saccharomyces* yeast for maintaining culturability in the process. Kemsawasd et al. (2015b) reported that some amino acids had beneficial effect on growth, glucose consumption and ethanol production

of non-*Saccharomyces* yeasts in single pure fermentation such as serine for *L. thermotolerans*, alanine for *H. uvarum*, alanine and asparagine for *M. pulcherrima*. Whether these amino acids favor the growth of non-*Saccharomyces* in mixed fermentations needs future studies to make it clear. In terms of ethanol toxicity alone, a slow decrease of *H. uvarum* culturability after 48 h was seen and with high ethanol concentration (12%) a decrease of culturability of one log unit was seen after 24 h (Chapter 4). This slow reduction indicated its relative high ethanol tolerance. The ethanol tolerance of other non-*Saccharomyces* yeast was not analyzed, but we can presume similar tolerance or even higher. Interestingly, *Starm. bacillaris* was reported to be more ethanol tolerant to *S. cerevisiae* based on the minimum inhibitory concentration for yeast growth (Salvadó et al. 2011). The application of high ethanol tolerant strains will highlight other factors involved in yeast interaction and it could be a future experimental strategy.

The presence of toxic effects of *S. cerevisiae* exudates was indicated by our system when we compare the growth of non-*Saccharomyces* yeasts in presence of *S. cerevisiae* supernatants and in synthetic must with the main metabolites (sugar, ethanol and nitrogen) mimicking these supernatants, because the former induced faster culturability decrease than the latter (Chapter 4 and 5). The identity of the toxic effects have been recently determined as antimicrobial peptides, which corresponds to fragments of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein and correlates with GADPH encoding genes of *TDH1*, *TDH2* and *TDH3* (Branco et al. 2014). If we imagine that GAPDH protein breaks and is transformed into antimicrobial peptides after it finishes the role in glycolysis process, it relates the appearance and increase of antimicrobial peptides with the sugar depletion during alcoholic fermentation. This hypothesis could initially explain why supernatants from late stage of fermentation affected the culturability of non-*Saccharomyces* yeasts more and faster than early stages of fermentation (Chapter 4 and 5). Media with low sugar concentration did not induce (or it was much lower) growth inhibition of non-*Saccharomyces* yeasts, which we have observed (chapter 4) as well as reported in other studies (Pérez-Nevado et al. 2006, Williams et al. 2015). This might result from relative lower concentration of antimicrobial peptides when sugar is still available. Furthermore, for a good explanation of the formation process from GAPDH protein to antimicrobial peptides, it would be also interesting to analyze the downstream product of GAPDH protein in related non-*Saccharomyces* yeast species.

Although recent studies consistently indicated that the presence of toxic compounds (antimicrobial peptides) in *S. cerevisiae* exudates mainly accounted for the interaction between *Saccharomyces* and non-*Saccharomyces* yeasts, the incentive for their production is still unknown. It could be self-defensive strategy used by *S. cerevisiae* (Albergaria and Arneborg 2016), which have been domesticated by competition in nature. This can explain why *S. cerevisiae* produces the toxin in single pure fermentation without the existence of competitors — other non-*Saccharomyces* yeast species (chapter 4 and 5). Furthermore, all *S. cerevisiae* strains tested until now have been reported to be able to produce the toxic compounds alone despite that the production ability varied among *S. cerevisiae* strains (Kemsawasd et al. 2015a). *S. cerevisiae* strains with different production ability might be further studied at genetic level to understand the secretion process. In addition, these antimicrobial peptides have a potential to be developed as reagents for microbial control in mixed fermentation, because purified antimicrobial peptides not only showed wide fungicide or fungistatic effect on non-*Saccharomyces* but also bactericide or bacteriostatic effects on *Oenococcus oeni* (Branco et al. 2014).

Cell contact was reported as a probable mechanism used by *S. cerevisiae* to interact with other yeast species (Nissen et al. 2003, Renault et al. 2013). Nevertheless, this mechanism seems to be strain-dependent according to recent studies (Kemsawasd et al. 2015a). The *S. cerevisiae* strain used in this study did not show the effect of cell contact, because culturability loss of non-*Saccharomyces* still happened when cell contact with *S. cerevisiae* was avoided by compartmented apparatus (Chapter 4). *S. cerevisiae* Saint Georges S101 has been the only strain reported until now with obvious contact-dependent mechanism in yeast interaction, and interestingly its production ability of antimicrobial peptides is relative low (Kemsawasd et al. 2015a). Future study might consider determining the relationship between the contact-dependent mechanism and low toxic compounds production.

In addition to environmental factors, toxic compounds and cell contact, this thesis also found species and strain specificity in the interaction between *S. cerevisiae* and non-*Saccharomyces* (Chapter 5). It is apparent that *S. cerevisiae* is the most resilient yeast which is able to grow well in the fluctuating wine fermentation. The growth of non-*Saccharomyces* species is impaired by environmental changes and metabolites from *S. cerevisiae*, with *Starm. bacillaris* and *T. delbrueckii* less affected than *H. uvarum* and *M. pulcherrima*. Compared to species specificity, strain

variation seems like more complicated. The observation of high-tolerant strains within the same species is expected. Nevertheless, instead of a long coexistence with *S. cerevisiae*, the high-tolerant strains showed a faster culturability decrease in mixed fermentation. According to this phenomenon, we hypothesize that the existence of high-tolerant strains probably induces some regulation mechanism of *S. cerevisiae* in yeast interaction. We have tried to find some clues for the probable regulation mechanism in the consumption of main metabolites and faster consumption of glucose, fructose, alanine, ammonium, arginine or glutamine depending on species was seen (Chapter 5). Therefore, it is possible that the competition for nutrients in non-*Saccharomyces* induce more production of toxic compounds from *S. cerevisiae*. Future studies at genetic level will help to explain the possible regulatory mechanism.

So, we have observed that the lack of culturability does not mean the cell lysis due to stable cell concentrations checked by microscopy. Thus, it was interesting to know how yeast interaction affects the viability of non-*Saccharomyces*. One of the sensitive parameters could be the RNA, because DNA is more stable molecule. The existence of rRNA from these non-*Saccharomyces* could be verified by culture-independent techniques. In our study, 10^5-10^6 cells/mL of *H. uvarum* and *Starm. bacillaris* were quantified with stable rRNA and non-compromised membrane (Chapter 2, 3 and 4). Purified antimicrobial peptides not only decreased the culturability and membrane permeability but also disturbed intracellular pH homeostasis of *H. guilliermondii* cells (Branco et al. 2015), which underlines the main influence of yeast interaction on integrity of cell membrane. Furthermore, when stress was removed by providing favorable conditions, some non-*Saccharomyces* could resuscitate (Chapter 5, Branco et al. 2015). These phenomena also demonstrated that some non-*Saccharomyces* yeast cells existed at viable but non-culturable (VBNC) state after losing their culturability in the interaction with *Saccharomyces*.

The VBNC states found at late stage of alcoholic fermentation challenge our definition on “live or dead” cells. It is generally recognized that membrane integrity and rRNA stability are two key indexes for distinguish live or dead cells. However, some cells with compromised membrane could resuscitate (Chapter 5) and EMA-qPCR required a cell recovery step to include live cells, which were permeable to EMA (Andorrà et al. 2010a). The stability of rRNA in yeast cells under stress also

stated the complexity: culturable *S. cerevisiae* and *Starm. bacillaris* showed rRNA decrease at the end of fermentation, whereas dead cells still kept low level of rRNA (Chapter 3). Likewise, Andorrà et al. (2011) doubted that RNA degradation speed could change due to different death treatments and yeast species involved. Therefore, future studies analyzing physiological states of cells during the process from culturable to death might be helpful for our understanding (Díaz et al. 2010). The resuscitation ability of VBNC states should also be further analyzed considering their probable impact on wine quality.

Investigation of yeast interaction was limited due to the methodological biases of culture-dependent techniques in the past. The development of real-time techniques for yeast diversity investigation correlates with the ability of directing yeast dynamics, which represent an advance in winemaking field (Bisson and Joseph 2009). In this thesis we used different culture-independent techniques to follow yeast interaction including massive sequencing, FISH, qPCR and DGGE. Small modifications have been made in some techniques for better analysis of yeast viability. Probable interference caused by phenol and pigments was considered when applying these techniques and final yeast identification and/or quantification was directly performed from wine samples (Chapter 1, 2 and 3). Their advantages and limitations are well discussed in the context of viable yeast identification and quantification. Noteworthy, the combined use of these techniques enables more comprehensive investigation.

