INFLUENCE OF NON-SACCHAROMYCES YEAST ON WINEMAKING AND QUALITY

Jéssica Lleixà Daga

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Influence of non-*Saccharomyces* yeasts on winemaking and quality

JESSICA LLEIXÀ DAGA

DOCTORAL THESIS
2019

Jessica Lleixà Daga

Influence of non-*Saccharomyces* yeasts on winemaking and quality

DOCTORAL THESIS

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Universitat Rovira I Virgili

UNIVERSITAT ROVIRA i VIRGILI

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Departament de Bioquímica I Biotecnologia
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WE STATE that the present study, entitled "**Influence of non-*Saccharomyces* yeasts on winemaking and quality**", presented by **Jessica Lleixà Daga** for the award of the degree of Doctor, has been carried out under our supervision at the Department of Biochemistry and Biotechnology of this university.

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Tarragona, April 2019

Als meus pares, Juan José i Estrella

A les meves germanes, Patrícia i Meritxell

Al Joan

La gent que en circumstàncies normals ja és extraordinària, en casos extraordinaris aconsegueix el miracle que tot sembli normal.

Carles Capdevila

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OBJECTIVES AND OUTLINE

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF NON-SACCHAROMYCES YEAST ON WINEMAKING AND QUALITY
Jéssica Lleixà Daga

The present work in fulfilment of my PhD degree was performed at Rovira i Virgili University (URV) from 2015 to 2019. This work was developed in the Oenological Biotechnology group at the Department of Biochemistry and Biotechnology. During this period, I held a pre-doctoral fellowship from the Rovira i Virgili University (2016PVF-PIPF-8). Furthermore, in order to get the distinction of international program I conducted a research at the Australian Wine Research Institute (AWRI) in Adelaide (Australia) from March to July of 2017. The investigation performed as a visitor PhD student resulted in a scientific report included in the appendix of the manuscript.

The research carried out in this thesis is framed within two projects. The first one is the WILDWINE EU Project (EU contract 315065) and its main objective was to determine the microbial fingerprint of different Appellations of Origin (AOC) from different European wine regions. The final goal of the project was to develop original mixtures of indigenous yeast and lactic acid bacteria (LAB) to produce high quality wines taking advantage of the specific *terroir* of its region. The second project was METACONVIN (AGL2015-73273-JIN), entitled: "Application of metagenomics and metatranscriptomics to microbiological control of aging wines". The aim of this project was to control the microbial communities of aged wines from different regions using massive sequencing techniques. Taking all this together, a better understanding of the indigenous yeast and bacterial communities present in the different wine elaboration stages would be achieved using new technologies that provide new information and knowledge.

Within this framework, the thesis hypothesis was as follows: **Certain non-*Saccharomyces* yeasts can exert a positive effect on alcoholic fermentation and final wine quality depending on the available nutrients during fermentation and the interaction of these non-*Saccharomyces* yeasts with other microorganisms intrinsically present or inoculated in the must.**

In order to demonstrate this hypothesis, the general objective was to study the effect of biotic and abiotic factors on yeast and bacterial communities during alcoholic fermentation and its effect on the resulting wine. This general objective splits up in the following specific objectives:

OBJECTIVE 1: To assess the influence of grape health on non-*Saccharomyces* yeasts load and diversity during spontaneous fermentations.

The health state of grapes is critical for wine production and it causes great losses in winemakers from all over the world. The alteration of grape status alters both the chemical and microbiota composition affecting alcoholic fermentation and the final wine.

In this study, we followed the fermentation dynamics of Macabeo must from different health status: healthy (i) and affected by sour rot (ii) or *Botrytis* infection (iii). The aim of this work was to establish a relationship between the health state of the grape with specific changes on the grape microbiota using culture dependent and independent techniques.

This objective is described in Chapter 1: **Microbiome dynamics during spontaneous fermentations of sound grapes in comparison with sour rot and *Botrytis* infected grapes.** Results published in International Journal of Food Microbiology (2018) 281, 36-46.

OBJECTIVE 2: To study the nutrient competition between *Saccharomyces* and non-*Saccharomyces* yeasts during alcoholic fermentation.

As we have already mentioned, the use of non-*Saccharomyces* yeasts for wine production has gained interest. However, the use of mixed or sequential cultures of non-*Saccharomyces* and *Saccharomyces* yeasts can be not as good as we have expected depending on many factors. Some of the main elements that can affect the fermentation performance and yeast dynamics are the nutrients availability, specially nitrogen and sugar, or the inoculation time of *S. cerevisiae*.

To better understand the impact of these aspects in alcoholic fermentation, we carried out mixed fermentations of *Saccharomyces* and four non-*Saccharomyces* yeasts under different nitrogen and sugar conditions together with the sequential addition of *S. cerevisiae* at different time points. We finally suggested the most suitable inoculation option depending on the nutrients conditions.

This objective is described in Chapter 2: ***Saccharomyces* and non-*Saccharomyces* competition during microvinification under different sugar and nitrogen conditions.** Results published in Frontiers in Microbiology (2016) 7, 1959.

OBJECTIVE 3: To analyze the effect of non-*Saccharomyces* yeasts inoculation on the fermentation performances of grape musts and their resulting wines.

In recent years, we have observed a growing interest in the use of non-*Saccharomyces* yeasts as starter cultures for alcoholic fermentation. These yeasts have been described to dispose many

positive aptitudes that lead to more complex and distinctive wines. *Hanseniaspora vineae* is a non-*Saccharomyces* yeast of special interest mainly for its positive contribution to the aroma profile of wines.

In this study, we compared the fermentation dynamics and the final wines obtained from Macabeo and Merlot natural musts inoculated with *H. vineae* or *Saccharomyces cerevisiae*. The fermentations were performed in semi-industrial conditions and yeast population evolution was analyzed using either culture dependent and independent techniques. Finally, the volatile compounds of the resulting wines were determined and a sensorial analysis was also undergone by a specialized panel.

This objective is described in Chapter 3: **Comparison of fermentation and wines produced by inoculation of *Hanseniaspora vineae* and *Saccharomyces cerevisiae*.** Results published in Frontiers in Microbiology (2016) 7, 338.

OBJECTIVE 4: To characterize the nitrogen regulation mechanisms in the non-*Saccharomyces* yeast *Hanseniaspora vineae*.

H. vineae has become a promising non-*Saccharomyces* yeast for winemaking considering its positive contribution to the aroma profile of wines. However, as we have previously expounded, nutritional factors like nitrogen content can negatively alter the fermentation performance and the establishment of the inoculated yeast. The main driver of alcoholic fermentation, *S. cerevisiae*, displays a nitrogen regulation mechanism called Nitrogen Catabolite Repression (NCR) to select the best nitrogen source available in the environment for growth. This mechanism is thoroughly described in this species. However, little is known in non-*Saccharomyces* yeasts entailing a big field to explore which can become of great interest to improve mixed and sequential fermentations approaches.

In this work, we evaluated the presence of the nitrogen catabolite repression (NCR) mechanism in *H. vineae*. To achieve this purpose we did laboratory-scale fermentations using synthetic must with a specific nitrogen concentration. Finally, we monitored both the expression of selected ortholog genes regulated under NCR mechanism and the amino acid and ammonium consumption along the fermentation.

This objective is described in Chapter 4: **Analysis of the NCR mechanisms in *Hanseniaspora vineae* and *Saccharomyces cerevisiae* during winemaking.** Results published in Frontiers in Genetics (2019) 9, 747.

OBJECTIVE 5: To decipher the genetic and phenotypic diversity of the spoiler yeast *Brettanomyces bruxellensis.*

Brettanomyces bruxellensis is a non-*Saccharomyces* yeast considered as the main responsible of aging wines spoilage. Its versatility to develop in hostile environments and its capability to produce volatile phenols emphasize the threat that supposes for winemakers. This yeast also displays a great genetic and phenotypic diversity regarding to volatile phenols production and sulphite tolerance, among others.

Taking into account these aspects, we aimed to determine the genetic and phenotypic diversity of *B. bruxellensis* strains isolated from different Catalan wine regions. The genetic variability of the isolates was studied using the intron splice sites (ISS-PCR) technique. After the genetic clustering of the strains, selected isolates were evaluated for sulphite tolerance and volatile phenols production.

This objective is described in Chapter 5: **Genetic and phenotypic diversity of *Brettanomyces bruxellensis* strains from wine regions of Catalonia.** Results submitted to International Journal of Food Microbiology.

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INTRODUCTION

1. From grape to wine: microorganisms at the different winemaking stages

1.1. Wine production

The elaboration of fermented beverages is one of the most ancient practices of human history. In this sense, wine is one fermented beverage that has played a major role in human civilization. The first evidence of wine production dates back to 2000 years before present in Ancient Persia (Jagtap et al., 2017; Pretorius, 2000) and it could be considered as the origin of biotechnology. However, we have to move until 1863, after 200 years of the microscopic observation of yeasts by Antonie van Leeuwenhoek, when Louis Pasteur recognized yeasts as the responsible of wine fermentation (Barnett, 2000).

The winemaking process consists in different steps that can vary depending on the type of wine desired, for example red or white wine (Figure 1). Despite the differences on the elaboration, the alcoholic fermentation is an essential process to produce any type of wine.

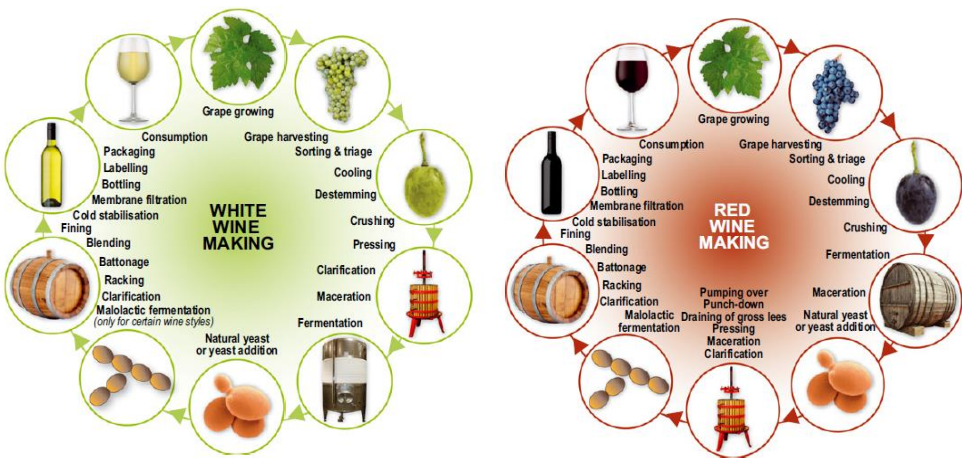


Figure 1. The main steps in white and red wine production (Jolly et al., 2014).

By and large, alcoholic fermentation consists on the biotransformation of the sugars present in grapes (mainly glucose and fructose) into alcohol and carbon dioxide (Ribéreau-Gayon et al., 2006). In addition to sugar, grapes contain other elements like amino acids, polyphenols or acids. Some of these compounds are also metabolized by yeasts into volatile metabolites which have an impact on the aroma and flavor of wine (Pretorius, 2016; Swiegers et al., 2005).

Saccharomyces cerevisiae has been universally considered as the *wine yeast*. This yeast species has taken this consideration due to its ability to prevail along the winemaking process despite the

hard conditions of the environment (low pH, high osmotic pressure, high ethanol concentration, etc.). Other than that, *S. cerevisiae* is one of the best fermentation performers since it transforms rapidly and efficiently the sugars present in the must. However, this is not the only yeast species we find during winemaking process. Other yeast species share their habitat with *S. cerevisiae* in some points of the wine production. This group of yeasts are commonly known as non-*Saccharomyces* yeasts (Fleet, 2008).

Moreover, other organisms like bacteria and fungi are also cohabitants with yeasts on grape berry surfaces or in winery environments. All these microbial communities interact and evolve during winemaking process yielding a complex succession of microorganisms. Depending on various conditions, the population dynamic would progress in different ways resulting in high quality and complex wines, uniform wines or spoiled wines. Throughout this introduction, we will cover the normal population's progress during the different stages of winemaking, the different techniques to study this population dynamic, and also which factors can affect the development of specific microorganisms and the fermentation performance.

1.2. Grape berry

One of the first steps in wine production begins with grape harvesting. The berry soundness and the ripening state at harvest will have a great impact on the microbial communities present on the grape surface and, therefore, on the correct evolution of the alcoholic fermentation.

Initially, the grape surface is inhabited by a community of aerobic fungi, yeasts and bacteria. However, this aerobic community will be displaced by fermentative organisms as the fruit matures and the sugar content increases (Bisson and Walker, 2015). Overall, the microorganisms dwelling the berries are mostly species from the soil that have colonized the different plant tissues through the roots (Zarraonaindia et al., 2015). This is not the only way to reach the grape surface, microorganisms can also come from the vineyard, from the agricultural tools used or they can even be spread by the air and by insect vectors or birds (Barata et al., 2012c; Loureiro and Malfeito-Ferreira, 2003; Madden et al., 2017).

Regarding to the population size of the main microbial groups of oenological interest in the grape berry, yeasts are the predominant organisms ranging populations of 10^4 – 10^6 cfu/g when grapes are mature (Fleet, 2003). On the other hand, acetic acid bacteria (AAB) and lactic acid bacteria (LAB) reach populations up to 10^2 – 10^3 cells/g (Barbe et al., 2001) and 10^3 cfu/g (Bae et

al., 2006), respectively in sound grapes. However, the damage of the berry is associated with an increase load of yeasts and AAB together with the alteration of the species diversity (Barata et al., 2012a).

The ecology of the grape berry has been studied by many researchers worldwide showing similar results which demonstrate the ubiquitous nature of the microbial species colonizing the grape berry and their strong relationship with the vineyard environment (Table 1). Taking into account all this information, Barata et al. (2012b) proposed to classify the yeast grape microbiota in three groups depending on their behaviour on grapes. Therefore, the classification is the following:

1. Oligotrophic, oxidative basidiomycetous yeasts (*Cryptococcus* spp., *Sporobolomyces* spp., *Rhodotorula* spp., *Filobasidium* spp., etc.) and the yeast-like fungus *Aerobasidium pullulans*. These are the dominant yeasts pre-veraison and during early ripening when the nutrient availability is poor. Apart from grape surfaces, this group is also prevalent in other plant tissues related to the vineyard environment (soil, bark and leaves) (Barata et al., 2012b; Bisson and Walker, 2015; Gilbert et al., 2014; Loureiro and Malfeito-Ferreira, 2003; Pinto et al., 2014).
2. Copiothrophic ascomycetes yeasts including oxidative (some *Candida* spp.), weakly fermentative apiculate (*Hanseniaspora* spp.), film-forming (*Pichia* spp.) and fermentative (*Starmerella bacillaris, Metschnikowia* spp.) yeast species. These ascomycetous yeasts succeed the basidiomycetous as the fruit ripens and the juice is released raising nutrients. Moreover, ecological studies have pointed out *Hanseniaspora* as the prevailing genus on grape berries since its species represent between 50-75% of all the yeasts population (Romano et al., 2006). However, the application of next-generation sequencing (NGS) techniques have shown that the presence of this genus in grapes and musts depends on the vintage (Grangeteau et al., 2017) and it is much lower than previously detected by other techniques (Bokulich et al., 2014; Wang et al., 2015).
3. Copiothrophic strongly fermentative yeasts (*Saccharomyces* spp., *Torulaspora* spp., *Zygosaccharomyces* spp. and *Pichia* spp.). The high prevalence of this yeast group occurs when the grape berry is damaged and the environment is full of nutrients (Barata et al., 2008). Nevertheless, small populations of these genera have been also detected on sound grapes and musts (Grangeteau et al., 2017; Wang et al., 2015).

Furthermore, we can also find AAB and LAB in lower proportions on grape surfaces (Table 2). In the case of AAB, *Gluconobacter oxydans* is the most described species in sound grapes while in damaged grapes, AAB population increases and includes *Gluconobacter* spp. and *Acetobacter* spp. (Barbe et al., 2001). However, just some LAB species have been identified on berry surfaces and they mostly belong to *Lactobacillus* spp., despite the seldom isolation of *Pediococcus* spp. and *Oenococcus oeni* (Barata et al., 2012c; Bisson and Walker, 2015; Portillo and Mas, 2016). Finally, other aerobic bacteria are present on grape surfaces although they are not able to survive the subsequent fermentation conditions. Some of these bacteria are *Enterococcus* spp., *Enterobacter* spp., *Bacillus* spp., *Burkholderia* spp., *Serratia* spp., and *Staphylococcus* spp. (Barata et al., 2012a, 2012b).

The employment of NGS techniques has allowed the identification of greater microorganisms in vineyard environments. Stefanini and Cavalieri (2018) performed a list of the most abundant fungal and bacterial phyla and genera detected through this technology on grapes and musts, which in general supports and complements previous works (Table 3).

As the grapes are harvested and enter to the winery, the microbiota of the berry can change depending on the treatment applied (washing or filtration techniques) or through the contact with the winery equipment and surfaces containing indigenous yeast populations like *Saccharomyces* spp. Once in the winery, the grapes are crushed and the yeast from the berry, mainly non-*Saccharomyces* yeasts, enter in contact with the resulting grape juice for fermentation.

Table 1. Yeast species identified in healthy grape surface and grape must.

Genera	Species	Countries
Basidiomycetous yeast		
Cryptococcus	*C. laurentii*	Portugal[b], Australia[i], France[j]
	C. magnus	Portugal[b], China[g], Australia[i]
	C. flavescens	China[g], France[j]
	C. carnescens	China[g]
	C. oreinsis	Australia[i]
	C. albidus	France[j]
Sporobolomyces	*S. roseus*	Portugal[b], France[j]
Sporidiobolus	*S. pararoseus*	China[g], France[j]
	S. salmonicolor	France[j]
Rhodosporidium	*R. babjevae*	Australia[i], France[j]
Rhodotorula	*R. laryngis*	Australia[i]
	R. glutinis	France[j]
	R. graminis	France[j]
	R. mucilaginosa	France[j]
Ascomycetous yeast		
Aerobasidium	*A. pullulans*	Portugal[b], Spain[c,h], Italy[e], Australia[i], France[j]
Candida	*C. stellata*	Portugal[a], Italy[e], France[j]
	C. boidinii	Italy[f], France[j]
	Other Candida spp.	Portugal[a,b], Italy[e], France[j], Chile[d], China[g]
Starmerella	*S. bacillaris*	Portugal[a,b], Spain[c,h], Italy[e,f], China[g]
Hanseniaspora	*H. uvarum*	Portugal[a,b], Spain[c,h], Chile[d], Italy[e,f], China[g], France[j]
	H. guillermondii	Portugal[b], Spain[c,h], Italy[e,f], China[g], France[j]
	H. vineae	Spain[c]
	H. opuntiae	Italy[f], France[j]
	H. valbyensis	Spain[h]
Meschnikowia	*M. pulcherrima*	Portugal[a], Spain[c], Chile[d], Italy[e], China[g], France[j]
Pichia	*P. guilliermondii*	Portugal[a], China[g]
	P. burtonii	Portugal[b]
	P. anomala	Spain[c], France[j]
	P. glabrum	Italy[e]
	P. fermentans	France[j]
Torulaspora	*T. pretoriensis*	Portugal[a]
	T. delbrueckii	Spain[c], Chile[d]
Issatchenkia	*I. orientalis*	Spain[c], Italy[e], China[g], France[j]
	I. terricola	Italy[e,f], China[g], Spain[h], France[j]
Kluyveromyces	*K. thermotolerans*	Spain[c], Chile[d], Italy[e]
	K. marxianus	Italy[e]
	K. lactis	France[j]
Saccharomycopsis	*S. vini*	Portugal[b]
Saccharomyces	*S. cerevisiae*	Spain[c], Chile[d], Italy[e], France[j]
	S. boulardii	France[j]
Zygosaccharomyces	*Z. bailii*	Spain[c], Italy[f], China[g]
	Z. microellipsoide	Chile[d]
	Z. fermentati	China[g], Spain[h]
Debaromyces	*D. hansenii*	Chile[d], France[j]
Zygoascus	*Z. hellenicus*	Italy[e,f]
Yarrowia	*Y. lipolytica*	France[j]
Lipomyces	*L. spencermartinsiae*	France[j]

(a) Barata et al. (2008); (b) Barata et al. (2012a); (c) Clavijo et al. (2010); (d) Ganga and Martínez, (2004) (e) Tristezza et al. (2013); (f) Garofalo et al. (2016); (g) Li et al. (2010); (h) Padilla et al. (2016); (i) Prakitchaiwattana et al. (2004); (j) Renouf et al. (2005).

Table 2. Bacterial species identified in healthy grape surface and grape must.

Genera	Species	Countries
Acetic Acid Bacteria (AAB)		
Gluconobacter	*G. oxydans*	France[a,d], Portugal[c], Spain[e,i], Chile[f]
	G. cerinus	Greece[g]
	G. japonicus	Spain[j]
Gluconacetobacter	*Ga. saccharivorans*	Portugal[c], Spain[j]
	Ga. hansenii	Portugal[c], Spain[e]
	Ga. liquefaciens	Spain[i]
	Ga. xylinus	Spain[i]
	Ga. europaeus	Spain[i]
Acetobacter	*A. malorum*	Portugal[c], Spain[j]
	A. aceti	Spain[e]
	A. cerevisiae	Chile[f], Spain[j]
	A. pasteurianus	Spain[j]
	A. tropicalis	Spain[j]
Lactic Acid Bacteria (LAB)		
Lactobacillus	*L. casei*	France[a]
	L. sanfransiensis	France[a,d], Spain[h]
	L. lindneri	Australia[b], Spain[h]
	L. kunkeei	Australia[b]
	L. plantarum	France[d], Greece[g], Spain[h]
	L. brevis	Chile[f]
	L. mali	Spain[h]
Lactococcus	*L. lactis*	Australia[b]
Leuconostoc	*L. mesenteroides*	France[a,d]
Pediococcus	*P. parvulus*	France[a,d]
	P. acidilactici	France[d]
	P. damnosus	France[d]
	P. pentosaceus	Spain[h]
Oenococcus	*O. oeni*	France[a,b], Spain[h]
Weisella	*W. paramesenteroides*	Australia[b], France[d]
Other bacterial species		
Serratia	*S. rubidaea*	France[a,d]
	Serratia spp.	Chile[f]
Enterobacter	*E. gergoviae*	France[a,d]
	Enterobacter spp.	Chile[f]
	E. ludwigii	Greece[g]
Pseudomonas	*P. jessenii*	France[a,d]
Bukholderia	*B. vietnamiensis*	France[a,d]
Leyfsonia	*L. xyli*	France[a,d]
Bacillus	*B. mycoides*	France[a]
	B. subtilis	Greece[g]
Enterococcus	*E. faecium*	France[a,d]
	E. durans	Australia[b], Portugal[c]
	E. avium	Australia[b]
Staphylococcus	*Staphylococcus* spp.	Chile[f]
	S. saprophyticus	Portugal[c]
	S. epidermidis	Greece[g]
Acinetobacter	*Acinetobacter* spp.	Chile[f]
Klebsiella	*K. oxytoca*	Greece[g]
Tatumella	*T. ptyseos*	Greece[g]

(a) Renouf et al. (2005); (b) Bae et al. (2006); (c) Barata et al. (2012a); (d) Renouf et al. (2007); (e) González et al. (2005); (f) Prieto et al. (2007); (g) Nisiotou et al. (2011); (h) Franquès et al. (2017); (i) González et al. (2004) (j) Valera et al. (2011)

Table 3. Most abundant genera and phyla identified in grapes and musts using NGS techniques and classified as "fermenting", "innocent", "spoilage" and "unknown effect" microorganisms (Stefanini and Cavalieri, 2018).

Grapes	Fungi	Bacteria
Fermenting	*Hanseniaspora, Saccharomyces*	Firmicutes, Lactobacillales
Innocent	*Candida, Debaryomyces, Hanseniaspora, Metschnikowia, Pichia*	Firmicutes, Proteobacteria, *Bacillales, Bacillus, Enterobacteriales, Pseudomonadales*
Spoilage	*Botryotinia, Cladosporium, Pichia, Torulaspora, Zygosaccharomyces, Saccharomycodaceae*	Firmicutes, Proteobacteria, Rhodospirillales
Unknown effect	*Alternaria, Aureobasidium, Cryptococcus, Erysiphe, Issatchenkia, Itersonilia, Monilinia, Mucor, Phoma, Sporidiobolus, Starmerella, Dothioraceae, Pleosporaceae, Dothideomycetes*	Pasteurellales, Bacteroidales, Actinobacteria, *Lysinibacillus, Sporosarcina*
Musts	**Fungi**	**Bacteria**
Fermenting	*Hanseniaspora, Saccharomyces*	Lactobacillales, *Oenococcus*
Innocent	*Candida, Hanseniaspora, Lachancea, Metschnikowia, Pichia*	Rhodospirillales
Spoilage	*Aspergillus, Botryotinia, Cladosporium, Saccharomycodes, Penicillium, Pichia*	Bacillales, Enterobacteriales, Pseudomonadales
Unknown effect	*Aureobasidium, Davidiella, Erysiphe, Saccharomycopsis, Saturnispora, Sphingomonas, Starmerella, Yarrowia*	*Propionibacter, Corynebacterium*

1.3. Alcoholic fermentation

A great diversity of yeast genera and species is initially present in the grape must. As previously mentioned, the origin of these yeasts is mainly the grape berry. Nevertheless, some yeasts also come from the resident microbiota of the winery. The vast majority of them belong to non-*Saccharomyces* yeast species while *S. cerevisiae*, which is a typical inhabitant of the winery environment, is nearly absent on the berry surface and beginning of the fermentation (Fleet, 2003).

The microbiota present in the grape must changes along the alcoholic fermentation process. Indeed, the initial grape juice composition supports the growth of just certain microbial species favouring the development of fermentative yeasts. The high sugar content and the low pH are the main responsible of this selective pressure (Bisson and Walker, 2015). Also the imbalance between carbon and nitrogen sources is relevant in the selectivity of the grape must (Mas et al., 2014). Moreover, the addition of sulphur dioxide, an antioxidant and antimicrobial agent, can also exert an additional pressure especially towards oxidative microorganisms. As the fermentation

progresses, the environment becomes more restrictive with increasingly anaerobic conditions, higher levels of ethanol and less availability of nutrients that tighten up the number of microbial species able to survive (Pretorius, 2000; Ribéreau-Gayon et al., 2006).

Overall, *Hanseniaspora, Candida* and *Metschnikowia* genera begin the fermentation process. Occasionally, species of *Pichia, Issatchenkia* and *Kluyveromyces* can also develop during this stage. These yeast species may grow up to 10^6-10^7 cfu/ml until mid-fermentation when their population sharply decay. This is the point in which *S. cerevisiae* becomes predominant, reaching populations of 10^7-10^8 cfu/ml, until it completes the fermentation. Nevertheless, the microbial succession occasionally can lead to stuck or sluggish fermentations as a result of an excessive propagation of non-fermentative yeasts that consume the nutrients needed for the development of the fermentative ones (Ciani et al., 2006; Medina et al., 2012; Padilla et al., 2016a).

Despite the heterogeneous microbial communities present in grape must, *S. cerevisiae* became the desired wine yeast to impose over the other species in order to lead the alcoholic fermentation (Reed and Peppler, 1975). Nowadays, thanks to Müller-Thurgau who introduced in 1890 the concept of inoculating wine fermentations with pure yeast cultures, most industrial wineries use this practice to inoculate a selected yeast strain, mainly belonging to *S. cerevisiae,* to ensure the completion of the fermentation and the uniformity of wines (Pretorius, 2000).

This brings us to the concepts of spontaneous and inoculated fermentations. While the inoculated fermentations guarantee wine production and quality, the result of spontaneous fermentations is unpredictable. Despite of the spoilage threaten, the final wines from spontaneous fermentations can be more complex and integrate more flavours than the inoculated ones (Jolly et al., 2014). In fact, recent trends are pushing winemakers to produce wines through spontaneous or "natural" fermentations. Again, this is taking wineries to a loss of microbiological control that can end up in the problems previously mentioned, such as sluggish or stuck fermentations and the production of unpleasant compounds.

Therefore, to take advantages of both inoculated and spontaneous approaches, mixed and sequential fermentations are catching winemaker's attention and interest. Using these strategies, we can benefit from non-*Saccharomyces* and *S. cerevisiae* metabolism obtaining more interesting and distinctive wines (Comitini et al., 2017; Padilla et al., 2016a, 2017).

1.3.1. *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is the dominant yeast species in wine fermentations and many of its strains are commercialized in the form of active dry yeast as starter cultures. The physiological aptitudes exhibited by this yeast makes it the most desired driver of fermentation. Thus, not only it is able to grow under wine fermentation conditions (high ethanol and organic acids levels, low pH values, scarce oxygen, resistance to SO_2 and absence of certain nutrients) (Bisson, 1999) but also it is one of the fastest yeast in consuming sugar and producing ethanol even in the presence of oxygen (Cabtree effect) (Albergaria and Arneborg, 2016).

Recently, strategies like whole-genome sequencing and systems biology are being evaluated to achieve a *Saccharomyces* yeast with the suitable properties to face the current viticulture and consumer challenges, being climate change one of the most concerning issues (Table 4) (Borneman et al., 2013; Jagtap et al., 2017). Apart from these strategies, other *Saccharomyces* species like *S. uvarum* and its hybrids with *S. cerevisiae* (*S. cerevisiae* x *S. uvarum)* have gained interest for their potential to unravel these winemaking challenges (Pérez-Torrado et al., 2017).

Table 4. Desirable properties of wine yeast (Pretorius, 2000).

Fermentation properties	Technological properties
Rapid initiation of fermentation	High genetic stability
High fermentation efficiency	High sulphite tolerance
High ethanol tolerance	Low sulphite binding activity
High osmotolerance	Low foam formation
Low temperature optimum	Flocculation properties
Moderate biomass production	Compact sediment
	Resistance to desiccation
	Zymocidal (killer) properties
	Genetic marking
	Proteolytic activity
	Low nitrogen demand
Flavour characteristics	**Metabolic properties with health implications**
Low sulphide/DMS/thiol formation	Low sulphite formation
Low volatile acidity production	Low biogenic amine formation
Low higher alcohol production	Low ethyl carbamate (urea) potential
Liberation of glycosylated flavour precursors	
High glycerol production	
Hydrolytic activity	
Enhanced autolysis	
Modified esterase activity	

1.3.2. Non-*Saccharomyces* yeasts

Lately, the new tendencies in winemaking have brought non-*Saccharomyces* yeasts in the spotlight of oenological field. Their positive contribution to the wine have largely displaced the negative background associated to this yeast group. Therefore, many studies conducting mixed and sequential fermentations have been done in the last few years (Varela, 2016).

Due to the interest of the market in premium and high quality wines together with great acceptance among consumers of wines presenting distinctive autochthonous peculiarities, we should introduce the concept of *terroir*. Wine *terroir* refers to the relationship between the features of wine with vineyard environment (Leeuwen and Seguin, 2006). *Terroir* is influenced by regional, varietal and climatic factors that in turn condition the microbial biogeography of grapes (Bokulich et al., 2014). In that sense, the WILDWINE Project was a European project with the participation of my research group, conducted from 2012 to 2015 that explored the concept of *terroir* and proposed the use of indigenous yeasts and bacteria as starter cultures. Therefore, the microbial fingerprint of different European wine regions was established and the species and strains that were isolated were evaluated for their aptitudes in wine production (Mas et al., 2016). For example, in Priorat region, the main species isolated in the vineyard were the yeast-like fungus *A. pullulans* and *Hanseniaspora uvarum,* while in the must, *H. uvarum* was the predominant followed by *S. cerevisiae* and *S. bacillaris.* Other yeast species like *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* were also identified but in lower proportions (Mas et al., 2016; Padilla et al., 2016a). Therefore, in order to get a wine with "Priorat *terroir*" we should industrially reproduce the described microbial fingerprint with the inoculation of these species at their respective proportions in the grape must.