Massive sequencing exhibits good fungal diversity analysis in our study due to its main technical advantages: the high throughput analysis, which allows the detection of minor yeast population (Chapter 1). The main limitation of massive sequencing is that it only provides a qualitative assessment and thus should be paired with quantitative techniques for target group enumeration (Bokulich and Mills 2012). Meanwhile, current operation as mentioned in recent extensive research are all based on rDNA amplification and thus cannot exclude the detection of dead cells and DNA remnant in our samples (Mayo et al. 2014). The use of massive sequencing with cDNA or dye treatment can be considered in the next step as we tried in this study for qPCR and DGGE. Furthermore, a reliable and complete bioinformatics interpretation of the large numbers of sequences obtained is still on the way to meet our need for yeast identification at species level, such as the

identification of no hit reads and close-relative species as the "uncultured *Hanseniaspora*/*Saccharomyces*" that we detected.

FISH is considered promising technique for the investigation of viable yeast species in wine samples, because the hybridization probe direct combines with intracellular rRNA without the need of RNA extraction (Andorrà et al. 2011). A critical step for employing FISH into direct wine sample analysis is the development of specific hybridization probes with good fluorescence intensity. Hybridization probes were designed in this study, one for eight species of *Hanseniaspora* and the other targeting *Starm. bacillaris*, and both have been successfully used for analysis of wine samples (Chapter 2). FISH combined with fluorescence microscopy acts as a spatiotemporal snapshot, visually presenting target yeast cells within a mixed population, and FISH combined with flow cytometry can improve detection limits with the record of thousands of cells in several seconds. Therefore, as a technique for target yeast analysis, its application on investigation of other yeast species will depend on the development of corresponding hybridization probes.

EMA treatment and reverse transcription of extracted RNA have been combined with qPCR and DGGE in this study for examining viable yeast population, which might involved yeast interaction at late stage of alcoholic fermentation. Both treatments demonstrated their advantages but also showed their limitations especially for the interference from dead cells. Dead cells higher than 10^7 cells/mL in wine sample will interfere with the detection of viable cells, which is lower than 10^3 cells/mL when EMA-QPCR is used. For techniques based on reverse transcription of extracted RNA, the relative stability of rRNA after death treatment (75% ethanol 24h) could interfere with the determination of minor viable yeast population. Similar reports were also obtained by studies using heat treatment to kill cells (Hierro et al. 2006, Andorrà et al. 2011). The relative stability of rRNA, as we have stated in FISH and RT-QPCR analysis (Chapter 2 and 3) is being analyzed in different cell states, which could provide some references for related techniques. In addition, other techniques such as fluorescence ratio imaging microscopy (FRIM) for pH gradient analysis (Branco et al. 2015) and rRNA precursors (pre-rRNA) analysis after nutrient stimulation (Cangelosi and Meschke 2014) could be considered in the future for a better estimation of yeast viability and measurement of yeast interaction.

To sum up, our results verify initial hypothesis that yeast population dynamic largely depends on the interaction between *S. cerevisiae* and non-*Saccharomyces* and that the combination of different culture-independent techniques could be the appropriate tools to understand that interaction.

Our research used main indigenous strains isolated from Priorat region and related findings especially on yeast interaction will be of great value for applying them in controlled mixed fermentation. The metabolites from *S. cerevisiae* could be developed as microbial control reagents in winemaking field. In addition to the application significance, our study also highlights the role of interaction between *S. cerevisiae* and non-*Saccharomyces* in population dynamics and cell states. The developed culture-independent techniques promote our understanding of cell viability. What is important is this thesis opens the doors to research in the future with the new questions posed:

(i) What kinds of metabolism exist in VBNC yeast cells and how they impact on wine quality?

(ii) Does the synthesis of toxic compounds from GAPDH protein only exist in *S. cerevisiae*? If there is a positive answer, why?

(iii) What is the incentive for toxin production in *S. cerevisiae* strains?

(iv) Do regulation mechanisms exist to adjust the production of toxic compounds when *S. cerevisiae* is mixed with other non-*Saccharomyces* strains during alcoholic fermentation? Is consumption of nutrients involved in these regulation mechanisms?

(v) How antimicrobial peptides affect cell states of different non-*Saccharomyces*?

(vi) Does nutrient consumption contribute to the high tolerance of non-*Saccharomyces* species in harsh environments?

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GENERAL CONCLUSIONS

GENERAL CONCLUSIONS

1. Culture-independent techniques not only enable a rapid identification and/or quantification of different wine yeast but also allow an evaluation of viable but non-culturable yeast status (VBNC) during alcoholic fermentation. Each culture-independent technique presents advantages and limitations. Therefore, the combined use of different culture-independent techniques and even traditional culture-dependent techniques are advised to have a comprehensive observation of yeast diversity and viability.

2. Grape must from three vineyards in Priorat region showed distinct and rich fungal diversity. *Starm. bacillaris* and *H. uvarum* were two main non-*Saccharomyces* yeast detected in grape must and subsequent alcoholic fermentation.

3. Quantifiable non-*Saccharomyces* yeast cells existed at viable but non-culturable state during late stage of alcoholic fermentation.

4. Contact-dependent mechanism did not correlate with the culturability loss of non-*Saccharomyces* in our mixed fermentation. Environmental changes such as ethanol, sugar and nitrogen concentration changes indeed affect culturability of non-*Saccharomyces*. However, the interaction between *S. cerevisiae* and non-*Saccharomyces* due to metabolites secreted from *S. cerevisiae* during the alcoholic fermentation had a main role in the culturability loss of non-*Saccharomyces*.

5. The interaction between *Saccharomyces* and non-*Saccharomyces* yeasts is species and strain dependent.

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1. Culture media

YPD medium

YPD medium (Yeast extract peptone dextrose medium) is a general medium for yeast growth.

Glucose 20 g/L, peptone 20 g/L, yeast extract 10 g/L, agar 20 g/L (solid medium)

Lysine medium

Lysine medium is a selective medium which supports the growth of non-*Saccharomyces* yeast.

Lysine medium 66 g/L (Oxoid, USA), lactate potassium 4 mL/L (18 mL lactic acid 85% and 14 g KOH in 100mL distilled water), bring to the boil to dissolve completely; When the medium cools down to around 50 °C, add 1 mL lactic acid 10% to adjust the pH at 5.

WL nutrient medium

WL nutrient medium (Wallerstein Laboratories nutrient medium) is an identification medium on which yeasts form different morphologies and colors for discrimination (Cavazza et al. 1992).

WL nutrient agar 75 g/L (Biogenetics, Italy), bring to the boil to dissolve completely and then autoclaved.

Synthetic must

Sugar (g/L): glucose 100, fructose 100;

Acid (g/L): DL-malic acid 5, citric acid 0.5, tartaric acid 3;

Mineral salts (g/L): KH_2PO_4 0.75, K_2SO_4 0.5, $MgSO_4 \cdot 7H_2O$ 0.25, $CaCl_2 \cdot 2H_2O$ 0.155, NaCl 0.2;

Nitrogen: NH4Cl 0.46 g/L, amino acid mother solution 10 mL/L;

Micronutrient mother solution 1 mL/L;

Vitamins mother solution 10 mL/L;

Solutions with sugar, acid, mineral salts and ammonium nitrogen dissolved is firstly autoclaved, then all the mother solutions are added after autoclaving and finally pH is adjusted to 3.3 with NaOH. After the volume adjustment until the required level, synthetic must is filtered by a 0.22 µm filtration system.