The employment of non-*Saccharomyces* yeasts not only improves wine complexity and flavour through their enzymatic activities that influence primary and secondary aroma compounds (Jolly et al., 2014; Padilla et al., 2016b). These yeasts also contribute to wine stability through the production of extracellular metabolites that prevent the growth of spoilage microorganisms (Comitini et al., 2004; Mehlomakulu et al., 2017; Mehlomakulu and Africa, 2015; Villalba et al., 2016). Moreover, their potential in lowering ethanol content in wine is one of the most recent and interesting features explored to face both consumer preferences and the greater maturity of grapes as a consequence of climate change (Contreras et al., 2015; Quirós et al., 2014; Varela et al., 2017).

Table 5. Positive oenological characteristics of non-*Saccharomyces* yeast.

Yeast genera	Oenological features	References
Torulaspora	Reduction of ethanol concentration Low volatile acidity and acetaldehyde Increase production of 2-phenylethanol, lactones and glycerol Decrease fatty acids and ethyl esters content Consumption of malic acid Few production of higher alcohols High mannoproteins production Release of TdKT killer toxins against spoilage yeasts	Bely et al. (2008), Azzolini et al. (2012, 2015), (Belda et al., 2015) , García et al. (2017), González-Royo et al. (2015), Villalba et al. (2016)
Metschnikowia	Reduction of ethanol and acetaldehyde concentration High production of esters and glycerol Increase levels of the thiol 4-MSP (4-methyl-4-sulfanylpentan-2-one), 2-phenyl ethyl alcohol and 2-phenyl acetate Low volatile acidity	Contreras et al. (2014), Varela et al. (2016, 2017a), Ruiz et al. (2018), Escribano et al. (2018)
Hanseniaspora	Increase levels of acetate esters, ethyl esters compounds, medium-chain fatty acid ethyl esters, terpens and fatty acids High production of 2-phenylethyl acetate, glycerol, acteladehyde and vitisin B Low volatile acidity Synthesis of benzenoid compounds	Medina et al. (2013, 2016), Hu et al. (2018b, 2018a), Lombardi et al. (2018), Martin et al. (2016b, 2016a), Tristezza et al. (2016)
Candida/Starmerella	Reduction of ethanol concentration Low volatile acidity High production of glycerol, esters, aldehydes, ketones, terpenes and C13-norisoprenoids Decrease ethyl acetate, volatile fatty acids and malic acid compounds Production of volatile organic compounds (VOCs) with antifungal activity agains *B. cinerea*	Englezos et al. (2015, 2016a, 2016b, 2018), Lemos Junior et al. (2016), Nisiotou et al. (2018)
Kluyveromyces/Lachancea	Reduction of the pH and acetaldehyde and higher alcohols content. Increase of lactic acid, glycerol and 2-phenylethanol Low volatile acidity Release of Kwkt killer toxins against *Brettanomyces/Dekkera*	Balikci et al. (2016), Benito et al. (2015), Comitini et al. (2004a), Comitini and Ciani (2011), Gobbi et al. (2013), Kapsopoulou et al. (2005, 2007)
Issatchenkia	Reduction of malic acid and acetaldehyde Higher production of phenols, monoterpens and norisoprenoids	Kim et al. (2008), González-Pombo et al. (2011), Ovalle et al. (2018)
Pichia	Increase of the thiol 3-mercaptohexyl acetate (3MHA) and acetaldehyde Enhancement of vinylphenolic pyranoanthocianins Release of killer toxins with antifungal activity High content of polysaccharides	Anfang et al. (2004), Benito et al. (2011), Błaszczyk et al. (2015), Domizio et al. (2011)

Yeast genera	Oenological features	References
Zygosaccharomyces	Low production of acetic acid, H_2S and SO_2 Degradation of malic acid High production of polysaccharides	Domizio et al. (2011), Jolly et al. (2014b)
Schizosaccharomyces	Consumption of malic acid Decrease urea content High production of polysaccharides Increase pyruvic acid and vinylphenolic pyranoanthocyanin content	Loira et al. (2018), Morata (2013), Mylona et al. (2016), Romani et al. (2018)

1.4. Malolactic fermentation

Malolactic fermentation is performed after alcoholic fermentation by lactic acid bacteria (LAB). This process is commonly applied in red wines and also in certain white wines with remarkable acidity. The biological process consists in the conversion of the dicarboxylic L-malic acid into the monocarboxylic L-lactic acid and carbon dioxide. As a result, there is an increase of the pH of the wine in 0.1-0.2 units and a decrease of the titratable acidity (Liu, 2002).

Oenococcus oeni is the most common driver of malolactic fermentation. This bacterium outcompetes other LAB species due to its ability to develop in harsh environments with high ethanol concentrations, low pH and limited nutrient content. Despite this fact, other LAB species found in wine belong to the genera *Lactobacillus, Leuconostoc* and *Pediococcus* (Table 6) (Bartowsky, 2005).

Table 6. Lactic acid bacteria genera and species isolated from wine (Bartowsky, 2005).

Lactobacillus	*Leuconostoc*	*Oenococcus*	*Pediococcus*
Heterolactic	**Heterolactic**	**Heterolactic**	**Homolactic**
Lb. brevis, Lb. cellobiosis	*Lc. mesenteroides*	*O. oeni*	*P. pentosaceus,*
Lb. buchneri, Lb. casei Lb.			*P. damnosus*
hilgardii, Lb. jensenii			*(P. cerevisiae)*
Lb. kunkeei, Lb. nagelii			*P. parvulus*
Lb. trichodes, Lb. sakei			
Lb. curvatus			
Lb. delbrueckii			
Lb. fermentum			
Lb. fructivorans			
Lb. vermiforme			
Homolactic			
Lb. plantarum			

The same as yeasts, the selection of indigenous LAB strains is of interest to get starter cultures for malolactic fermentation and obtain wines with specific *terroir* (Mas et al., 2016). In the WILDWINE Project, Franquès et al. (2017) identified and typified several LAB strains from grapes and wine of Priorat region. Whereas in grape samples *Lb. plantarum* was the main species

recovered, in wine, *O. oeni* was the predominant one representing almost the 96% of all the LAB isolates.

Finally, considering the new role of non-*Saccharomyces* yeast in alcoholic fermentation, many studies about the effect of non-*Saccharomyces* yeast on LAB and malolactic fermentation are being performed to face the new oenological scenarios (Balmaseda et al., 2018).

1.5. Aging and bottling

After alcoholic or malolactic fermentation, wine can be directly bottled or undergo an aging period in barrels. This post-fermentation stage is a critical point in winemaking since the occurrence of some microorganisms can alter the quality of the final product.

The main microorganisms that represent a risk in this stage are acetic acid bacteria (AAB), particularly *Acetobacter* species, and the yeast *Brettanomyces bruxellensis* (Bartowsky, 2009; Loureiro and Malfeito-Ferreira, 2003). Nevertheless, LAB and other *Saccharomyces* and non-*Saccharomyces* yeasts can also develop during the aging stage and spoil the wine (du Toit and Pretorius, 2000). The growth of AAB supposes an important concern since it results in the production of acetic acid by the oxidation of ethanol. In order to avoid AAB is necessary to ensure the absence of oxygen during storage as they are aerobic bacteria (Bartowsky and Henschke, 2008). On the other hand, *B. bruxellensis* presence can occur even under oxygen absence and its threat is mostly related to its ability to produce volatile phenols that confer unpleasant aromas to the wine (Loureiro and Malfeito-Ferreira, 2003).

There are three possible points in which these organisms can enter in the winemaking process: from the raw material including grapes and winery equipment, during alcoholic and malolactic fermentation, and finally, during aging in oak barrels and bottling. The aging stage takes place during several months allowing the slow growth of spoilage microorganisms coming from previous stages or already present in the barrels and bottles. Indeed, wines stored in barrels are more exposed to *Brettanomyces* spoilage since they are used several times and *B. bruxellensis* has been found 8 mm down within the wood barrel staves (Malfeito-Ferreira, 2005). Therefore, an appropriate cellar sanitation, the exclusion of oxygen and the supplementation with antimicrobial agents are extremely important in these post-fermentation processes to prevent the development of spoilage yeasts and bacteria (du Toit and Pretorius, 2000).

Additionally, these microorganisms can also proliferate after bottling causing turbidity or haze related to the microbial growth, producing off-flavours and releasing carbon dioxide. Thus, the amount of residual nutrients and microorganisms should be taken in consideration to avoid microbial development and ensure the wine entirety (Bisson and Walker, 2015).

2. Approaches to assess the microbiome diversity during wine elaboration

The study of the microbial populations during winemaking, from grapes to wine, is a field of interest since Pasteur determined that alcoholic fermentation was a biological process (Barnett, 2000). Initially, the microbiota diversity was basically assessed by culture-dependent techniques. However, the limitations of culturing methods and the diversity discrepancy between culturable and *in situ* microorganisms has turned out the attention to culture-independent techniques, basically based on molecular biology approaches.

2.1. Culture-dependent techniques

Several culture media and conditions are used to recover the vast majority of the species in the environment or a specific microorganism. Therefore, the choice of the media will determine the characterisation of the community.

In oenological field, some culture media are commonly employed to isolate microorganisms associated with the winemaking process. Actually, plating in a non-selective rich medium like YPD (Yeast-Peptone-Dextrose) is the most usual practice to follow yeast populations during fermentation. However, the fast growth of *Saccharomyces* species restricts the development of other yeast species belonging to non-*Saccharomyces* group. To face this problem, samples are also plated on Lysine Agar where *Saccharomyces* grows slowly in comparison to non-*Saccharomyces* yeasts when lysine is used as the only nitrogen source (Bisson and Walker, 2015; Egli et al., 1998). Other strategies to isolate specific yeast species have been developed. For example, the addition of cycloheximide in the medium selects some non-*Saccharomyces* species and it is specially applied for *Brettanomyces* isolation (Barnett et al., 1990). In addition, WL (Wallersteins Laboratory Nutrient Agar) medium is a very interesting tool to assess the known community of a fermentation because it is a non-selective medium that allows yeast identification by colony colour and morphology (Pallmann et al., 2001). However, the application of this medium for ecological studies can be questioned as some species or genera share similitudes of morphology. Thus, the use of this medium has to be accompanied by other identification methods.

Regarding to LAB and AAB recovery, MRS and GYC media are respectively used for the selective isolation of each microbial group. In fact, MRS is a complex broth containing different nutrient sources to support LAB growth (De Man et al., 1960). Moreover, this medium has been

optimized to recover these bacteria from hostile environments like wine providing it with additional salts, amino acids or antimicrobial and antifungal agents (Bordas et al., 2013). Finally, GYC (Glucose-Yeast Extract-$CaCO_3$) is described for AAB isolation in wine (Ruiz et al., 2000) and it has become one of the preferential media for many researchers. This culture medium enables a great strains recovery and a quick and visual identification of AAB, since their production of acetic acid degrades the $CaCO_3$ resulting in a clear halo on the plate (Vegas et al., 2010).

After isolation and growth of the different microorganisms in each medium, isolates are then identified using molecular techniques mainly based on the polymerase chain reaction (PCR) of different ribosomal DNA regions. For example, the restriction enzyme digestion of internal transcribed spacers (ITS) amplicons from 5.8S ribosomal DNA region (Esteve-Zarzoso et al., 1999; Guillamón et al., 1998) is the leading technique for yeast identification together with rDNA sequencing analysis (Kurtzman and Robnett, 1998). Furthermore, restriction analysis of different rDNA regions (PCR-RFLP of 16S rDNA; PCR-RFLP of 16S-23S rDNA ITS or PCR-RFLP of 16S-23S-5S rDNA) are also proposed for identification of AAB at species level (Mas et al., 2007). Similarly to yeast and AAB, the characterisation of LAB from oenological environments usually revolves around the restriction analysis of the amplified 16S rDNA, known as 16S-ARDRA method (Rodas et al., 2003).

The application of these culture-dependent techniques has allowed the isolation and identification of more than 70 genera of fungi and up to 40 yeast and 50 bacterial species associated with winemaking (Morgan et al., 2017). Despite the successful results of culture based methods, its application is time consuming and sometimes ends up with biased results (Andorrà et al., 2008; Rantsiou et al., 2005). Moreover, the culture media and conditions only allow the growth of some species, commonly the most abundant and fast-growing ones. So, these techniques fail in reproducing the ecological niches since they just show a small fraction of the community leaving low abundance species as well as viable but non-culturable (VBNC) cells behind (Andorrà et al., 2008; Millet and Lonvaud-Funel, 2000; Nocker et al., 2007; Torija et al., 2010) . Nowadays, in order to have a better image of the microbial diversity and community composition of wine related samples, the use of culture-independent techniques is required.

2.2. Culture-independent techniques

At the end of the 20th Century, researchers started using culture-independent techniques to study the microbial ecology in fermented foods. Most of these techniques rely on molecular methods that use nucleic acids, DNA or RNA, as a target molecules (Cocolin et al., 2013). As it has been mentioned, these techniques provide a most accurate and faster tool to decipher the microbial community of a specific environment removing the bias of culture-based methods. Nowadays, their employment in food microbiology can replace or complement the traditional techniques (Galimberti et al., 2015).

The molecular approaches used for microbial ecology can be classified in targeted tools or community profiling tools (Bokulich and Mills, 2012). A targeted tool would be any technique that detects a taxonomically defined group of microbes using a selected set of primers or probes. They cannot be applied to characterize the microbial community; however, they are used to follow important microorganisms of a certain environment. With regard to community profiling tools, these techniques use universal primers targeting universal-taxonomically defined groups (usually kingdom-level phyla) to amplify heterogeneous DNA sequences that will proportionate a whole community landscape. Nevertheless, the results provided by these methods are qualitative or semiquantitative. To solve this issue, these techniques should be simultaneously applied with targeted tools to get quantitative results.

Currently, there are many targeted and profiling community methods available. Despite this fact, it will be further discuss the techniques employed in the present thesis: quantitative PCR (qPCR), PCR combined with denaturing gradient gel electrophoresis (PCR-DGGE) and Next Generation Sequencing (NGS) technologies (Table 7).

Table 7. Comparison of the molecular techniques used in this work adapted from Bokulich and Mills (2012). Additional information collected from Loman et al. (2012), Metzker (2010) and Quail et al. (2012).

Tool	Target size (bp)	Sensitivity*	Run time	Advantages	Disadvantages
qPCR	100-150	10^1-10^3	4 hours	Sensitive, quantitative	Targeted technique
DGGE	100-300	10^3	12 hours	Single-nucleotide fragment separation	Technically difficult
Next Generation Sequencing (NGS)					
Ion Torrent					
314 chip	≈ 200	10^5	3 hours	Fast throughput (80-100 Mb/h) and short run time. Low instrument cost.	Short reads and high error rates in homopolymers repeats.
316 chip	≈ 200	10^6	3 hours		
318 chip	≈ 200	10^6	3 hours		
Illumina					
MiSeq	150	10^7	27 hours	High throughput per run and low error rate. Lowest sequencing cost.	Short reads length.
GAIIx	150	10^8	10 days		
HiSeq 2000	150	10^9	11 days		
454 Life Sciences					
GS FLX+	500-600	10^6	23 hours	Long fragment length reads. Theoretically higher taxonomic resolution.	Lower throughput and homopolymeric errors. Expensive
GS Junior	300	10^5	11 hours		

*Sensitivity meaning the detection limit for qPCR and DGGE (cells/ml) or reads per run for Ion Torrent, Illumina and 454 Life Sciences.