- Amino acid mother solution (g/L): Tyrosine 1.95 (heated to 100 °C), Tryptophan 17.42 (70 °C), Isoleucine 3.25 (70 °C), Aspartic Acid 4.42 (disengagement of CO_2), Glutamic acid 11.96 (disengagement of CO_2), Arginine 36.79, Leucine 4.81 (increase T °C until dissolve), Threonine 7.54, Glycine 1.82, Glutamine 49.92, Alanine 14.56, Valine 4.42, Methionine 3.12, Phenylalanine 3.77, Serine 7.8, Histidine 3.38, Lysine 1.69, Cysteine 2.08, Proline 59.93, dissolved in 20 g/L $NaHCO_3$ solution, filtered by 0.2 µm filter and preserved at -20 °C.
- Micronutrient mother solution (mg/L): $MnSO_4 \cdot H_2O$ 4, $ZnSO_4 \cdot 7H_2O$ 4, $CuSO_4 \cdot 5H_2O$ 1, KI 1, $CoCl_2 \cdot 6H_2O$ 0.4, H_3BO_3 1, $(NH_4)_6Mo_7O_{24}$ 1, dissolved in distilled water, filtered by 0.2 µm filter and preserved at 4 °C.
- Vitamins mother solution (g/L): myo-inositol 2, calcium panthothenate 0.15, nicotinic acid 0.2, chlorohydrate thiamine 0.025, pyridoxine 0.025, biotine 3mL (100 mg/mL solution), dissolved in distilled water, filtered by 0.2 µm filter and preserved at -20 °C.

2. Chemical analysis of grape must or synthetic must during alcoholic fermentation

Density analysis

Density analysis is commonly used in wineries for monitoring fermentation, because the decrease of density is directly proportional to the decrease of sugar during fermentation. Automatized densimeter (Densito 30PX Portable Density Meter, Mettler Toledo, Spain) was used for density analysis.

Weight loss analysis

Weight loss analysis is used in laboratories for fast monitoring fermentations, because the decrease of weight caused by CO_2 emission is proportional to the sugar decrease during grape must fermentation. Analytical Balance (Cobos precision, Spain) was used for weight loss analysis.

Glucose, fructose and ethanol analysis

Concentrations of glucose, fructose and ethanol were separately analyzed by Ultrospec 2100 pro UV/Visible Spectrophotometer (GE Healthcare, USA) using enzymatic kits from Roche Diagnostics (Germany).

Acetic acid and glycerol analysis

Concentrations of acetic acid and glycerol were separately tested by Miura One Multianalyzer (TDI, Spain) using the enzymatic kit from Biosystems S. A. (Spain).

Amino acids and ammonium analysis

Concentrations of individual amino acids and ammonium were analyzed by HPLC according to Andorrà et al. (2012). Samples preparation was performed at 80 °C for 2 h with a mixture of 400 μL sample, 700 μL 1M borate buffer (pH 9), 300 μL methanol, 10 μL L-2-aminoadipic acid (Internal standard, 1 g/L) and 15 μL diethylethoxymethylenemalonate (Fluka, Germany). Agilent 1100 Series HPLC (Agilent Technologies, Germany) were used for analysis 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 × 250 nm, 5 μm ACE C18-HL column (Symta, Spain) with a guard column (ACE5C18-HL) through a binary gradient (phase A, 25 mM acetate buffer pH=5.8 with 0.02% sodium azide, phase B, 80:20 mixture of acetonitrile and methanol, 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.

3. Plate counting

Appropriate dilutions from samples are prepared and spread onto a medium on which each cell forms a colony, and thus by counting colonies we obtain yeast concentration in colony form units (cfu)/mL. Whitley Automatic Spiral Plater (AES Laboratoire, France) was used for plating and ProtoColHr automatic colony counter (Microbiology International, USA) was used for yeast quantification.

4. The extraction of DNA and RNA from yeast in wine samples

DNA extraction using the DNeasy plant minikit (Qiagen, USA, Hierro et al. 2006)

DNA was extracted following manufacturer's instruction which contained cell lysis, removal of RNA and other cell constituents (cell debris, protein and polysaccharide), DNA cleaning and collection. Cell lysis was performed in a mini bead-beater (Biospec Products Inc., Okla) with presence of 700 µL **buffer AP1** and 1 g glass beads (0.5 mm diameter) using three times of 1 min at maximum velocity with intervals of 1 minute on ice. RNA treatment was performed at 65 °C for 10 min with 4 µL **RNase A** in cell suspension and other cell constituents were placed in a **QIAshredder Mini spin column** using 130 µL **buffer AP2** in cell suspension at 4 °C. Then DNA was collected in **DNeasy Mini spin column**, washed by 675 µL **buffer AP3** and 1 mL **buffer AW** and finally dissolved in 100 µL AE buffer. Reagents in bold are the original ones from the commercial kit.

EMA treatment (Andorrà et al. 2010a)

EMA treatment was performed before DNA extraction to bind EMA to the DNA from dead cells. Cells' membrane was firstly recovered in YPD broth at 13 °C for 2 h in order to remove the ethanol interference. Cells suspension in distilled water was added EMA solution (2 µL 5 g/L into 1 mL suspension, final concentration 24 µM)

and incubated in dark for 10 min. Then two exposures for 30 seconds under the light (650 W halogen lamp at 20 cm from the samples), with an interval of 1 minute on ice were used for photolysis. Finally, cells was washed with sterile distilled water to remove the unstained EMA and collected for further DNA extraction.

RNA extraction using the Masterpure complete DNA and RNA purification kit (Epicenter, USA)

RNA was extracted following manufacturer's instruction which contained cell lysis, total nucleic acid precipitation and DNA removal (Reagents in bold are the original ones from the commercial kit). Cell lysis was performed at 65 °C for 15 min with addition of 300 μL of **tissue and cell lysis solution** and 1 μL of **proteinase K** (25 ng/μL) to cell pellet from samples by centrifugation. Total nucleic acid precipitation was further carried out by protein removal (add 150 μL **MPC protein precipitation reagent** to lysed samples on ice, vortex for 10 seconds, pellet the debris by centrifugation at 4 °C for 10 min at 14000 rpm and collect the supernantant), pellet total nucleic acids (add 500 μL isopropanol to supernatant, mix well, centrifuge at 4 °C for 10 min at 14000 rpm and discard isopropanol), cleaning total nucleic acids using 70% ethanol and final suspending the total nucleic acids in 29.5 μL of DNase and RNase-free water. Total nucleic acids was treated with 6 U TURBO DNase (Ambion, USA) in 3.5 μL 10× buffer at 37 °C for 3 h or overnight (if needed) to completely remove DNA.

Reverse transcription

RNA was further synthesized into cDNA by reverse transcription using the following procedures. Promega (Italy). 0.5 μL of RNA was mixed with 4 μL of DNase and RNase-free water and 0.5 μL of random primers (500 ng/μL, Promega, Italy). After incubation at 72 °C for 5 min, they were chilled on ice for 5 min, then added with 7.5 μL of mixture (2.5 μL of M-MLV RT 5× buffer, 2.5 μL of dNTP mix (10 mM each dNTP), 20 U of RNase inhibitor, 100 U of M-MLV reverse transcriptase and 1.5 μL of DNase and RNase-free water, all from Promega, Italy) and incubated at 42 °C for 1 h with a final step of 72 °C for 10 min to stop the reaction.

5. Molecular techniques for yeast identification and/or quantification

5.8S-ITS-RFLP analysis

5.8S-ITS-RFLP was used for yeast species identification comprising amplification with primers ITS1-ITS4, digestion with restriction enzymes (*Hinf*I, *Hae*III, *Cfo*I, *Dde*I and *Mbo*I) and restriction profile analysis according to Esteve-Zarzoso et al. (1999) and Csoma and Sipiczki (2008). The main reaction parameters were listed as follows.

(1) Primers:

ITS1 5'-TCCGTACGTGAACCTGCGG-3'

ITS4 5'-TCCTCCGCTTATTGATATGC-3'

(2) PCR mixture (50 μL):

1 μL of 10 μM primer ITS1, 1 μL of 10 μM primer ITS4, 1 μL of dNTP mix (10 mM each dNTP), 3 μL of 50 mM $MgCl_2$, 5 μL of 10× buffer without Mg^{2+}, 2.5 U of Taq DNA polymerase, 5 μL of DNA, sterile Milli-Q water until 50 μL.

(3) PCR programs:

95 °C 5 min; 40 cycles of 95 °C 30 s, 52 °C 1 min and 72 °C 1 min; and a final extension of 72 °C 10 min.

(4) 1.5 % agarose gel electrophoresis to obtain profile of PCR products.

(5) Digestion at 37 °C overnight with 20 μL of mixture:

1 μL of enzyme, 2 μL of buffer, 7 μL of sterile Milli-Q water, 10 μL of PCR products.

(6) 3 % agarose gel electrophoresis to obtain restriction profiles with comparison to 100 bp of molecular weight marker (Invitrogen, USA).

26S rDNA D1/D2 domain sequencing analysis

26S rDNA D1/D2 domain sequencing analysis was used for yeast species identification comprising amplification with primers NL1-NL4 (Kurtzman and Robnett 1998), sequencing by ABI3730 XL DNA sequencer (Macrogen, Korea) and performing BLAST analysis using sequence alignment against the NCBI database (http://blast.ncbi.nlm.nih.gov/). The main reaction parameters were listed as follows.

(1) Primers:

NL1 5'-GCATATCAATAAGCGGAGGAAAAG -3'

NL4 5'-GGTCCGTGTTTCAAGACGG-3'

(2) PCR mixture (50 µL):

1 µL of 10 µM primer NL1, 1 µL of 10 µM primer NL4, 1 µL of dNTP mix (10 mM each dNTP), 1.5 µL of 50 mM $MgCl_2$, 5 µL of 10× buffer without Mg^{2+}, 1,25 U of Taq DNA polymerase, 1 µL of DNA, sterile Milli-Q water until 50 µL.

(3) PCR programs:

95°C 5min; 36 cycles of 94 °C 1 min, 52 °C 2 min and 72 °C 2 min; and a final extension of 72 °C 5 min.

(4) 1.5 % agarose gel electrophoresis to verify profile of PCR products and then PCR products is sent to Macrogen (Korea) for purification and sequencing as we mentioned above.

DGGE

DGGE was used for yeast species diversity analysis. It comprises amplification with primers $U1^{GC}$-U2 (Andorrà et al. 2008) or $NL1^{GC}$-LS2 (Cocolin et al. 2000), band separation by a polyacrylamide gel with a linear gradient of DNA denaturants, and then sequencing of the different bands eluted by ABI3730 XL DNA sequencer (Macrogen, Korea) and sequence alignment by BLAST from the NCBI database (http://blast.ncbi.nlm.nih.gov/). The complete procedures followed the description in Andorrà et al. (2008) and main parameters were listed as follows.

(1) Primers:

$U1^{GC}$ 5’-GCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCGTGAAATTGTTGAAAGGGAA-3’

U2 5’-GACTCCTTGGTCCGTGTT-3’

$NL1^{GC}$ 5’-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCCATATCAATAAGCGGAGGAAAAG-3’

LS2 5’-ATTCCCAAACAACTCGACTC-3’

(2) PCR mixture (50 µL):

1.5 µL of 5 µM primer $U1^{GC}$, 1.5 µL of 5 µM primer U2, 0.1 µL of dNTP mix (10 mM each dNTP), 1 µL of 50 mM $MgCl_2$, 5 µL of 10× buffer without Mg^{2+}, 1,25 U of Taq DNA polymerase, 1 µL of DNA, sterile Milli-Q water until 50 µL.

1 µL of 10 µM primer $NL1^{GC}$, 1 µL of 10 µM primer LS2, 1 µL of dNTP mix (10 mM each dNTP), 4 µL of 25 mM $MgCl_2$, 5 µL of 10× buffer without Mg^{2+}, 1,25 U of Taq DNA polymerase, 2 µL of DNA, sterile Milli-Q water until 50 µL.

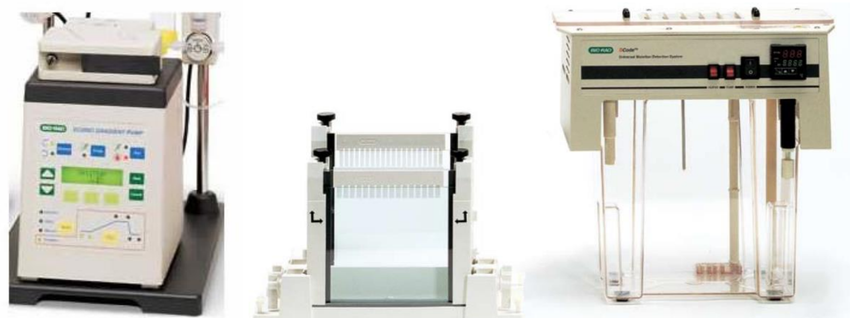
(3) PCR programs:

U1^{GC}-U2: 94°C 4min; 35 cycles of 94 °C 30 s, 57 °C 1 min and 72 °C 1 min; and a final extension of 72 °C 7 min.

NL1^{GC}-LS2: 95 °C 5 min; 35 cycles of 95 °C 1 min, 52 °C 45 s and 72 °C 1 min; and a final extension of 72 °C 7 min.

(4) The polyacrylamide gel with gradient from 35% to 55% was built By Econo Gradient Pump (Bio-Rad, USA) using solution A (0% denaturing solution) and B (100% denaturing solution). 20 mL of both solutions was added 20 µL TEMED (N, N, N, N'-tetra-methyl-ethylenediamine) and 200 µL ammonium persulphate (0.1 g/mL) and then used for building the gel with pump speed of 10 mL/min and pump programme of T 0 s 55 % B, T 30 s 55 % B, T 3 min 35 % B and T 3.5 min 35 % B. After polymerization for 1 h, samples (20 µL DNA and 10 µL gel loading buffer) were loaded to the gel and electrophoresis was run at 60 °C in 1× TAE buffer for 4 h with a constant 170 V in DCode universal mutation detection system (Bio-Rad, USA). Then the gel was strained with ethidium bromide solution (1 µg/mL) or Sybr Green (25 µL in 250 µL 1×TAE buffer) for 15 min. The visualization was realized by MiniBis Pro (DNR Bio-Imaging System Ltd., Israel) and target band was excised and kept in 50 µL of sterile water overnight for 4 °C. Re-amplification of 1 µL eluted DNA was done with same primers but without GC clamp and identified by sequencing analysis.

- 0% denaturing solution: 10 mL 40% Acrylamide/BisAcrylamide, 1 mL 50× TAE, distilled water until 50 mL.
- 100% denaturing solution: 21 g urea, 20 mL formamide, 10 mL 40% Acrylamide/BisAcrylamide, 1 mL 50× TAE, distilled water until 50 mL.
- Gel loading buffer: 2.5 mL dye solution (0.05 g bromophenol blue, 0.05 g xylene cyanol, 10 mL 1× TAE buffer), 7 mL glycerol.
- 50× TAE buffer: 242 g/L trizma base, 57.1 g/L acetic acid glacial, 100 mL/L 0.5 M EDTA (186.12 g/L at pH 8.0).



Massive sequencing

Massive sequencing was used for fungi identification comprising amplification with modified NL1-NL4 primers, sequencing by a 454 Roche platform with the Genome Sequencing FLX System and sequence alignment using database of 26S rDNA sequences obtained from GenBank of NCBI. The whole process was conducted by LifeSequencing S. L. (Spain) with main parameters listed as follows.

(1) DNA extraction was conducted as indicated in 4.