2.2.1. Quantitative PCR

Quantitative PCR or Real Time PCR is one of these targeted tools that consists in a PCR able to determine the amount of product generated along the reaction. This is possible thanks to the emission of fluorescence by the fluorescent dyes or probes present in the mix reaction. Therefore, the fluorescence produced correlates with the amount of the delivered product. Furthermore, considering a maximum amplification efficiency, which means that the number of the molecules duplicates in each amplification cycle, it is possible to calculate the DNA molecules present in the original sample.

One of the most commonly used dye is SYBR Green, which unspecifically binds to the double-stranded DNA. As more double-stranded DNA is present, more dye is bounded and, consequently the fluorescence emitted increases proportionally to the DNA concentration. During the first cycles, the fluorescence produced is very low and it cannot be distinguished from the background. While the product generated is being accumulated, the fluorescence signal starts

to exponentially increase until the exhaustion of some of the reaction components like primers, dNTPs or the fluorescent dye. In that point, the signal levels off and saturates (Kubista et al., 2006). The number of cycles needed to achieve the established fluorescence threshold is known as Ct value. This value is important because it is necessary for the quantitative analysis of the sample (Agilent Technologies, 2012).

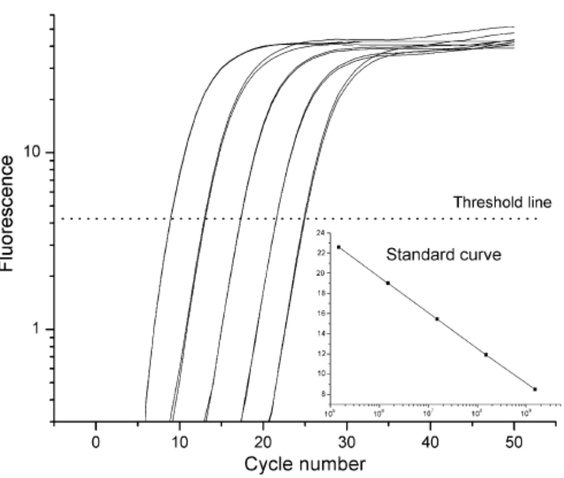


Figure 2. qPCR standard curve shown in logarithmic scale. The Ct values for each dilution are plotted against the logarithm of the initial template copy number in the standard sample (Kubista et al., 2006).

qPCR has been broadly applied to detect and follow microorganisms of oenological interest in wine. Among others reasons, this technique stands out for its specificity and sensitivity aiming to detect even 10 cells/ml (Hierro et al., 2007). Notwithstanding, an appropriate primer design for each microorganism or microbial group is needed. In this sense, many primer sets are available to detect and quantify different wine microorganisms (Table 8).

Table 8. Primers designed for the detection and quantification of some wine related microorganisms using qPCR.

Target	Primers	Ribosomal gene region	References
Total yeast	200F/324R	26S rRNA	Hierro et al. (2006)
Saccharomyces spp.	CESPF/SCERR	ITS2 and 5.8S rRNA spanning region	Hierro et al. (2007)
Hanseniaspora spp.	CESPF/HUVR	ITS2 and 5.8S rRNA spanning region	Hierro et al. (2007)
S. bacillaris	AF/200R	D1/D2 (26S rRNA)	Andorrà et al. (2010)
T. delbrueckii	TODSL2/TODSR2	ITS (between 18S rRNA and 26S rRNA)	Zott et al. (2010)
Metschnikowia spp.	MP5FW/MP3BW	26S rRNA	Díaz et al. (2013)
B. cinerea	BC3F/BC3R	ITS (between 18S rRNA and 28S rRNA)	Suarez et al. (2005)
B. bruxellensis	DBRUXF/DBRUXR	D1/D2 (26S rRNA)	Phister and Mills (2003)
Z. bailii	ZBF1/ZBR1	D1/D2 (26S rRNA)	Rawsthorne and Phister (2006)
LAB	WLAB1/WLAB2	16S rRNA	Neeley et al. (2005)
AAB	AQ1F/AQ2R	16S rRNA	González et al. (2005b)

2.2.2. Denaturing Gradient Gel Electroporesis (DGGE)

DGGE is a profiling community tool that allows the differentiation of DNA fragments of the same size but with different sequences. It is basically an electrophoretic method where samples are separated in a denaturing gradient gel depending on their denaturation melting point.

The samples load on DGGE gels are PCR products containing a 30-40 bp GC clamp, previously added to one of the primers, to avoid the complete denaturation of the sample. Furthermore, the preparation of these acrylamide denaturing gels relies in the use of denaturing compounds like urea and formamide. Usually, a mix of high and low denaturing solutions are incorporated generating an increasing linear denaturing gradient (from less to higher denaturing conditions). As the samples move through the gel and the denaturing conditions increase, the double-strand DNA partially melts until it achieves the lowest melting domain, depending on the DNA sequence, and it stops. After that, the gel bands are revealed, excised and re-amplified using primers without GC-clamp for further sequencing and identification (Ercolini, 2004).

PCR-DGGE has been widely applied to monitor yeast and bacterial diversity and dynamics during alcoholic fermentation since Cocolin et al. (2000) employed it in wine fermentation for the first time. The main primer pairs used to amplify fungi and yeast are $NL1^{GC}$/NL2 (Cocolin et al., 2000) and $U1^{GC}$/U2 (Meroth et al., 2003a). Moreover, the current primers used for general bacteria are $341f^{GC}$/518r (Muyzer et al., 1993) while to specifically amplify LAB and AAB, $L1^{GC}$/HDA2 (Meroth et al., 2003b) and $WBAC1^{GC}$/WBAC2 (Andorrà et al., 2008) primers can be respectively utilized.

The detection limit of this method to observe a clear band on DGGE gel has been established to 10^3 cells/ml (Cocolin et al., 2000, 2001). However, Prakitchaiwattana et al. (2004) demonstrate that in a species mixture where many of the species displayed a population of at least 10^6 cfu/ml, this may impede the detection of other species that are present at 10^4 cfu/ml or less. Therefore, this situation represents one of the most important limitations of the method for describing the microbial community. Other issues concerning this technique is that is very time-consuming, difficult and only a small number of samples can be run in a gel. Apart from that, the co-migration of band or the induced errors by the PCR (like amplification bias or chimera formation) can alter the results.

Despite the limitations of the DGGE, it still is an interesting tool to profile the microbial ecology during wine fermentation. Additionally, thanks to this technique many researchers have

provide a wider view of wine ecology from grapes to wine (Andorrà et al., 2010; Nisiotou et al., 2007, 2011; Prakitchaiwattana et al., 2004; Renouf et al., 2007; Wang et al., 2015).

2.2.3. Next-generation sequencing (NGS)

Next-generation sequencing (NGS) comprise several technologies that massively sequence heterogeneous DNA fragments (Bokulich and Mills, 2012). Sanger et al. (1977) was the pioneer developing the first-generation DNA sequencing technology commonly known as Sanger's "chain-termination" or dideoxy technique. However, the term NGS refers to the technologies developed after Sanger's approach, specifically including second and third generation sequencing techniques (Türktaş et al., 2015).

Currently, all works related to wine microbiome are done using second-generation DNA sequencing (SGS) techniques. This approach as well as Sanger's is based in sequencing by synthesis. Despite this common characteristic, SGS uses natural nucleotides and can be read in real time. Moreover, this technology is based in a luminescent method to measure pyrophosphate synthesis that relies in a two-enzyme process (Heather and Chain, 2016). Nevertheless, these techniques still have some limitations, such as errors from DNA amplification, loss of synchronization and the duration of completion (Morgan et al., 2017; Schadt et al., 2010). Therefore, to cope with these drawbacks third-generation sequencing (TGS) techniques capable of sequencing single molecules without DNA amplification are being developed.

From the SGS technologies, Illumina and 454 pyrosequencing are the most used platforms for grapevine ecology approaches (Morgan et al., 2017). Although in lower proportion, Ion Torrent technology is also employed. Besides, this approach has been placed between second and third sequencing technologies. In the next paragraphs, the basis of these platforms will be further explained:

- **454 pyrosequencing**

In 454 pyrosequencing, DNA molecules are attached to beads through adapter sequences before undergoing bridge water-in-oil emulsion PCR (emPCR). After that, DNA-coated beads are distributed over a picoliter reaction plate where each bead is fixed into one well. This is the point where sequencing by synthesis occurs. After the addition of dNTPs, the two-enzyme process

takes place and the release of pyrophosphate is measured using a charged couple device (CCD) allowing the identification of bases (Heather and Chain, 2016; Morgan et al., 2017).

- **Illumina**

Illumina sequencing process consists on the bridge amplification of adaptor-ligated DNA fragments on the surface of a glass (Figure 3A). Thereafter, bases are determined by a cyclic reversible termination strategy, which sequences the template strand one nucleotide at a time through progressive rounds of base incorporation, washing, imaging and cleaning. In this strategy, fluorescently-labeled dNTPs are used to pause the polymerization reaction enabling unincorporated bases removal and the identification of the added bases by fluorescent scanning with a CCD camera. Then, the hole process is repeated (Morgan et al., 2017; Reuter et al., 2015).

- **Ion Torrent**

Ion Torrent is also known as the first "post-light" sequencing technology since it not uses fluorescence or luminescence. Similarly to 454 pyrosequencing, beads containing clonal populations of DNA fragment, obtained from emulsion PCR (emPCR), are distributed into a picowell plate where the sequencing-by-synthesis reaction occurs. Unlike pyrosequencing, Ion Torrent consists in a complementary metal-oxide-semiconductor (CMOS) technology that measures the nucleotide incorporation through pH changes induced by the release of hydrogen ions during DNA polymerization (Figure 3B). Since no optical scanning is needed, the speed sequencing runs and costs are much lower than pyrosequencing (Liu et al., 2012; Morgan et al., 2017; Reuter et al., 2015).

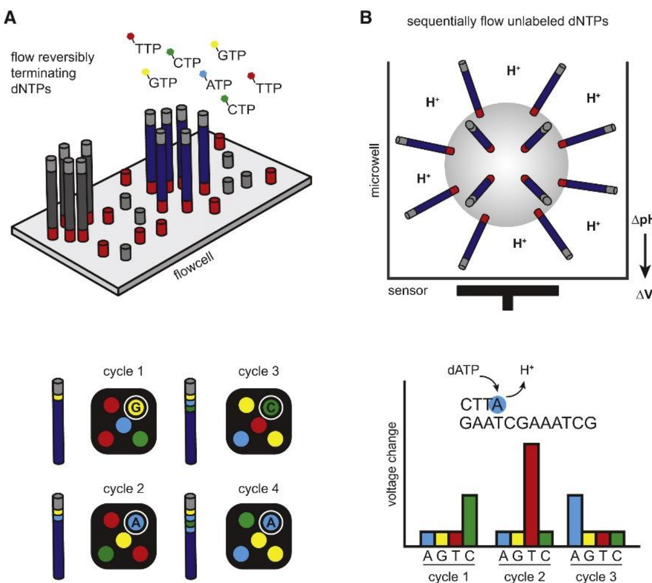


Figure 3. Schematic view of Illumina (A) and Ion Torrent (B) sequencing platforms basis (Reuter et al., 2015).

After introducing the main employed technology platforms, it has to be mentioned one of the most important decision for sequencing, the choice of target genes or regions. Different results would be obtained depending on the region sequenced. The same as qPCR and DGGE, ribosomal RNA genes are the most suitable target genes since are universally present in all species and contain highly conservative fragments for designing primers that cover all the community but also present variable regions that allow species discrimination (Cocolin et al., 2013).

For fungi, ITS regions are the most commonly targeted gene regions for fungal diversity description (Bokulich et al., 2012, 2016; Setati, 2015) followed by D1 and D2 regions of the 26S rRNA (Holland et al., 2014) and the partial 18S rRNA gene (De Filippis et al., 2017, 2018; Portillo and Mas, 2016). In the case of bacteria, 9 hypervariable regions (V1-V9) of the 16S rRNA have been targeted for bacterial diversity estimation (Piao et al., 2015; Pinto et al., 2015; Portillo et al., 2016).

Regarding to fungal community targets, Pinto et al. (2014, 2015) compared the results obtained with ITS2 region with those achieved using the D2 domain of the 26S rRNA region. Despite obtaining similar taxonomic depth with both regions, these regions only shared a small portion of the identified taxonomies. Additionally, a higher number of taxa were identified using ITS2 region. Moreover, performances of several ITS primers were also evaluated and ITS1 was the region that identified the highest number of taxa (Bokulich and Mills, 2013). Thus, the most promising target for fungi seems to be ITS1 region.

Finally, some comparisons between the different hypervariable regions have been done for bacteria analysis. For example, Bokulich et al. (2012) found that V4 and V5 regions resulted in a similar bacterial composition obtaining greater taxonomic depth for certain species using V4. Furthermore, Campanaro et al. (2014) were able to detect 89 bacteria genera targeting both V3-V4 and V5-V6 regions. However, from these genera just 31 were commonly found in both target regions indicating the need of covering four hypervariable regions to get a more detailed view of the microbial composition.

Finally, one of the most significant points of the sequencing techniques is to handle with all the data generated and analyze it. Nowadays, several open source pipelines like mothur, QIIME or MG-RAST are available and provide tools for basic data analysis (Kioroglou et al., 2018). The analysis of this data usually consist in the following steps (Figure 4) (Morgan et al., 2017; Stefanini and Cavalieri, 2018):

i. Quality trimming and de-noising. Eliminates erroneous and low-quality reads obtained by the sequencing platform. Removes also artifacts like chimeras and contaminant sequences.

ii. OTU-picking/clustering. Minimize the amount of data identifying the Operational Taxonomic Units (OTUs) of the samples. For clustering, a pairwise comparison of the sequences is undergone using an established percentage identity threshold. Finally, a representative sequence for each cluster is selected.

iii. Taxonomic assignment. Assign taxonomic identities to the selected OTUs through the comparison of these sequences to those present in reference databases, such as Greengenes, UNITE, SILVA, NCBI, etc.

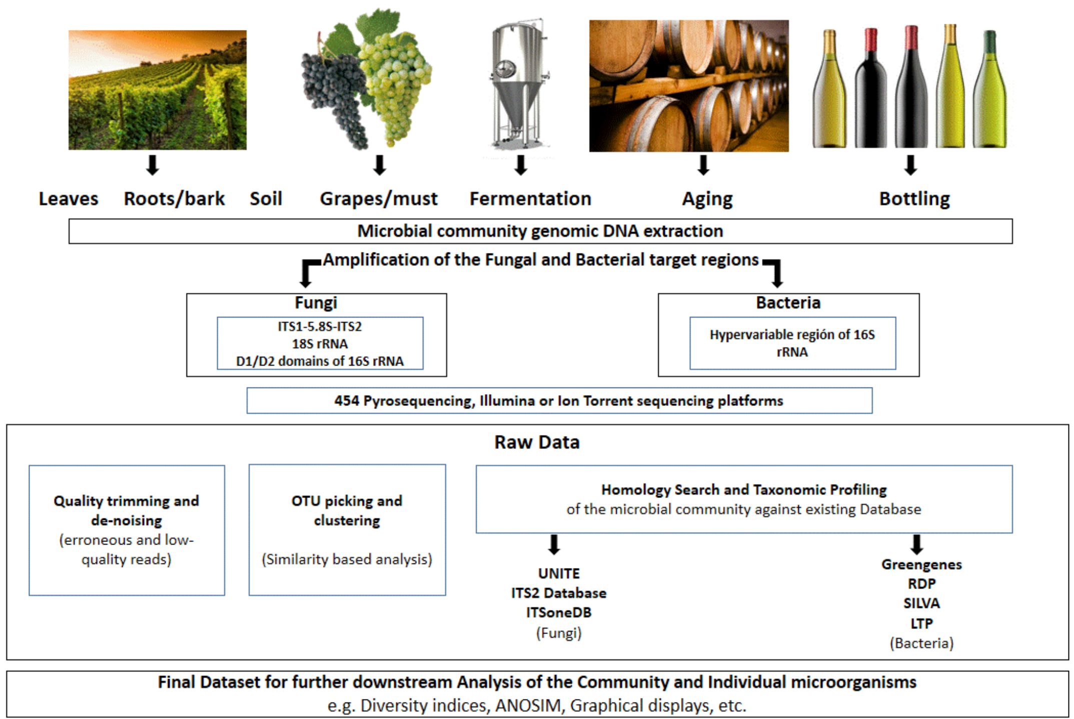


Figure 4. Schematic representation of the main steps comprised in next-generation sequencing (NGS). Figure adapted from Morgan et al. (2017).