(2) Primers:

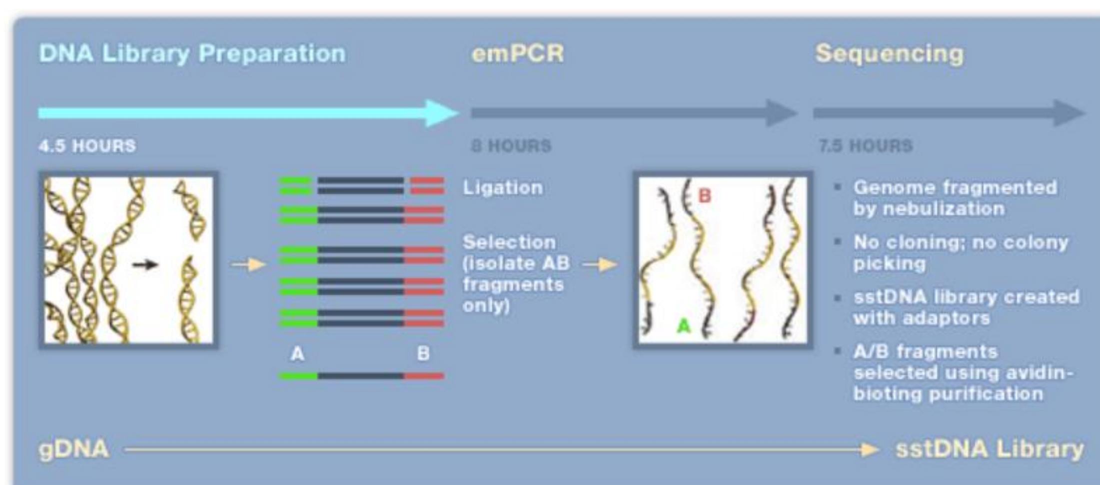
Modified NL1:

5'-forward adaptor-MIDs for each sample-GCATATCAATAAGCGGAGGAAAAG -3'

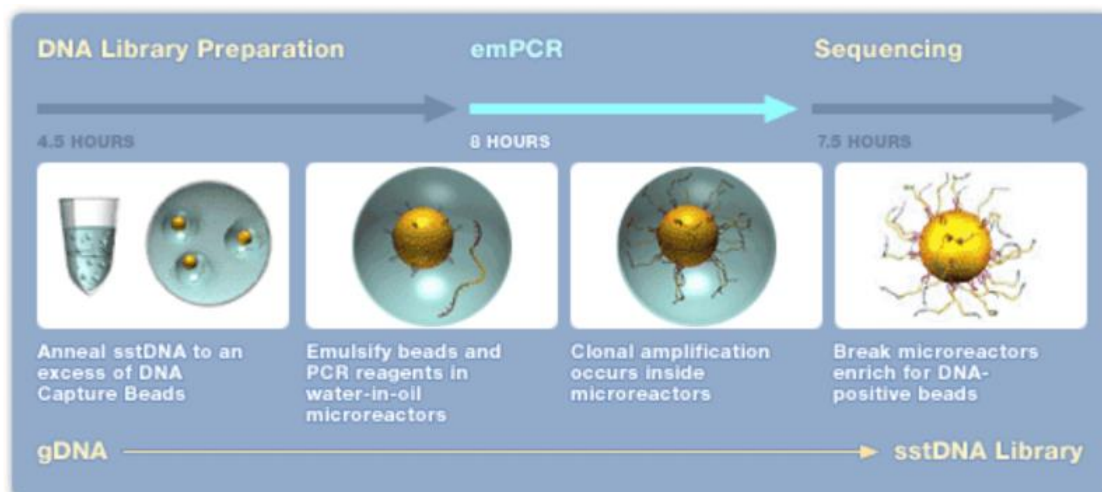
Modified NL4: 5'-reverse adaptor-GGTCCGTGTTTCAAGACGG-3'

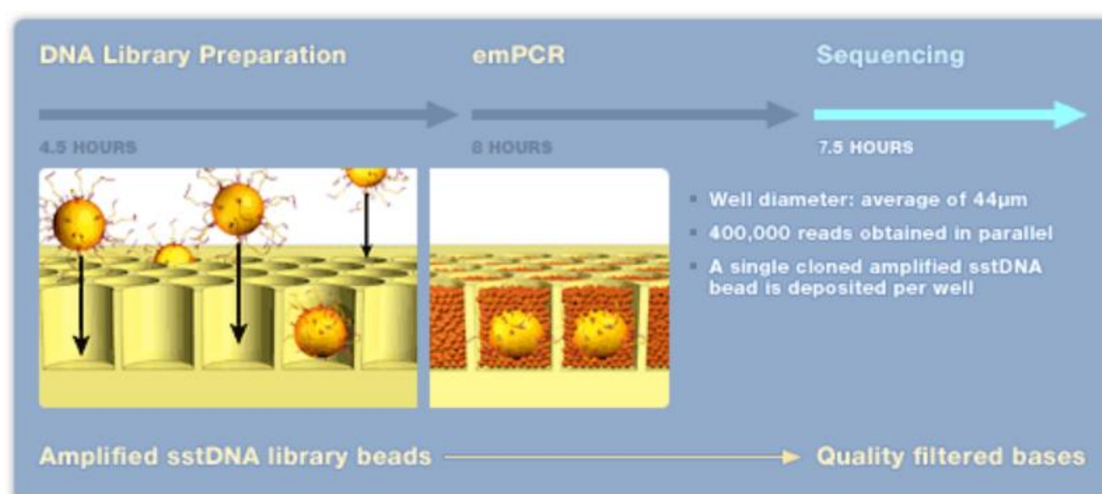
(3) DNA libraries were built for each sample by PCR using the improved primers, cleaned by primer-dimmer removal protocol and sequenced by a 454 FLX Roche sequencer. Subsequently, sequences with low quality or length lower than 300 nt were removed, and sequences with high quality were aligned with a confidence cutoff value of 80 % and an e-value of 10^{-5}.

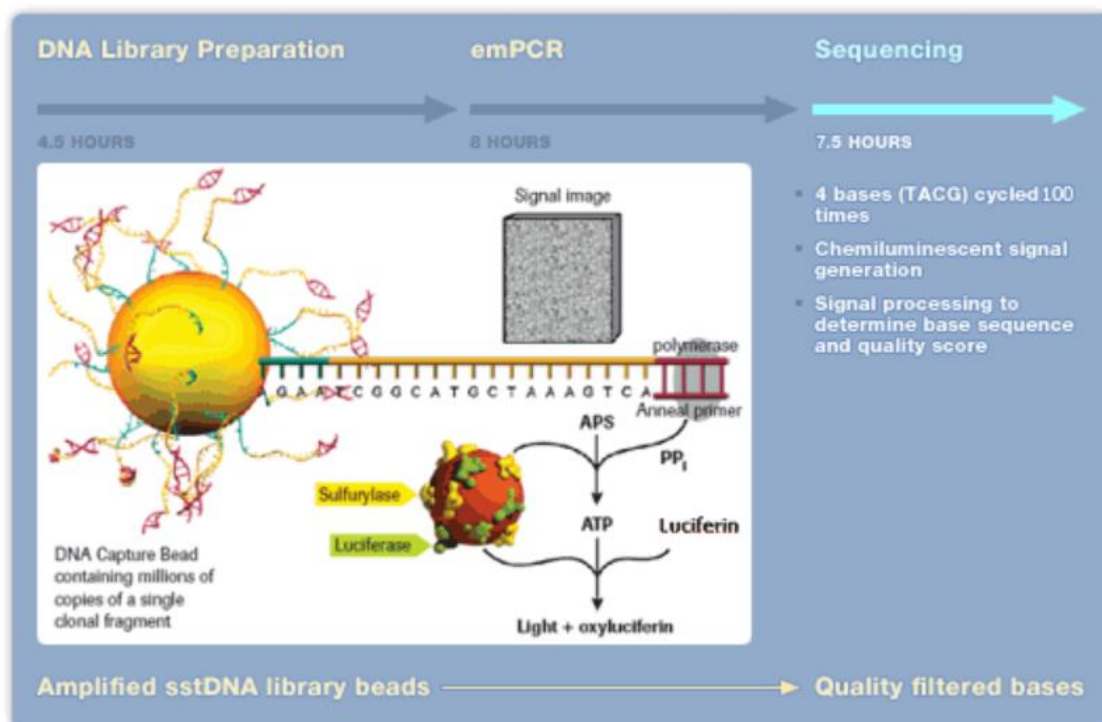
Estimate S v9.1.0 (Colwell, 2013) was used to further analyze biodiversity in single sample (Shannon diversity, Simpson diversity and estimated species richness) and similarity between two samples (Jaccard Classic and Bray-Curtis).



Annex I







qPCR

qPCR was used for yeast quantification comprising establishment of standard curves and analysis of unknown samples. Standard curves were built between Ct value and a series of cell concentration known (10^7, 10^6, 10^5, 10^4, 10^3, 10^2 cells/mL). Then Ct value obtained from unknown samples was calculated into cell concentration based on well-built standard curves.