3. Factors influencing wine microbiota and quality

The microbial communities present along the winemaking process can be affected by several factors classified in biotic and abiotic factors. Consequently, these microbiota changes can lead into alterations on the fermentation performance and the resulting wine. Along this section, both biotic and abiotic factors will be explained in detail.

3.1. Biotic factors

Biotic factors refer to the interaction between the different microorganisms present along the winemaking process or to the development of non-desired microorganisms at different points of this process. These microbial interactions comprise from the competition for nutrients to the production of killer toxins, antibiotic or quorum sensing molecules.

Furthermore, external conditions can favour the over-growth of some microorganisms and define the interactions of the different microbial populations present in the environment. Therefore, exists a reciprocal relationship between biotic and abiotic factors (Barata et al., 2012b). In the following section, it will be introduced a non-*Saccharomyces* yeast of oenological interest studied in the present thesis. Later, attention will be addressed to the most common grape and wine spoiler microorganisms followed by the interaction between the different yeast species, focusing specifically in *Saccharomyces* and non-*Saccharomyces* interactions.

3.1.1. *Hanseniaspora vineae*

Hanseniaspora vineae is an apiculate yeast that belongs to the bipolar budding genus of *Hanseniaspora* (anamorph *Kloeckera*). Species of *Hanseniaspora* are the main yeasts present on grapes and their contribution to fermentation is currently under study.

Different from other *Hanseniaspora* species, *H. vineae* is difficult to isolate from berries and it is mainly recovered during early fermentation stages. This situation could be explained by its fermentation capacity, which differs from the general *Hanseniaspora* group, reaching ethanol concentrations of 10%, together with the high homology of the pyruvate kinase gene with *S. cerevisiae* (Martín, 2016).

Recently, several genomes of the different *Hanseniaspora* species have been sequenced. In the case of *H. vineae*, up to now there is only available the genome sequence of a strain isolated from

Uruguayan red grape variety (Giorello et al., 2014). Interestingly, *H. vineae* is the *Hanseniaspora* species with the highest number of alcohol dehydrogenases, which would explain its better fermentation ability (Martin et al., 2018).

The good fermentation behaviour is not the only attractive feature displayed by *H. vineae.* Actually, it mainly contributes to wine aroma through different secondary metabolic pathways and enzymatic activities (Figure 5). This yeast exhibits several enzymes of oenological interest like esterases, β-glycosidases, lipases or proteases. However, it essentially stands out by its prominent β-glycosidase activity, that release flavor from non-volatile glycosylated precursors (López et al., 2015). Moreover, sequential fermentations with *H. vineae* resulted in increased amounts of 2-phenetyl acetate that gives fruity aroma to wine (Lleixà et al., 2016; Medina et al., 2013; Viana et al., 2011b). This fact can be correlated with the higher production of this ester compared to *S. cerevisiae* (Figure 6) (Martin et al., 2016a).

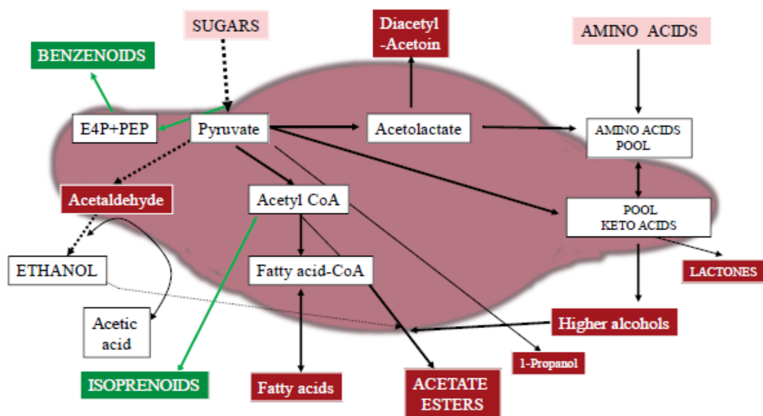


Figure 5. Flavour compounds produced by *H. vineae* during wine fermentation (Martin et al., 2018).

Furthermore, *H. vineae* has shown to synthetize the benzenoid compounds benzyl alcohol and p-hydroxybenzyl alcohol in the absence of Phenylalanine ammonia-lyase and Tyrosine ammonia-lyase pathways. These pathways are typically present in plants and the derived metabolites of these routes confer floral and fruity aroma to wine. Martin et al. (2016b) have proposed two alternative routes for benzenoids formation from sugars or from active Phenylalanine catabolism. Besides, this species has also demonstrated to contribute to the red colour of wine thanks to the production of vitisin B pigment and the increment of acetaldehyde content, necessary for visitin B formation (Medina et al., 2016).

Therefore, considering all the features exhibited by this yeast species, we can understand the increasing number of research studies focused on *H. vineae* and also the great interest on its application in oenology.

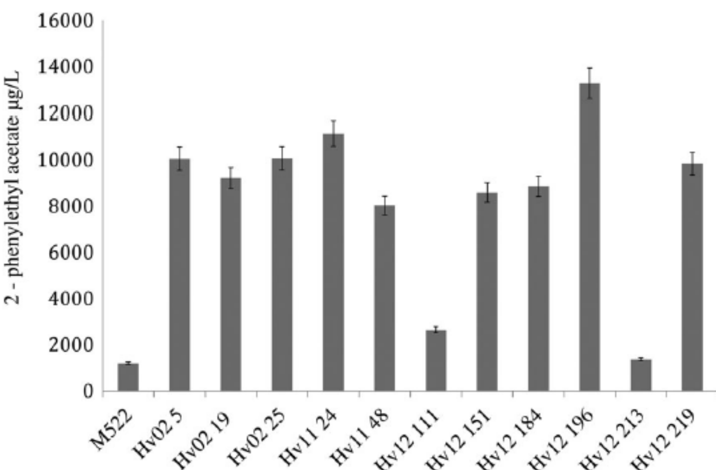


Figure 6. Production of 2-phenetyl acetate by different *H. vineae* strains in comparison to *S. cerevisiae* M522 (Martin et al., 2016a).

3.1.2. Spoiler microorganisms

Several microorganisms can develop along the winemaking process carrying problems such as the alteration of microbial population or the production of compounds that result in unpleasant flavors or aromas. Therefore, they are commonly known as spoiler organisms. In this section, three common spoiler microorganisms belonging to fungi, yeast and bacteria groups are described.

➢ ***Botrytis cinerea***

B. cinerea (teleomorph *Botryotinia fuckeliana*) is a filamentous fungus frequently found to infect mature berries responsible for grey rot or *Botrytis* bunch rot. This fungus is a non-host-specific necrotrophic pathogen that occurs worldwide and it can infect a broad range of plants. In vineyards, it usually develops under cool and high rainfall conditions during ripening period. Moreover, this fungus can survive in the soil and expand on the plant parts scattered on the vineyard floor. Under excessive wet conditions, the berry skin can rupture increasing the possibilities of *B. cinerea* development in the plant. Besides, when cold nights are combined with warm dry days, this fungus can develop the noble rot syndrome that consists on the dehydration of the berry concentrating the sugar and generating glycerol (Steel et al., 2013). Despite being

considered a spoiler organism, the presence of *B. cinerea* is desired for the production of some sweet wines. For this reason, grapes go through an extended ripening before harvesting and through a prolonged period of drying before crushing to promote the development of *B. cinerea* (Stefanini et al., 2016).

B. cinerea displays some oxidative and esterase activities that degrades phenolic compounds affecting the wine colour (proanthocyanidins, catechin and epicathequin), transforms terpenes in less odorous compounds, hydrolyses ethyl esters of fatty acids and produces glucans that difficult wine clarification (Barata et al., 2012b). In addition, it also releases some compounds related to off-flavours and aromas in wine, described as mushroom or earthy odours. These compounds include 2-methylisoborneol, geosmin, 1-octen-3-one, 1-octen-3-ol, 2-octen-1-ol, and 2-heptanol (La Guerche et al., 2006). Moreover, *B. cinerea* also affects fermentation performance since it uses ammonium, amino acids and vitamins like thiamine for growth. Therefore, to avoid stuck fermentations and the production of hydrogen sulphide by fermentative yeasts it is necessary to supplement the must from *Botrytis*-infected grapes with nitrogen and vitamins (Bell and Henschke, 2005).

- ***Brettanomyces bruxellensis***

B. bruxellensis (anamorph of *Dekkera bruxellensis*) is a spoiler yeast commonly isolated from wine, especially from aging wines in barrels. This yeast is able to live in harsh environments like wine, which contains scarce nutrient availability, low pH and high ethanol and sulphite concentrations. Indeed, it has developed several mechanisms to survive in these hostile environments enabling it to use many carbon and nitrogen substrates for growth, such as ethanol, glucose and fructose or amino acids, nitrates and nitrites (Smith and Divol, 2016).

The contamination of wine with *B. bruxellensis* is commonly originated from unsanitary treated winery material, especially from wood barrels. In addition, the ability of this yeast to form biofilms hinders an appropriate disinfection of the barrels (Oelofse et al., 2008). As well as good sanitary practices should be applied, an effective concentration of sulphite should be added to avoid *B. bruxellensis* development since many of its isolates have been described as high sulphite-tolerant (Curtin et al., 2012).

The main fault associated with *B. bruxellensis* is the production of volatile phenols, basically 4-ethylphenol and 4-ethylguaiacol, from its hydroxycinnamic acids, *p*-coumaric acid and ferulic

acid respectively. These compounds confer unpleasant aromas to the wine known as "Brett" character and described as phenolic, leather, horse sweat or stable odours. Furthermore, these hydroxycinnamic acids are mainly present in grape seeds and are transformed by *B. bruxellensis* to volatile phenols through two-enzyme process (Figure 7) (Curtin et al., 2015). The sensorial threshold of 4-ethylphenol and 4-ethylguaiacol is considerable small being 230 µg/l and 47 µg/l, respectively (Chatonnet et al., 1990). Therefore, low quantities of these volatile phenols are easy appreciable by wine consumers and represent a concerning issue for the producers.

Figure 7. Formation process of ethylphenols from their hydroxycinnamic precursors (Suárez et al., 2007).

- **Acetic Acid Bacteria (AAB)**

The spoiler role of AAB can occur mainly in two steps of the winemaking process: in grapes before vintage, known as sour rot, or during aging in barrel or bottle storage. First of all, sour rot is a grape disease that affects late-ripening vineyards with dense and thin-skinned berry bunches. This type of rot is induced by yeasts with the collaboration of AAB (*Acetobacter* spp. and *Gluconobacter* spp.) and it has detrimental effects on wine quality basically through the production of acetic acid (Barata et al., 2012b).

Sour rot disease begins with the skin damage of the berry by biotic (e.g. birds or molds) or abiotic factors (e.g. rain or berry abrasion). This skin injury can be healed by the plant. However, if insects like *Drosophila* sp. are present, the berry surface can evolve to a disease situation since it transports yeasts and AAB. Firstly, yeasts would ferment berry sugars producing ethanol followed by the development of *Gluconobacter* and *Gluconoacetobacter* spp. with the release of gluconic

and acetic acid. Finally, *Acetobacter* spp. would oxidize the ethanol increasing the amounts of acetic acid. The volatile compounds produced by these organisms will attract more insect populations which would further spread sour rot to other injured berries (Barata et al., 2012c). The main drawbacks of wines produced from sour rot grapes consist in the presence of higher volatile acidity and residual sugars (Barata et al., 2011).

The other points of potential AAB spoilage are barrel aging and bottle storage. Similarly to *B. bruxellensis*, unsterilized or ineffective cleaned material represent the main sources of AAB in these steps. However, oxygen is the main responsible factor for their development. Thus, if the anoxic environment is disrupted, the present AAB would proliferate and spoil the wine (Bartowsky and Henschke, 2008). Moreover, the trend in reducing SO_2 content in wine together with non-filtration step before bottling in red wines can contribute to the development of the resident AAB populations, especially when oxygen is able to enter through the bottle cork in vertically-stored wine bottles (Bartowsky et al., 2003).

3.1.3. *Saccharomyces* and non-*Saccharomyces* yeasts interactions

Nowadays, winemaker's interests are especially focused on taking advantage of both *Saccharomyces* and non-*Saccharomyces* yeasts. On the one hand, the development of non-*Saccharomyces* yeasts is expected during first fermentation stages to provide complexity to the wine. Meanwhile, *S. cerevisiae* imposition from the middle to the end of the fermentation is desired to ensure the success of the process. Thus, the study of the interactions between *Saccharomyces* and non-*Saccharomyces* yeasts is gaining attention.

Interactions between *Saccharomyces* and non-*Saccharomyces* yeasts are mainly based on nutrient competition, the release of antimicrobial compounds or cell-to-cell contact mechanism (Figure 8). Considering the high competitiveness of *S. cerevisiae*, the inoculum size ratio of *Saccharomyces* and non-*Saccharomyces* yeasts have to be considered, especially in mixed fermentations when non-*Saccharomyces* contribution is desired (Andorrà et al., 2012; Viana et al., 2011a). Moreover, performing sequential fermentations seems to be a useful way to ensure the activity of non-*Saccharomyces* yeasts (Azzolini et al., 2012; Viana et al., 2011a).

Among all the competition factors, ethanol concentration is broadly known to provide *S. cerevisiae* dominance over the other yeast species (Pretorius, 2000). Nevertheless, competition for nitrogen and vitamins also plays a key role in yeasts interaction (Medina et al., 2012; Taillandier et al., 2014).

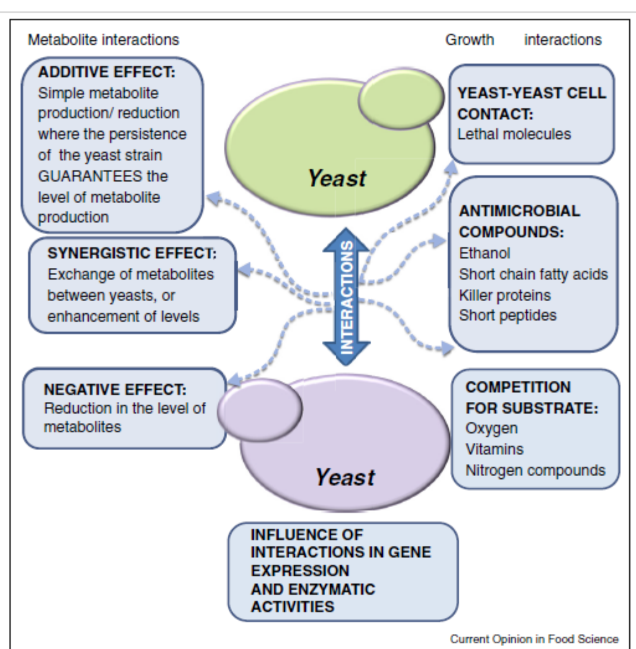


Figure 8. Possible yeast interactions during mixed fermentations (Ciani and Comitini, 2015) .

In recent years, researchers are focusing on the determination of the nitrogen preferences of the different non-*Saccharomyces* yeast to assess their impact on *Saccharomyces* (Gobert et al., 2017; Prior et al., 2019). Additionally, this interaction is not always negative since non-*Saccharomyces* yeasts have shown to not only release nitrogen and support *S. cerevisiae* activity (Prior et al., 2019) but also to stimulate nitrogen and sugar metabolism of *S. cerevisiae* (Curiel et al., 2017).

Other environmental factors like oxygen availability and temperature also take part in these yeast interactions. While *S. cerevisiae* is able to rapidly grow under anaerobic conditions, most non-*Saccharomyces* yeasts poorly develop in these conditions becoming an advantage for *S. cerevisiae* (Albergaria and Arneborg, 2016). In the case of temperatures, low temperatures have been proven to favor non-*Saccharomyces* over *Saccharomyces* yeasts (Ciani and Comitini, 2006).

The release of bioactive yeast metabolites also defines the interaction and dominance of yeast species. Branco et al. (2014) identified the antimicrobial peptides produced by *S. cerevisiae* as fragments of the protein glyceraldehyde-phosphate deshydrogenase. These peptides are toxic for specific non-*Saccharomyces* species, such as *K. marxianus, K. thermotolerans, H. guillermondii* and *B. bruxellensis* (Albergaria et al., 2010). Moreover, Wang et al. (2016) demonstrated that the effect of the metabolites produced by *S. cerevisiae* on the culturability of non-*Saccharomyces* yeasts also depends on yeast species and strain. As well as *S. cerevisiae,* non-*Saccharomyces* yeasts also produce killer toxins against other yeast species (Table 5). Indeed, some of these strains have been

postulated as biocontrol agents to prevent the growth of spoiler microorganisms like *B. bruxellensis* (Comitini and Ciani, 2011; Villalba et al., 2016).

Lastly, cell-to-cell contact is another mechanism that has shown to influence yeast interactions (Ciani et al., 2016). Renault et al. (2013) studied this phenomenon in mixed fermentations with *T. delbrueckii* and *S. cerevisiae* and found that *S. cerevisiae* induced *T. delbrueckii's* dead when they were in physical contact. However, when these yeasts where physically separated, *T. delbrueckii* maintained its viability and metabolic activity.

3.2. Abiotic factors

Abiotic factors are environmental aspects that can affect the development of microorganisms and, consequently, the microbiota dynamics during the winemaking. Thus, these factors include, among others, from vineyard management and climate to substrate availability, pH or temperature (Figure 9). In the following sections, attention will be focused on some of these factors of special relevance.

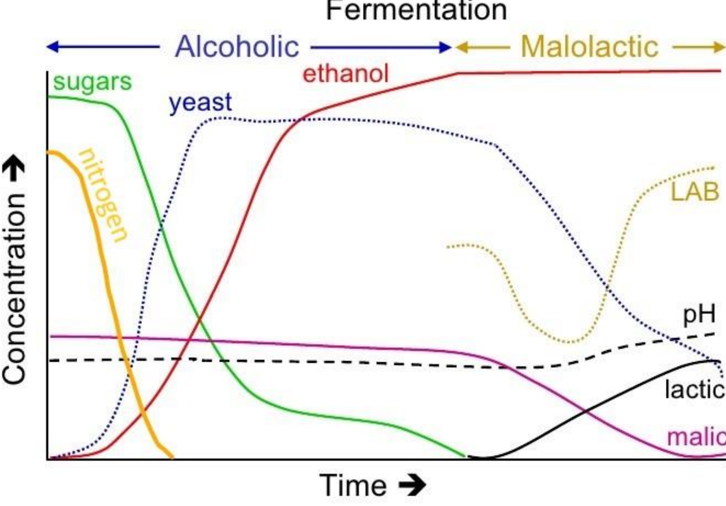


Figure 9. Progress of the main parameters during alcoholic and malolactic fermentation. Adapted from Jiranek (2010).

3.2.1. Climate and vineyard management

The microbiota present in grapes has been broadly studied for many years. These works have concluded that grape microbiota depends on the age, size and location of the vineyard and also on grape variety and vintage (reviewed by Barata et al. 2012b). Notwithstanding, all this research was performed using culture-dependent techniques and probably employing insufficient sample

numbers which can lead to unreliable conclusions. Therefore, during last years many researchers are applying NGS techniques to fill these gaps deciphering the grapevine, vineyard and grapes microbiome and the factors that shape these bacterial and fungal populations (Stefanini and Cavalieri, 2018).

On the one hand, vintage and climate modulate soil and grape microbiota. For example, Grangeteau et al. (2017) observed greater proportions of moulds in drier and warmer vintages. Moreover, aspects like precipitation, maximum temperature, humidity, latitude, longitude and vineyard orientation are the main responsible agents of grape microbial community divergences (Bokulich et al., 2014; Portillo et al., 2016). Regarding to grapevine plant, not only vine varietals dispose different bacterial and fungal populations (Bokulich et al., 2014), also the variations in grape clone background affect the bacterial microbiome (Zhang et al., 2018). Moreover, the microbial communities present in grapes are the ones lately found in fresh must. However, as the fermentation progresses, these populations have shown to resemble more to the ones found in the vine bark (Morrison-Whittle and Goddard, 2018).

Furthermore, related to soil vineyard microbiome, it has been demonstrated that the diversification of fungal population increase with geographic distance (Miura et al., 2017). Indeed, many wine-fermentation microorganisms come from uncultivated ecosystems outside the vineyards (Morrison-Whittle and Goddard, 2018). Nevertheless, the soil carbon and nitrogen pools would finally determine the structure of these fungal and bacterial communities (Burns et al., 2015, 2016).

Finally, phytosanitary practices also define grape microbiota. Grangeteau et al., (2017) observed a biodiversity decrease on those grapes with organic protection in comparison with grapes treated with conventional and ecophyto methods. Furthermore, they also found a relationship between the supplementation of SO_2 with the early implantation and dominance of *S. cerevisiae*.

Thus, the location of the vineyard together with grape varietal, climatic conditions and anthropogenic treatments will determine the microbiome of the vine and grapes.

3.2.2. Nutrients

The nutrients present in grape must and its evolution during the alcoholic fermentation determine yeast development. Therefore, yeast cells have developed different strategies to cope the excess or limitation of some key nutrients for their growth, such as sugar and nitrogen.

3.2.2.1. Sugar and ethanol

One of the first stresses that yeast have to face is the initial sugar content. Moreover, sugar concentration is directly related to ethanol toxicity since the sugars present in the grape juice will be transformed into ethanol during alcoholic fermentation. Thus, both parameters will be addressed in next paragraphs.

First of all, yeasts have to deal with the osmotic stress derived from the high glucose and fructose concentrations (≥ 200 g/l) of the initial grape must. When yeasts are exposed to this situation, there is a release of water from the cell, a decrease of turgor pressure and a reduction of water availability. Therefore, to counteract this osmotic shock, the yeast modifies the cell wall and the cytoskeleton together with the synthesis of glycerol to reestablish the osmotic balance. The synthesis of glycerol is mediated by the high osmolarity glycerol (HOG) signal transduction pathway (Bauer and Pretorius, 2000).

As fermentation proceeds, nutrients are consumed and ethanol concentration increases. One of the most evident effects of ethanol on yeast cells is the increase membrane permeability and fluidity. At the same time, this membrane permeability results in the intracellular acidification of the cell. Moreover, metabolic processes like proton-dependent transport, enzymatic activities and protein folding are also affected. Among others, the cellular response to this situation involves the synthesis of trehalose and the induction of heat shock proteins (Bauer and Pretorius, 2000; Querol et al., 2003). Additionally, to restore the membrane structure, the yeast increments the percentage of unsaturated fatty acids and modifies the sterol content (Arneborg et al., 1995). These effects can be observed in *S. cerevisiae* when ethanol content achieves 4% v/v while, in non-*Saccharomyces* yeasts, those can be observed even in 2% v/v ethanol concentrations.

Ethanol content is considered one of the major drivers of yeast population dynamics during wine fermentation. Therefore, the great dominance of *S. cerevisiae* during fermentation is broadly explained by its higher ethanol tolerance (Arroyo-López et al., 2010) over the non-*Saccharomyces* yeasts, which were considered to survive in ethanol contents up to 4-5% v/v (Albergaria and

Arneborg, 2016). Nevertheless, later studies have shown that several non-*Saccharomyces* yeast species are able to tolerate much higher ethanol levels than previously expected (Pina et al., 2004; Salvadó et al., 2011, Wang et al, 2015). Despite the role of ethanol on non-*Saccharomyces* yeasts survival, it has been demonstrated that other toxic compounds produced by *S. cerevisiae* seems to have a stronger influence on non-*Saccharomyces* disappearance in mixed fermentations (Pérez-Nevado et al., 2006).

Currently, climate change represents one of the major concerns in wine production since it modifies grape must composition. The main consequences on grapes are the increase of sugar content and the decrease of organic acids, especially malic acid (Querol et al., 2018). Consequently, the final wines exhibit greater ethanol content that exacerbates the yeast stress to ethanol during fermentation. This higher ethanol concentration also alters the perception of aroma complexity in wines (Dequin et al., 2017). In addition, the consumer preferences for lower ethanol wines makes the problem even worse. The case of Australia, where the ethanol concentration in red wines has increased around 1% v/v per decade since 1980 and cultivars like Shiraz are being harvested at high sugar concentration (>240 g/l), represents a good example of this situation (Varela et al., 2015). Indeed, it was unusual to find wines with more than 14% v/v few decades ago. Nowadays, it is common to encounter wines with an ethanol content of more than 16% v/v (Varela et al., 2015). Therefore, several strategies to reduce ethanol content in wines, such as the use of *Saccharomyces* hybrids or non-*Saccharomyces* yeasts, are being studied (Dequin et al., 2017; Varela et al., 2015).

3.2.2.2. Nitrogen

Nitrogen is one of the most critical nutrients for yeast growth during winemaking. The nitrogen sources used by yeasts during fermentation are known as yeast assimilable nitrogen (YAN) and consist in a mixture of ammonium ions and amino acids. In grape musts, the concentration of YAN is very variable ranging from 60 to 400 mg N/l depending on grape variety, environmental conditions and cultivar management (Bely et al., 1990).

The availability of nitrogen affects yeast growth, fermentation performance and the organoleptic characteristics of the resulting wine (Brice et al., 2014; Swiegers et al., 2005). In fact, low nitrogen levels can lead into stuck or sluggish fermentations (Bisson, 1999). Moreover, 300 mg N/l of YAN is the concentration needed to achieve an optimum fermentation kinetics (Marsit

and Dequin, 2015). Meanwhile, a YAN content of 140 mg N/l is considered as the minimum concentration to complete alcoholic fermentation by *S. cerevisiae* (Bell and Henschke, 2005). However, this condition depends on the amount of sugar present in the grape must, requiring large amounts of nitrogen under high sugar conditions (Martínez-Moreno et al., 2012). This indicates the importance of this nutrient, especially in recent days where musts exhibit higher sugar levels as a result of climate change conditions.

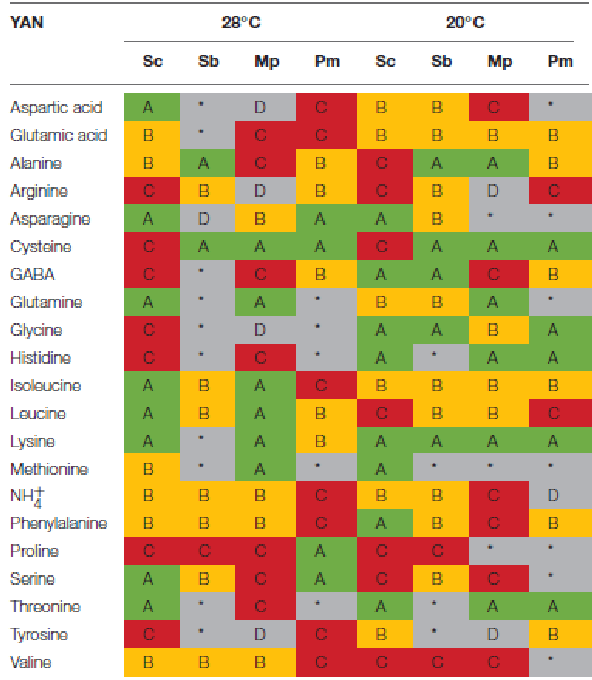
Likewise, exists a great variability in YAN uptake and nitrogen consumption profiles and preferences within wine and non-wine *S. cerevisiae* species and strains (Gutiérrez et al., 2012; Jiranek et al., 1995). Indeed, these divergences are the result of modifications in the regulation mechanisms of nitrogen uptake between strains (Gutiérrez et al., 2013). Nevertheless, Crépin et al. (2012) analyzed the consumption kinetics of 18 nitrogen compounds by 14 *S. cerevisiae* strains in synthetic grape-must medium and, despite the differences in the nitrogen consumption profile, all the strains consumed the different nitrogen sources in a similar order. Thus, these researchers were able to classify the nitrogen compounds according to the order in which they were consumed (Table 9).

Table 9. Consumption pattern of different nitrogen sources by *S. cerevisiae* strains under wine fermentation conditions (Crépin et al., 2012).

Prematurely consumed	Early consumed	Late consumed
Lysine	Asparagine, threonine, glutamic acid, leucine, histidine, methionine, isoleucine, serine, glutamine, phenylalanine	Ammonium*, valine, arginine, alanine, tryptophan, tyrosine

*Ammonium was observed to be rapidly consumed when the concentration of a preferred nitrogen sources like glutamine was low.

As it has been mentioned, many studies have focused on nitrogen metabolism, consumption and preferences between *S. cerevisiae* strains (Brice et al., 2018; Crépin et al., 2012; Gutiérrez et al., 2012; Jiranek et al., 1995). However, considering the increasing interest on mixed and sequential fermentations, little is known about non-*Saccharomyces* yeast nitrogen preferences. In fact, the rapid nitrogen depletion by non-*Saccharomyces* species, such as *T. delbrueckii, H. vineae* and *M. pulcherrima*, has been reported in sequential fermentations disabling *S. cerevisiae* development and resulting in sluggish and stuck fermentations (Medina et al., 2012; Taillandier et al., 2014).



YAN	28°C				20°C			
	Sc	Sb	Mp	Pm	Sc	Sb	Mp	Pm
Aspartic acid	A	*	D	C	B	B	C	*
Glutamic acid	B	*	C	C	B	B	B	B
Alanine	B	A	C	B	C	A	A	B
Arginine	C	B	D	B	C	B	D	C
Asparagine	A	D	B	A	A	B	*	*
Cysteine	C	A	A	A	C	A	A	A
GABA	C	*	C	B	A	A	C	B
Glutamine	A	*	A	*	B	B	A	*
Glycine	C	*	D	*	A	A	B	A
Histidine	C	*	C	*	A	*	A	A
Isoleucine	A	B	A	C	B	B	B	B
Leucine	A	B	A	B	C	B	B	C
Lysine	A	*	A	B	A	A	A	A
Methionine	B	*	A	*	A	*	*	*
NH_4^+	B	B	B	C	B	B	C	D
Phenylalanine	B	B	B	C	A	B	C	B
Proline	C	C	C	A	C	C	*	*
Serine	A	B	C	A	C	B	C	*
Threonine	A	*	C	*	A	*	A	A
Tyrosine	C	*	D	C	B	*	D	B
Valine	B	B	B	C	C	C	C	*

Figure 10. Nitrogen sources preferences at T_{50} (time point at which the 50% of the initial YAN concentration is consumed) on different temperature conditions (Gobert et al., 2017). *A* indicates preferred sources, *B* intermediate sources, *C* non-preferred sources, *D* very low levels of assimilation and * absence of assimilation. Yeasts evaluated correspond to *S. cerevisiae SLM* (Sc), *S. bacillaris BBMV5FA17* (Sb), *M. pulcherrima BB810* (Mp) and *P. membranifaciens BB3* (Pm).