(1) Universal primers for yeast (Hierro et al. 2006):

YEAST-F 5'-GAGTCGAGTTGTTTGGGAATGC-3'

YEAST-R 5'-TCTCTTTTCCAAAGTTCTTTTCATCTTT-3'

Primers for *Saccharomyces* (Hierro et al. 2007):

CESP-F 5'-ATCGAATTTTTGAACGCACATTG-3'

SCER-R 5'-CGCAGAGAAACCTCTCTTTGGA-3'

Primers for *Hanseniaspora* (Hierro et al. 2007):

CESP-F 5'-ATCGAATTTTTGAACGCACATTG-3'

HUV-R 5'-AACCCTGAGTATCGCCCACA-3'

Primers for *Starm. bacillaris* (Andorrà et al. 2010b):

A-F 5'-CTAGCATTGACCTCATATAGG-3'

200-R 5'-GCATTCCCAAACAACTCGACTC-3'

(2) PCR mixture (25 μL):

0.75 μL of 7 μM forward primer, 0.75 μL of 7 μM reverse primer, 12.5 μL of Power SybrGreen Master Mix (Applied Biosystems, USA), 5 μL of DNA, 6 μL sterile Milli-Q water.

(3) PCR programs:

95 °C 10 min; 40 cycles of 95 °C 15 s, 60 °C 1 min and 72 °C 30 s.

Commercial kit for viability test

A commercial kit (LIVE/DEAD BactLight™ Bacterial Viablility Kit, Molecular Probes Inc, USA) was used to test cell viability. 1.5 µL 3.34 mM SYTO 9 dye and 0.7 µL 20 mM propidium iodide (PI) was added to cell suspension in sterile Milli-Q water (cell concentration around 10^6 cells/mL) after a wash step, which was used to remove the interference from sample background (media or wine). The observation was performed by fluorescence microscope (Leica DM 4000B, Germany) with filter system I3 (for cells stained with SYTO 9, green fluorescence) and N2.1 (for cells stained with PI, red fluorescence).

FISH

FISH was used to yeast identification and quantification comprising fixation by which cell morphology was stabilized and cell membrane was permeabilized, hybridization targeting intracellular rRNA by fluorescein isothiocyanate (FITC) labeled probes, washing away the excess probe and final analysis by fluorescence microscopy and flow cytometry. The main procedure was based on Andorrà et al. (2011) with small adjustments.

(1) Probe for *S. cerevisiae* (Xufre et al. 2006):

Sce-FITC 5'-FITC-TGACTTACGTCGCAGTCC-3'

Probe for eight species of *Hanseniaspora* (*H. clermontiae*, *H. guilliermondii*, *H. lachancei*, *H. meyeri*, *H. opuntiae*, *H. pseudoguilliermondii*, *H. uvarum*, *H. valbyensis*, Wang et al. 2014):

H8b-FITC 5'-FITC-AGGTAATCCCAGTTGGTT-3'

H8b-com 5'-AGGCAATCCCGGTTGGTT-3' (H8b-com is an oligonucleotide competitor used together with probe H8b-FITC to increase the specificity).

Probe for *Starm. bacillaris* (Wang et al. 2014):

Sba-FITC 5'-FITC-CTCCATGGCGCTCCTTTC-3'

Probe design: Consensus sequences of related species were built by alignment by Clustal Omega (EMBL-EBI, http://www.ebi.ac.uk/Tools/msa/clustalo/), and then species-specific sequences were selected by comparison between consensus sequences and sequence of target species. Evaluation of these sequences was performed based on the accessibility map of *S. cerevisiae* (Inácio et al., 2003), general and mismatch analyses with mathFISH (Yilmaz et al., 2011, http://mathfish.cee.wisc.edu/index.html).

(2) **Fixation**: Collected cells were suspended in 100 µL 1× phosphate-buffered saline (PBS) and 300 µL 4% (v/v) of paraformaldehyde and kept at 4ºC for 1 h with 1000 rpm agitation in a shaker. Fixed cells could be stored at −20°C if we centrifuged to collect cells and re-suspended cells with a 1:1 solution of 1× PBS and absolute ethanol. **Hybridization**: Approximately 10^6 fixed cells were collected by centrifugation and hybridized in a 50 µL solution containing 40 µL fresh hybridization buffer and 10 µL probes (final concentration for each probe was 10 ng/µL in the solution). The hybridization was kept at 46°C for 3 h in the dark. **Washing**: Cells were collected by centrifugation, re-suspended in 100 µL fresh washing buffer and incubated at 48°C for 30 min in the dark. **Cell suspension preparation**: Cells were collected and re-suspended in 100 µL 1× PBS, and analyzed immediately.

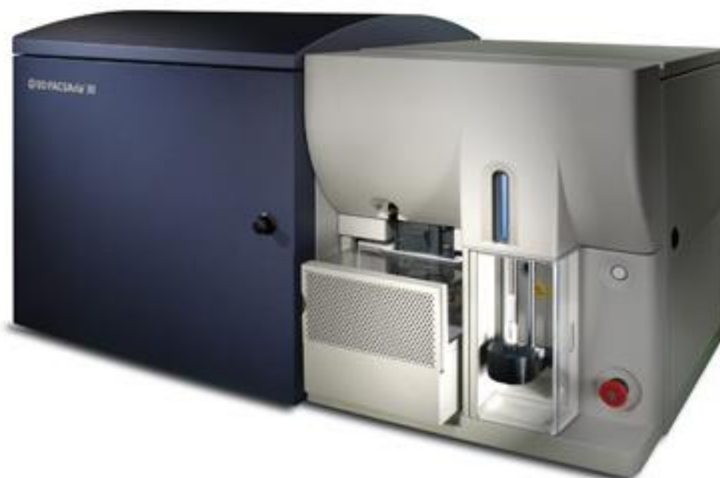
- 10× PBS (g/L): Na_2HPO_4 14.24, KH_2PO_4 2.04 g, NaCl 80.3, KCl 2.01, adjust the pH to 7.2, 0.2 µm filtration.
- 4 % Paraformaldehyde (200 mL): paraformaldehyde 8 g, Milli-Q water 190 mL, 2 drops of 1 M NaOH (the solution become transparent), 10 mL 10× PBS, 0.2 µm filtration, -20 °C storage.
- Hybridization buffer (1 mL): 900 µL 1M NaCl, 1 µL 10% (w/v) SDS, 20 µL 1M Tris-HCl (pH 8.0), 10 µL v/v formamide, 69 µL Milli-Q water, 0.2 µm filtration.
- Washing buffer (1 mL): 25 µL 1M Tris-HCl (pH 8.0), 500 µL 1M NaCl, 475 µL Milli-Q water, 0.2 µm filtration.

(3) Visualization by fluorescence microscopy

Epifluorescence microscope (Leica DM4000B, Wetzlar, Germany) equipped with filter I3 was used for cell observation and Neubauer chamber was used for yeast enumeration.

(4) Analysis by flow cytometry

BD FACSAria III flow cytometer (BD Biosciences, California, USA) equipped with a 15 mW, 488 nm argon-ion laser was used. Fluorescence was detected using a 530 (±30) nm band pass filter. At least 10,000 events were acquired and recorded in the linear mode for side scatter and in the log mode for fluorescent signals. The data were collected and analyzed using the FACSDiva software (BD Biosciences, California, USA).



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Annex II

Publications

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ORIGINAL RESEARCH
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Fungal diversity in grape must and wine fermentation assessed by massive sequencing, quantitative PCR and DGGE

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The diversity of fungi in grape must and during wine fermentation was investigated in this study by culture-dependent and culture-independent techniques. Carignan and Grenache grapes were harvested from three vineyards in the Priorat region (Spain) in 2012, and nine samples were selected from the grape must after crushing and during wine fermentation. From culture-dependent techniques, 362 isolates were randomly selected and identified by 5.8S-ITS-RFLP and 26S-D1/D2 sequencing. Meanwhile, genomic DNA was extracted directly from the nine samples and analyzed by qPCR, DGGE and massive sequencing. The results indicated that grape must after crushing harbored a high species richness of fungi with *Aspergillus tubingensis*, *Aureobasidium pullulans,* or *Starmerella bacillaris* as the dominant species. As fermentation proceeded, the species richness decreased, and yeasts such as *Hanseniaspora uvarum*, *Starmerella bacillaris* and *Saccharomyces cerevisiae* successively occupied the must samples. The "*terroir*" characteristics of the fungus population are more related to the location of the vineyard than to grape variety. Sulfur dioxide treatment caused a low effect on yeast diversity by similarity analysis. Because of the existence of large population of fungi on grape berries, massive sequencing was more appropriate to understand the fungal community in grape must after crushing than the other techniques used in this study. Suitable target sequences and databases were necessary for accurate evaluation of the community and the identification of species by the 454 pyrosequencing of amplicons.

Keywords: culture-independent techniques, pyrosequencing, SO_2 treatment, community diversity and composition, wine yeast

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INTRODUCTION

Investigating the fungal community in grape must and wine fermentation is relevant for understanding its relationship with the grape sanitary status and the final wine characteristics (Bokulich et al., 2014). Recently, the development of next-generation sequencing provided a useful tool for the description of prokaryotic and eukaryotic microbial communities that exist in grape leaves, berries, must and wineries (Bokulich et al., 2013, 2014; David et al., 2014; Pinto et al., 2014; Taylor et al., 2014; Valera et al., 2015). The common approach used in these studies was targeted metasequencing: generic target sequences were amplified by PCR to establish a library; then amplicons were sequenced; and identification was performed by comparison with known sequences in databases (Huggett et al., 2013; Mayo et al., 2014).

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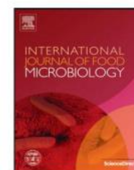

Monitoring of *Saccharomyces cerevisiae*, *Hanseniaspora uvarum*, and *Starmerella bacillaris* (synonym *Candida zemplinina*) populations during alcoholic fermentation by fluorescence *in situ* hybridization

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ABSTRACT

Various molecular approaches have been applied as culture-independent techniques to monitor wine fermentations over the last decade. Among them, those based on RNA detection have been widely used for yeast cell detection, assuming that RNA only exists in live cells. Fluorescence *in situ* hybridization (FISH) targeting intracellular rRNA is considered a promising technique for the investigation of wine ecology. For the present study, we applied the FISH technique in combination with epifluorescence microscopy and flow cytometry to directly quantify populations of *Saccharomyces cerevisiae*, *Hanseniaspora uvarum*, and *Starmerella bacillaris* during alcoholic fermentations. A new specific probe that hybridizes with eight species of *Hanseniaspora* genus and a second probe specific for *Starm. bacillaris* were designed, and the conditions for their application to pure cultures, mixed cultures, and wine samples were optimized. Single and mixed fermentations were performed with natural, concentrated must at two different temperatures, 15 °C and 25 °C. The population dynamics revealed that the *Sacch. cerevisiae* population increased to 10^7–10^8 cells/ml during all fermentations, whereas *H. uvarum* and *Starm. bacillaris* tended to increase in single fermentations but remained at levels similar to their inoculations at 10^6 cells/ml in mixed fermentations. Temperature mainly affected the fermentation duration (slower at the lower temperature) but did not affect the population sizes of the different species. The use of these probes in natural wine fermentations has been validated.

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1. Introduction

Wine fermentation is an ecologically complex process, and it is widely accepted that the yeast population changes as the fermentation proceeds (Fleet, 2008; Wang and Liu, 2013). Yeast species play important roles in the wine-making processes: transforming sugar to ethanol, producing specific secondary metabolites, and finally, contributing to wine flavor characteristics. The main yeast involved in this transformation process belongs to the *Saccharomyces* (*Sacch.*) genus, but other wine yeasts can also be isolated during the process, producing varying impacts on the wine composition (Andorrà et al., 2012; Fleet, 2003). Most of the knowledge generated concerning wine yeast ecology has been derived from plating, which has incorporated molecular analysis in the past 25 years (Ribéreau-Gayon et al., 2006). However, in the past 15 years, culture-independent techniques have provided a new view of microbial ecology during the wine production process.

The culture-independent molecular techniques employed to study the ecology of wine yeasts have mainly been based on the detection of DNA in wine samples (Andorrà et al., 2010a; Cocolin et al., 2000; Hierro et al., 2006). However, it is possible to detect DNA from dead cells when using these techniques (Andorrà et al., 2010b; Hierro et al., 2006). Furthermore, the presence of viable but non-culturable (VBNC) or injured cells in wine (Divol and Lonvaud-Funel, 2005; Millet and Lonvaud-Funel, 2000) introduces a new bias into the analysis of the wine yeast ecosystem. More recently, a small modification of the same culture-independent methods, QPCR coupled with ethidium monoazide (EMA)/PMA (Andorrà et al., 2010b; Rawsthorne and Phister, 2009), has been proposed to reveal the actual viable yeast population structure and resolve the differences observed between plate counting and molecular results. Other methodologies based on RNA detection, such as RT-PCR-DGGE (Mills et al., 2002) and RT-QPCR (Hierro et al., 2006), have also been proposed. A main hurdle of these techniques is that all of these methods rely on a high-quality DNA/RNA extraction and PCR/QPCR operation to provide accurate results. Fluorescence *in situ* hybridization (FISH) probes directly hybridize with intracellular ribosomal RNA at specific sites, and the target cells with fluorescent signals can be easily observed and recorded by epifluorescence microscopy and flow cytometry (Amann and Fuchs, 2008). In addition to its simplicity and rapidity, the ability to observe the cell morphology by a microscope and the high sensitivity obtained using a flow cytometer (ten fluorescent cells can be detected among ten million non-fluorescent cells, as the flow cytometer

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Viable and culturable populations of *Saccharomyces cerevisiae*, *Hanseniaspora uvarum* and *Starmerella bacillaris* (synonym *Candida zemplinina*) during Barbera must fermentation

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A B S T R A C T

The present study analyzed the viable and/or culturable populations of *Saccharomyces cerevisiae*, *Hanseniaspora uvarum* and *Starmerella bacillaris* (synonym *Candida zemplinina*) during laboratory grape must fermentation, in order to investigate the interaction between the three species considered. Firstly, population dynamics during wine fermentation were followed by culture-dependent techniques, and non-*Saccharomyces* yeast became non-culturable at late stages of fermentation when *S. cerevisiae* dominated. Four different culture-independent techniques were further applied to detect viable yeast cells at the late stage of fermentation. Both quantitative PCR techniques applied, namely ethidium monoazide bromide (EMA)-qPCR and Reverse Transcription (RT)-qPCR, detected *H. uvarum* and *Starm. bacillaris* at a concentration of 10^5 to 10^6 cells/mL. These non-culturable cells had membranes impermeable to EMA and stable rRNA. The background signals from dead cells did not interfere with the quantification of viable cells in wine samples by EMA-qPCR technique. As a qualitative culture-independent technique, DGGE technique was coupled with EMA treatment (EMA-PCR-DGGE) or with RT (RT-PCR-DGGE). With EMA-PCR-DGGE non-*Saccharomyces* species during fermentation were detected although it was limited by the predominance of *S. cerevisiae*.

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1. Introduction

Wine fermentations are complex ecological processes with a succession of variable yeast species that could provide different characteristics to the fermentation and final wine (Fleet, 2003). Considerable efforts have been devoted to investigate and monitor population dynamics during mixed fermentations with *Saccharomyces* and non-*Saccharomyces* species. According to culture-dependent analysis, the non-*Saccharomyces* species are only isolated from early stages and the fermentations are dominated by *Saccharomyces* at late stages (Fleet, 2008). Recently, using culture-independent techniques, it was highlighted that viable non-*Saccharomyces* populations could be quantified at late stages of fermentation (Andorrà, Landi, Mas, Guillamón, & Esteve-Zarzoso, 2008; Wang, Esteve-Zarzoso, & Mas, 2014) supporting their possible role also at the end of the transformation process. For these reasons, a thorough study is required to understand the states (culturable, live, injured or dead) of non-*Saccharomyces* during fermentation.