Recently, the preferential use of amino acids and ammonium by different non-*Saccharomyces* yeast strains has been assessed (Gobert et al., 2017; Prior et al., 2019). As stated in figure 10, each non-*Saccharomyces* yeasts display specific amino acid consumption profiles (Gobert et al., 2017).

Furthermore, there is an evident effect of temperature on YAN assimilation profiles. This situation has been previously observed in *S. cerevisiae* and it was suggested to be a consequence of the changes in plasma membrane to deal with lower temperatures, which simultaneously affect membrane permeases involved on YAN transport (Beltran et al., 2007).

As well as nitrogen consumption by non-*Saccharomyces* can interfere on *S. cerevisiae* growth in sequential fermentations, the depletion of some nitrogen sources also hinder the formation of some volatile compounds by *S. cerevisiae*. As an example, Gobert et al. (2017) reported that leucine consumption by non-*Saccharomyces* yeasts, which is the precursor of isoamyl acetate in *S. cerevisiae*, reduced the levels of this volatile compound in sequential fermentations when compared with pure cultures of *S. cerevisiae*.

3.2.2.2.1. Nitrogen catabolite repression

There are many nitrogenous compounds that *S. cerevisiae* is able to use for biomass production. However, not all these nitrogen sources promote its growth in the same way. Considering this feature, nitrogenous elements can be classified as preferred and non-preferred nitrogen sources. While growth in preferred nitrogen sources like glutamine or ammonium allow

high growth rate, *S. cerevisiae* exhibits slow growth in media with non-preferred nitrogen sources, such as proline or urea.

Therefore, *S. cerevisiae* displays a widely known regulation mechanism to select the best nitrogen sources for growth. This mechanism is called nitrogen catabolite repression (NCR) and acts at protein and transcriptional levels. The first consists in the inactivation, internalisation and degradation of the permeases that transport non-preferred nitrogen sources. Finally, the second one involves the regulation of the expression of the genes encoding for these permeases.

The responsible operators of transcriptional regulation include one regulatory protein (Ure2p) with two positive (Gln3p, Gat1p/Nil1p) and two negative (Dal80p, Gzf3p) GATA-binding transcriptional factors. During excess nitrogen conditions, the NCR activator Gln3p is in the cytoplasm bound to Ure2p, which prevents Gln3p entrance to the nucleus. Under limiting nitrogen conditions, Gln3p dissociates from Ure2p and moves to the nucleus where promotes the transcription of NCR-sensitive genes like some permeases as *GAP1, MEP2* and *PUT4* or genes related to nitrogen metabolism as *PUT2* or *GLN1* (Figure 11) (Magasanik and Kaiser, 2002; Ter Schure et al., 2000).

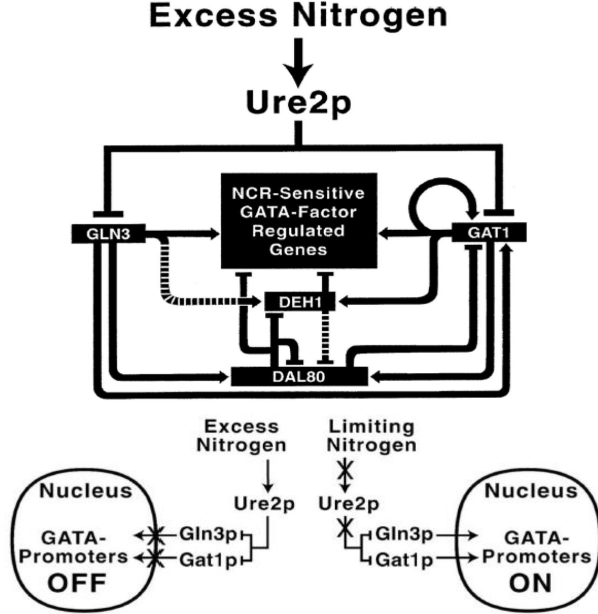


Figure 11. Model of NCR sensitive genes expression through GATA factors regulation under excess and limiting nitrogen content. Arrowheads and bars designate respectively positive and negative regulation. “X” indicates processes are not happening under the specific nitrogen conditions (Cooper, 2002).

Additionally, there is also a plasma membrane Ssy1-Ptr3-Ssy5 (SPS) amino acid sensor pathway that regulates the expression of specific amino acid permeases at the start of nitrogen consumption. This mechanism detects the presence of specific amino acids in the medium and induce the transcription of the specific amino acid permeases which elucidates the fast consumption of some amino acids during the beginning of the fermentation (Ljungdahl, 2009; Regenberg et al., 1999).

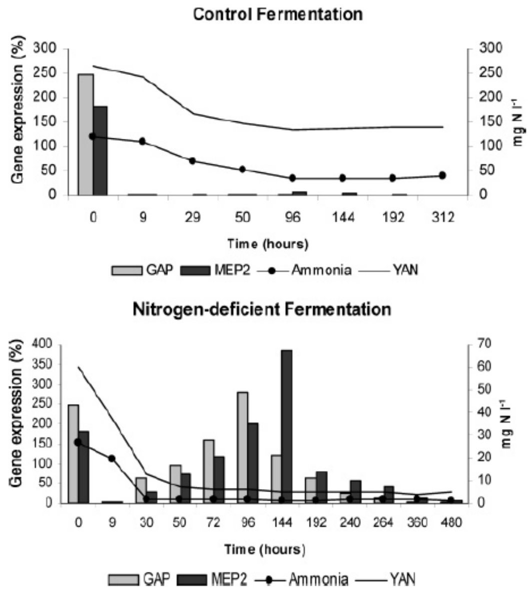


Figure 12. Gene expression of the general amino acid permease, *GAP1,* and the ammonium permease, *MEP2,* at time zero (before inoculation) and at different points of control and nitrogen-deficient fermentations (Beltran et al., 2005).

The specific nitrogen consumption and regulation of NCR mechanism has been deeply evaluated in *S. cerevisiae* in wine-like conditions (Brice et al., 2018; Gutiérrez et al., 2012; Tesnière et al., 2015). Specifically, the analysis of the expression of NCR-sensitive genes represents a good tool to assess the activation of this mechanism in different fermentation conditions (Beltran et al., 2004). Figure 12 is a good example of the expression changes by NCR-regulated permeases, *GAP1* and *MEP2,* under the presence or lack of nitrogen (Beltran et al., 2005). In the nitrogen-deficient fermentation, both genes start to express when almost all YAN and ammonia are consumed and they reach maximum expression levels after 4 and 6 days. Finally, after several days of nitrogen starvation, the expression of these genes decrease. When nitrogen is available, the transcriptional activators of NCR cannot enter to the nucleus and induce the expression of these permeases, which explains the repression of *GAP1* and *MEP2* throughout control fermentation.

3.2.3. Other abiotic factors: oxygen, temperature, pH and SO_2

Besides sugar, ethanol and nitrogen, other abiotic factors intercede on fermentation evolution and define the final wine composition. These factors include the availability of oxygen, the fermentation temperature, the addition of SO_2 and the pH of the must.

➢ **Oxygen**

Alcoholic fermentation occurs under oxygen-limited conditions and the availability of oxygen is decisive for the development and survival of the yeast species present along the fermentation. At the beginning of the fermentation there is a great availability of molecular oxygen. However, this amount of oxygen is rapidly consumed by the microorganisms present at the onset of the fermentation transforming it to an anaerobic environment (Albergaria and Arneborg, 2016).

While most of the yeast species known grow badly under strict anaerobic conditions, *S. cerevisiae* is able to grow rapidly in this situation giving it a great advantage over non-*Saccharomyces* yeast during wine fermentation (Visser et al., 1990). In fact, Hansen et al. (2001) showed how the low availability of oxygen caused *T. delbrueckii* and *L. thermotolerans* death in mixed fermentations while the increase of oxygen levels raised the survival rate of these yeast species. Moreover, the minimum oxygen consumption rate required for steady-state growth of *T. delbrueckii* is higher than in *S. cerevisiae* which would explain the poorer growth of *T. delbrueckii* under strict anaerobiosis (Hanl et al., 2005). Taillandier et al. (2014) also demonstrated the ability of *T. delbrueckii* to grow and complete alcoholic fermentation under anaerobic conditions. Despite this fact, *S. cerevisiae* has shown to grow and ferment more efficiently than *T. delbrueckii* and *L. thermotolerans* when low oxygen is available (Nissen et al., 2004).

Thus, the oxygen availability affects the development and fermentation capability of non-*Saccharomyces* yeasts. Nevertheless, this factor does not seem to be the main driver of their early death in mixed fermentations (Albergaria and Arneborg, 2016; Nissen et al., 2004; Nissen and Arneborg, 2003; Taillandier et al., 2014).

➢ **Temperature**

Temperatures higher than 15-20 °C together with the presence of ethanol decrease yeast growth through the disruption of membrane integrity and permeability (Albergaria and Arneborg, 2016). In fact, temperature has been proposed to be decisive in the niche construction hypothesis proposed by Goddard (2008). According to this theory, *S. cerevisiae* activities would modify its environment to give it an ecological advantage over its competitors. The vigorous sugar fermentation performed by this yeast releases an amount of energy responsible for the temperature increase during wine fermentation (up to 6 °C) (Goddard, 2008). This would be the

reason of the better performance of *S. cerevisiae* than other yeast species at higher temperatures (Arroyo-López et al., 2009; Salvadó et al., 2011).

Salvadó et al. (2011) assessed the effect of increasing ethanol concentration (0-25%) and temperature (4-46 °C) on *S. cerevisiae* competition with several non-*Saccaromyces* yeasts (*H. uvarum, T. delbrueckii, S. bacilaris, P. fermentans* and *K. marxianus*) during wine fermentation. They found that ethanol production by *S. cerevisiae* did not give a clear advantage when its content was below 9% v/v. However, an increase of temperature above 15 °C provides a considerable advantage to *S. cerevisiae* over non-*Saccharomyces* species. Indeed, several non-*Saccharomyces* and *Saccharomyces* non-*cerevisiae* yeasts show greater growth rate at low temperatures (Albertin et al., 2014; Alonso-del-Real et al., 2017; Charoenchai et al., 1998) and they are even able to persist and dominate over *S. cerevisiae* in wine fermentations performed at temperatures below 20 °C (Alonso-del-Real et al., 2017; Ciani and Comitini, 2006).

Therefore, the temperature at which vinification would take place will define the microorganisms present during the fermentation and their fermentation performance.

- **pH**

The initial pH of the must is also a determinant factor for yeast growth and survival. The grape must is an acidic environment and its pH usually ranges from 2.8 to 3.8. In fact, the ability of *S. cerevisiae* to survive in these acidic environments have been considered one the features for its dominance in wine fermentations (Pretorius, 2000). Nevertheless, climate change is unfolding a new situation for winemaking affecting grape maturation and resulting in musts with higher sugar concentrations and pH values (Querol et al., 2018).

The effect of pH has been evaluated in several non-*Saccharomyces* yeasts but no evident changes in the growth rate are observed (Charoenchai et al., 1998; Gao and Fleet, 1988). Besides, *Candida pulcherrima* showed a slight increase of fermentation ability in high pH musts (Jolly et al., 2003). Further research is needed to clarify the effect of pH on fermentation dynamics and also on non-*Saccharomyces* yeasts of oenological interest.

- **SO_2**

The addition of sulfur dioxide or SO_2 in grape musts is a common practice in wineries. The application of SO_2 aims to avoid oxidative reactions and inhibit the growth of the indigenous

microorganisms present in the must to ensure the implantation of the starter yeast cultures (Boulton et al., 1996). However, after dissolving in water, sulfur dioxide exists in equilibrium between molecular SO_2, bisulfite and sulfite species and the dominant form depends on the pH (Figure 13) (Fugelsang and Edwards, 2007). The molecular SO_2 is considered as the antimicrobial form and its inhibitory action relies on the decrease of the intracellular pH once the SO_2 enters within the cell and dissociates into SO_3^{2-} and HSO_3^- (Pretorius, 2000).

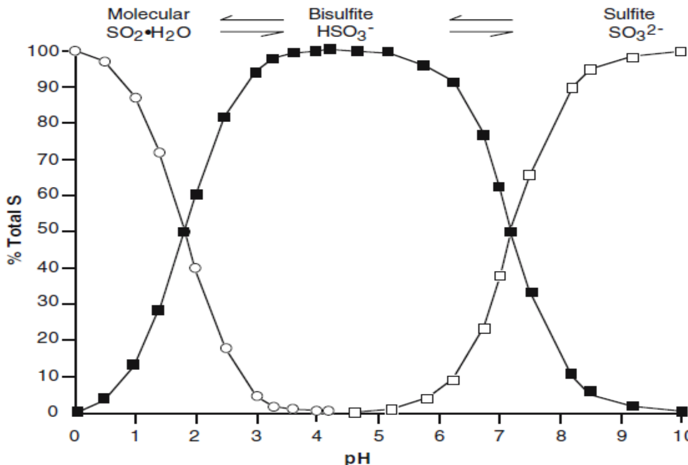


Figure 13. Relative abundance of molecular SO_2, bisulfite, and sulfite at different pH values (Fugelsang and Edwards, 2007).

Despite the high tolerance of *S. cerevisiae* to SO_2, an excessive addition of sulphite can lead to sluggish or stuck fermentations (Boulton et al., 1996). Moreover, in non-*Saccharomyces* yeast, the inhibitory action of SO_2 is increased by the ethanol produced during fermentation.

Several researchers have studied the addition of sulphite and its effect on yeast population dynamics during winemaking. Its inhibitory effect has been proved on non-*Saccharomyces* development (Constantí et al., 1998). However, this action was also observed with *S. cerevisiae* inoculation (Constantí et al., 1998) which demonstrated to confer a stabilizing effect itself without SO_2 addition (Bokulich et al., 2015). Furthermore, Cocolin and Mills (2003) demonstrated the persistence of *Hanseniaspora* and *Candida* populations, despite their loss of cultivability, in nonculturable state in wine fermenations treated with SO_2.

Furthermore, the tolerance to sulphite can vary widely between the different wine yeast species and strains (Curtin et al., 2012; Pretorius, 2000). Recent studies are evaluating the relationship between SO_2 tolerance and the yeast genotype to efficiently prevent spoilage with the minimal sulphite addition (Avramova et al., 2018).

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CHAPTER 1

Microbiome dynamics during spontaneous fermentations of sound grapes in comparison with sour rot and *Botrytis* infected grapes

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UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF NON-SACCHAROMYCES YEAST ON WINEMAKING AND QUALITY
Jéssica Lleixà Daga

Abstract

The main losses in viticulture around the world are normally associated with rotten grapes affecting both the chemical composition and the grape microbiota that later might affect the alcoholic fermentation. We analyzed the population in musts obtained from sour rotten, botrytized and healthy Macabeo grapes and the population dynamics during the spontaneous alcoholic fermentation by culture dependent and various culture independent methods including, for the first time, qPCR and massive sequencing. Grape health state affected the fermentation kinetics and also the microbial diversity and composition. Unexpectedly, the fermentation proceeded the fastest in the rotten must followed by the healthy and the botrytized grapes. As in previous studies, plate cell counts and qPCR results confirmed the increase in the number of both bacteria and fungi in the musts from damaged grapes. Massive sequencing detected higher biodiversity than the other techniques at each stage, with *Saccharomyces* and *Oenococcus* found already in the grape must. *Hanseniaspora osmophila* replaced to *Hanseniaspora uvarum* as the predominant yeast during the mid-fermentation stage for both damaged grapes. Furthermore, musts and beginning of fermentation from rotten and botrytized grapes consistently had a higher presence of the fungi *Zygosaccharomyces, Penicillium* and *Aspergillus* while high abundance of *Botrytis* was observed just for botrytized grapes. As expected, the acetic acid bacteria number increased in musts from rotten and botrytized grapes, mostly due to changes in proportion of the genus *Gluconoacetobacter* which remained more abundant during damaged grapes fermentation than during healthy ones. Interestingly, the presence of *Oenococcus oeni* at the end of the alcoholic fermentation was strongly affected by the health status of the grapes.