The application of culture-independent techniques in wine ecology studies is considered a valid approach to investigate the presence of viable but non-culturable (VBNC) cells (Cocolin, Alessandria, Dolci, Gorra, & Rantsiou, 2013). For the quantitative techniques, qPCR was firstly used to directly quantify yeast DNA from wine samples (Hierro, Esteve-Zarzoso, Mas, & Guillamón, 2007; Tofalo, Schirone, Corsetti, & Suzzi, 2012), but because of the stability of DNA, dead cells were also quantified resulting in an overestimation of yeast populations. To solve this problem, RT-qPCR, fluorescence in situ hybridization (FISH) and qPCR using ethidium monoazide bromide treatment or propidium monoazide bromide (EMA-qPCR or PMA-qPCR) were explored (Andorrà, Esteve-Zarzoso, Guillamón, & Mas, 2010; Hierro, Esteve-Zarzoso, González, Mas, & Guillamón, 2006; Rawsthorne & Phister, 2009; Shi et al., 2012; Vendrame, Manzano, Comi, Bertrand, & Iacumin, 2014; Wang et al., 2014). In RT-qPCR and FISH the cells with rRNA are considered viable, because the rRNA is less stable than DNA and, thus, is not quantified in dead cells (Andorrà, Monteiro, Esteve-Zarzoso, Albergaria, & Mas, 2011; Hierro et al., 2006; Wang et al., 2014). EMA-qPCR excludes cells with compromised membranes; EMA enters these cells and covalently combines with DNA which is not amplified by subsequent PCR reactions (Rudi, Moen, Drømtorp, & Holk, 2005). All of these quantitative techniques require specific primers, which increase the sensitivity for detection, avoiding the detection of non-targeted yeast species. As qualitative techniques, PCR-DGGE and RT-PCR-DGGE have been developed with universal primers to detect all probable yeast species without the need to know their sequences (Cocolin, Bisson, & Mills, 2000; Mills, Johannsen, & Cocolin, 2002). However, detection sensitivity of DGGE depended on the disparity of orders

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Interaction between *Hanseniaspora uvarum* and *Saccharomyces cerevisiae* during alcoholic fermentation

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ABSTRACT

During wine fermentation, *Saccharomyces* clearly dominate over non-*Saccharomyces* wine yeasts, and several factors could be related to this dominance. However, the main factor causing the reduction of cultivable non-*Saccharomyces* populations has not yet been fully established. In the present study, various single and mixed fermentations were performed to evaluate some of the factors likely responsible for the interaction between *Saccharomyces cerevisiae* and *Hanseniaspora uvarum*. Alcoholic fermentation was performed in compartmented experimental set ups with ratios of 1:1 and 1:9 and the cultivable population of both species was followed. The cultivable *H. uvarum* population decreased sharply at late stages when *S. cerevisiae* was present in the other compartment, similarly to alcoholic fermentations in non-compartmented vessels. Thus, cell-to-cell contact did not seem to be the main cause for the lack of cultivability of *H. uvarum*. Other compounds related to fermentation performance (such as sugar and ethanol) and/or certain metabolites secreted by *S. cerevisiae* could be related to the sharp decrease in *H. uvarum* cultivability. When these factors were analyzed, it was confirmed that metabolites from *S. cerevisiae* induced lack of cultivability in *H. uvarum*, however ethanol and other possible compounds did not seem to induce this effect but played some role during the process. This study contributes to a new understanding of the lack of cultivability of *H. uvarum* populations during the late stages of wine fermentation.

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1. Introduction

Wine fermentation is an ecologically complex process that involves diverse yeast species at different stages. These yeast species interact with each other and show a population succession, which is mostly characterized by large populations of non-*Saccharomyces* species at early stages and the dominance of *Saccharomyces* at late stages (Fleet, 2008). Research efforts to monitor this population succession have provided many hypotheses for this phenomenon. This process will be understood by testing these hypotheses and identifying the factors responsible for the dominance relations between yeast species.

Until now, investigations of yeast interactions have primarily emphasized four aspects during wine fermentations. First is the nutrient level, with carbon and nitrogen as the main limiting factors (Andorrà et al., 2012). The lack of equilibrium in natural grape musts between carbon and nitrogen is well known, and it is a limiting factor. *Saccharomyces* consumes both of them faster than non-*Saccharomyces*, causing nutrient depletion at the beginning of fermentation (Monteiro and Bisson, 1991; Albergaria et al., 2003). Furthermore, *Saccharomyces* is more efficient at producing biomass than non-*Saccharomyces* under fermentation conditions, i.e., non-*Saccharomyces* need higher nutrient concentrations to produce the same biomass (Andorrà et al., 2012). Thus, nutrient concentrations are regarded as an important factor for the dominance of *Saccharomyces*. Certain fermentation factors such as oxygen and temperature are a second factor correlated to yeast interactions related to the Crabtree effect and heat production, which is described as a fitness advantage of *Saccharomyces* in the niche construction theory (Goddard, 2008; Salvadó et al., 2011). SO_2 was also regarded as a selective fermentation factor because of different sensitivities from different yeast species (Ribéreau-Gayon et al., 2006). Thirdly, yeast metabolites such as ethanol, medium-chain fatty acids and killer toxins have been considered as well (Thomson et al., 2005; Piškur et al., 2006; Albergaria et al., 2010). Ethanol and medium-chain fatty acids are known to decrease the growth rate and even cause growth arrest of non-*Saccharomyces* yeasts due to their toxicity and the relatively inefficient regulation caused by these metabolites (Fleet, 2003; Thomson et al., 2005; Piškur et al., 2006). Killer toxins secreted by *Saccharomyces cerevisiae* are found to be death inducing factors for non-*Saccharomyces*, such as enzymes with glucanase activity (Magliani et al., 1997), proteinaceous compounds (Pérez-Nevado et al., 2006), and antimicrobial peptides (AMPs) derived from fragments of *S. cerevisiae* glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein (Albergaria et al., 2010; Branco et al., 2014). Finally, some direct factors such as quorum sensing phenomena (Granchi et al., 1998; Smid and Lacroix, 2013) and cell-to-cell contact (Nissen et al., 2003; Arneborg et al., 2005) might also

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The Interaction between *Saccharomyces cerevisiae* and Non-*Saccharomyces* Yeast during Alcoholic Fermentation Is Species and Strain Specific

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The present study analyzes the lack of culturability of different non-*Saccharomyces* strains due to interaction with *Saccharomyces cerevisiae* during alcoholic fermentation. Interaction was followed in mixed fermentations with 1:1 inoculation of *S. cerevisiae* and ten non-*Saccharomyces* strains. *Starmerella bacillaris,* and *Torulaspora delbrueckii* indicated longer coexistence in mixed fermentations compared with *Hanseniaspora uvarum* and *Metschnikowia pulcherrima*. Strain differences in culturability and nutrient consumption (glucose, alanine, ammonium, arginine, or glutamine) were found within each species in mixed fermentation with *S. cerevisiae*. The interaction was further analyzed using cell-free supernatant from *S. cerevisiae* and synthetic media mimicking both single fermentations with *S. cerevisiae* and using mixed fermentations with the corresponding non-*Saccharomyces* species. Cell-free *S. cerevisiae* supernatants induced faster culturability loss than synthetic media corresponding to the same fermentation stage. This demonstrated that some metabolites produced by *S. cerevisiae* played the main role in the decreased culturability of the other non-*Saccharomyces* yeasts. However, changes in the concentrations of main metabolites had also an effect. Culturability differences were observed among species and strains in culture assays and thus showed distinct tolerance to *S. cerevisiae* metabolites and fermentation environment. Viability kit and recovery analyses on non-culturable cells verified the existence of viable but not-culturable status. These findings are discussed in the context of interaction between non-*Saccharomyces* and *S. cerevisiae*.

Keywords: contact-dependent interaction, culturability loss, excreted compounds, viable but not-culturable (VBNC), wine

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INTRODUCTION

Spontaneous wine fermentation is driven by a succession of different yeast species. A great variety of non-*Saccharomyces* yeast species originate from grape berries and survive during the early stages of fermentation, such as species from the genera *Candida, Hanseniaspora, Lachancea, Metschnikowia, Pichia,* and *Torulaspora* (Fleet, 2003). Some species such as *Starmerella bacillaris* and *Hanseniaspora uvarum* grow to a high density (10^5–10^7 cells/mL) and dominate other non-*Saccharomyces* species