Keywords: wine, sour rot, *Botrytis*, massive sequencing, lactic acid bacteria, acetic acid bacteria

Introduction

The grape berry surface hosts a microbiota of filamentous fungi, yeast, and bacteria that can have an impact on grape and wine quality (Fleet, 2003; Ribéreau-Gayon et al., 2006). When the grape surface is altered (e.g. by damaged skin of the berry, highly compact bunches, excess of humidity, phytopathogen infections) the diversity and the population sizes of the microbiota are affected and can lead to the spoilage of the berry. Grape damage of the harvested bunches and the alteration of the grape ecological balance may compromise the vinification process and the final wine quality typically adding off-flavors (Steel et al., 2013). Thus, it is important to further investigate the microbiota diversity changes in damaged grapes and its influence on the alcoholic fermentation.

Sour rot and *Botrytis* infection are the most common causes of heavy grape berry crop losses. The sour rot affects mostly dense bunches close to harvesting and is typically characterized by vinegar odour and brown berries (Barata et al., 2012b). Disease aetiology is related with the skin rupture of the berry caused by physical factors (e.g. rain, hail, berry abrasion) or biological factors (e.g. insects, birds, moulds). The injuries on grape skin contribute to the development of yeasts and bacteria considered as the main responsible agents of this rot (Huber et al., 2011). Moreover, insects are an important source of microorganisms that can colonize grapes and proliferate once the injury in the skin is done (Barata et al., 2012c). *Botrytis* infection (also known as grey mold) is frequent in vineyards exposed to cold and wet conditions during the ripening period (Nigro et al., 2006). In the case of sweet wines, where the presence of *Botrytis cinerea* is desired, the grapes are subjected to an extended ripening before harvesting and to a prolonged period of drying before crushing to enhance the abundance of *B. cinerea* (Stefanini et al., 2016).

Previous studies have documented the microbiota in sound and damaged grapes, including sour rotten and *Botrytis*-affected grapes (Barata et al., 2008, 2012c; Mateo et al., 2014; Nisiotou et al., 2007, 2011). The results described how grape spoilage affects the grape microbiota, with damaged grapes harboring the highest yeast and acetic acid bacteria (AAB) population (Barata et al., 2008, 2012a; Mateo et al., 2014). However, most of these studies use culture based techniques probably leading to underestimation of the microbial species involved. Currently, it is accepted that culture-isolated microorganisms are not necessarily representative of the microbial diversity (Amann et al., 1995; Rantsiou et al., 2005). Thus, the reported species selected during grape

damaged by sour rot or *Botrytis* might be biased by the composition of culture media and the capacity of the microbes to grow on them (Cocolin et al., 2000; Millet and Lonvaud-Funel, 2000).

Recently, several culture-independent methods based on the genetic background have been used to analyze the microbial diversity from grapes to wine (reviewed in Cocolin et al., 2011). Generally, the use of molecular biology methods has not only endorsed the traditional results but has also been able to identify higher microbial diversity than previously expected (Nocker et al., 2007). Despite the potential of molecular techniques, we have just found one work where these were applied to study the microbial diversity of *Botrytis*-affected grapes (Nisiotou et al., 2011). Specifically, these authors used PCR-DGGE to monitor the yeast population changes during spontaneous fermentations of sound and *Botrytis*-affected grapes. The results included the detection of some bacterial genera not detected before in sour rot or botrytized musts like *Enterobacter, Bacillus* and *Staphylococcus*, some of them capable to survive in fermenting musts (Nisiotou et al., 2011).

Among molecular methods, massive sequencing (MS) technologies are becoming a widely used methodology to characterize more precisely the microbial community of complex environmental ecosystems, including food samples (Ercolini, 2013). For example, MS technologies have allowed metagenomic analysis of vineyard and wine microbiome deciphering which microorganisms are present with higher sensitivity than previous techniques and how their communities are affected by several magnitude factors (reviewed in Morgan et al., 2017).

In this study, we aim to establish the relationship between the sour rot and *Botrytis* infection affecting Macabeo grapes with specific changes on the grape microbiota. In order to achieve this objective, sound and damaged grapes were harvested and their microbial diversity monitored during subsequent spontaneous alcoholic fermentations by both culture dependent and independent methods including PCR-DGGE, qPCR and MS to weigh the biases introduced by the techniques in an effort to estimate the community changes introduced by sour rot and *Botrytis* infection.

Materials and methods

Grape samples and experimental wines

During 2016 vintage, grape clusters from the experimental vineyard of the Faculty of Enology (Mas dels Frares, Tarragona, Spain) were collected. The sampled vineyard plot produced Macabeo

cultivars. Samples were collected using gloves, ethanol, sterilized scissors and sterile plastic bags. Between 10 and 12 replicate grape clusters from different plants within the plot were collected from each grape state in order to capture the heterogeneity present in the sampled plot. Samples without damaged grapes or infection signals were denominated healthy or "H", grape clusters presenting brown, damaged grapes (typical from sour rot) were denominated rotten or "R" and grape clusters with grey mold in the surface (typical from the *Botrytis* affected grapes) were denominated botrytized or "B". H and R grapes were collected just before normal harvest, at the beginning of September and the B ones were collected two weeks later. Samples were immediately transported to the experimental cellar located 100 m away from the sampled plot and were crushed by a manual press, skins and seeds were removed by using a sieve resulting in approximately 3 litres of each grape health state. 50 ml of grape juice was directly sampled corresponding to "Must" samples. Afterwards, as a normal procedure in the cellar, 80 mg/l potassium metabisulphite (40 ppm SO_2) was added to the rest of the juice. Must samples and the rest of the juice were transported refrigerated to the laboratory within the next hour. Part of the must samples was directly used for microbiological culture and the rest of the must was stored at -80 °C until DNA extraction. The sulphited juice was incubated during 24 h at 4 °C to allow clarification. From each health status juice, triplicates of 400 ml clarified juice were incubated at 23 °C under agitation of 120 rpm in 500 ml flask and allowed to ferment spontaneously without inoculation.

Sampling and monitoring during spontaneous fermentations

The fermentation kinetics was followed considering the time needed to consume the 50% (t_{50}) and the 90% (t_{90}) of sugars. In order to easily monitor the fermentations, the density was measured daily with Densito 30PX Portable Density Meter (Mettler Toledo, Spain).

Glucose and fructose concentration was daily measured by Miura One Multianalyzer (TDI, Barcelona, Spain) using the enzymatic kit from Biosystems S. A. (Barcelona, Spain). Acetic acid and ethanol were just evaluated during late fermentation, in the last juice sampling point (when the juice density was below 1000 g/l and stable for two consecutive days). Acetic acid content was analyzed by Miura One Multianalyzer (TDI, Barcelona, Spain) using the enzymatic kit from Biosystems S. A. (Barcelona, Spain). In the case of ethanol, due to volume limitation, it was

measured on the last sampling point by enzymatic method using Ethanol Boehringer Mannheim kit (R-biopharm).

Samples for plating, qPCR, PCR-DGGE and massive sequencing were taken from the must, the beginning of the fermentation (24 h after the incubation), middle fermentation (juice density between 1050-1040 g/l) and, finally, late fermentation when the juice density was below 1000 g/l and stable for two consecutive days.

Plate culturing

Samples for plating were serially diluted in sterile MilliQ water (Millipore Q-PODTM Advantage A10), plated on (i) YPD medium (Glucose 2%, Peptone 2%, Yeast Extract 1%, Agar 1.7%) and (ii) lysine agar medium (Oxoid, England) plates incubated at 28 °C for 48 h; (iii) MRS Agar medium (De Man et al., 1960) supplemented with 4 g/l L-malic acid, 5 g/l fructose, 0.5 g/l L-cysteine, 100 mg/l nystatin and 25 mg/l sodium azide adjusted to pH 5.0 and incubated at 28 °C in a 10% CO_2 atmosphere and (iv) GYC Agar (glucose 5%, yeast extract 1%, $CaCO_3$ and agar 2%, pH 6.3) supplemented with 100 mg/l natamycin and incubated at 28 °C for 3–5 days under aerobic conditions. Appropriate dilution plates were counted. The YPD medium provided the total yeast counts, whereas the lysine agar medium is considered to provide the non-*Saccharomyces* cell counts since most *S. cerevisiae* strains have limited growth using lysine as a unique nitrogen source (De Angelo and Siebert, 1987; Heard and Fleet, 1986). However, it has to be considered that probably not all the non-*Saccharomyces* yeasts related to wine environment are able to use lysine as nitrogen source (Jolly et al., 2006). MRS medium and GYC provided LAB and AAB counts, respectively.

DNA extraction, qPCR and PCR-DGGE

Genomic DNA was extracted from grape must and spontaneous fermentation stages using the recommended procedure for the DNeasy Plant Mini kit (Qiagen, Hilden, Germany), including three bead-beating steps for 3 min in a FastPrep-24 bead beater (MP Bio, Solon, OH) to homogenize the samples. Extracted DNA concentration was measured by Nanodrop, adjusted with molecular grade water to a concentration of 50 ng/µl and stored at -20 °C until further processing.

Quantitative PCR (qPCR) was performed in an Applied Biosystems 7300 Fast Real-Time PCR System (Applied Biosystems). SYBR Premix Ex Taq (Tli RNase H Plus) was used according to the manufacturer's instructions (Takara). An ABI PRISM96 well optical plate was used for the reaction. This instrument automatically determined the Ct. Yeast quantification was performed using the primers YEASTF/YEASTR for total yeast (Hierro et al., 2006), CESPF/SCERR for *Saccharomyces* genus, CESPF/HUVR for *Hanseniaspora* genus (Hierro et al., 2007), AF/200R for *Starmerella bacillaris* (Andorrà et al., 2010), TodsL2/TodsR2 for *Torulaspora delbrueckii* (Zott et al., 2010), Mp5-fw/Mp3-bw for *Metschnikowia* spp. (Díaz et al., 2013) and Bc3F/Bc3R (Suarez et al., 2005) for *B. cinerea.* Bacterial quantification was performed using AQ1F/AQ2R primers for general AAB (González et al., 2006) and WLAB1/ WLAB2 for general LAB (Neeley et al., 2005). All the primers anneal the ribosomal gene region. Standard curves were calculated for each type of microorganism in triplicate samples using serial dilutions of purified DNA (Supplementary Table 1).

For the PCR-DGGE analysis, the primer pairs $U1^{GC}$/U2 and $341f^{GC}$/518r were used to amplify the specific U1/U2 of the 28S ribosomal region of yeast (Meroth et al., 2003) and the 16S ribosomal region of bacteria (Muyzer et al., 1993), respectively. The DGGE procedure followed the description in Lleixà et al. (2016). DNA from excised bands was re-amplified with the same primer pair without the GC-clamp and sequenced by Macrogen Company (South Korea). The BLASTN algorithm was applied to the GenBank database to identify the closest relative at species level. However, the taxonomic identification at species level is not accurate due to the length of the sequences.

Sequencing library construction

The library construction was done with the amplification of 1 sample for each of the musts and 2 samples for each of the fermenting points in the case of bacterial library. In the case of fungal library, 1 sample for each of the must and 1 sample of each of the fermented points were taken. The universal primer pairs 515F/806R (Caporaso et al., 2011) and FR1/FF390 (Prévost-Bouré et al., 2011) with adapters for the sequencing by the equipment PMG from Ion Torrent with chips 318 were used to amplify a region of the 16S and 18S ribosomal gene of bacteria and fungi, respectively. The use of 18S as taxonomic marker for eukaryotic genera is considered limited because many yeast species have no 18S sequence available in the databases, thus we used SILVA

(v119) database as described later on because it is more updated and includes more eukaryotic genera than other databases. The universal forward primers included a 10-bp barcode unique to each amplified sample. PCR reactions contained 5–100 ng DNA template, 1X GoTaq Green Master Mix (Promega), 1 mM $MgCl_2$, and 2 pmol of each primer. Reaction conditions consisted of an initial 94 °C for 3 min followed by 35 cycles of 94 °C for 45 s, 50 °C (for Bacteria) or 52 °C (Fungi) for 60 s, and 72 °C for 90 s, and a final extension of 72 °C for 10 min. PCR reactions were performed in triplicate for each sample replicate, pooled by sample and cleaned using a GeneRead Size Selection kit (Qiagen, Hilden, Germany). Cleaned PCR products were submitted to Centre for Omic Sciences (Reus, Spain) where their quality was checked by a Bionalyzer and their quantity adjusted for sequencing.

Data analysis

Raw sequences were demultiplexed and quality filtered using QIIME v1.9.1 (Caporaso et al., 2010b). Reads were discarded if the length of the read was <200 or >1000 and if any read contained one or more ambiguous base calls. Additionally, reads were truncated at any site containing 3 or more consecutive bases receiving quality score below 10 and reevaluating the remaining length with the aforementioned length rule. After quality filtering, 3,672,972 sequences remained with an average of 306,081 sequences per sample (Supplementary Table 2). Operational taxonomic units (OTUs) were picked by using QIIME's open-reference pipeline, where Greengenes (13_8) and SILVA (v119) were used as reference databases for 16S and 18S rRNA sequences correspondingly, at a 99% similarity threshold. The same databases and threshold have also been used for sequence alignment using PYNAST (Caporaso et al., 2010a) and OTU taxonomy assignment (Quast et al., 2013). The taxonomic assignment up to level species is not accurate for such a small fragment of DNA so the genera level was indicated except when the species was confirmed by qPCR and PCR-DGGE analysis. A final OTU table was created, excluding singletons (sequences observed just once), sequences detected by less of 0.001 abundance and sequences matching plant mitochondria or chloroplast. To avoid biases generated by differences in sequencing depth, bacterial and eukaryotic reads were rarefied to an even depth of 790 and 84,000 sequences per sample, respectively.

Alpha diversity (within-sample species richness) estimates were calculated by analyzing the observed OTUs, the phylogenetic diversity by the PD whole tree index, and Shannon and Simpson diversity indexes.

Statistical analysis

Beta-diversity (between-sample microbial community dissimilarity) estimates were calculated within QIIME using weighted UniFrac distances (Lozupone and Knight, 2005) between samples for bacterial sequences and eukaryotic sequences. Principal coordinate analysis (PCoA) was used to summarize and visualize patterns in species composition. ANOSIM (an analogue of univariate ANOVA which tests for differences between groups of samples) was performed in QIIME to determine significant differences in phylogenetic or species diversity among experimental factors (grape health state and fermentation stage). Kruskal-Wallis test was used to determine which taxa differed between sample groups. Taxonomic groups were considered to present significant differences in abundances across samples when False discovery rate (FDR)-corrected P values were lower than 0.05 for bacteria and $P < 0.05$ for fungi with no FDR correction due to the lack of replicated samples.

Results

Effect of grape health state on fermentation kinetics

Clarified musts from each health state, healthy (H), rotten (R) and botrytized (B), were divided into three biological replicates and allowed to ferment spontaneously (with no yeast or bacteria inoculation, Figure 1). Sugar concentration in R and B initial musts was higher than in the H one (Table 1). Despite the higher sugar content, R fermentations were the faster to consume the 50% (t_{50}) and the 90% (t_{90}) (Table 1). Both fermentations from H and B grapes consumed the 50% of the sugars in 5 days. However, H fermentations reached t_{90} in 11 days, 5 days earlier than the B ones that resulted in the slowest fermentations.

We also evaluated the sugar, ethanol and acetic acid concentration of the last sampling point, when density reached 1000 mg/l. In the case of H grapes, the fermentation was not complete on this time point considering the high sugar and low ethanol concentration. On the other hand, the low sugar and amount of ethanol suggested that R and B fermentations were almost finished at the last measured point. Interestingly, H and damaged grape juices presented similar acetic acid content (Table 1).

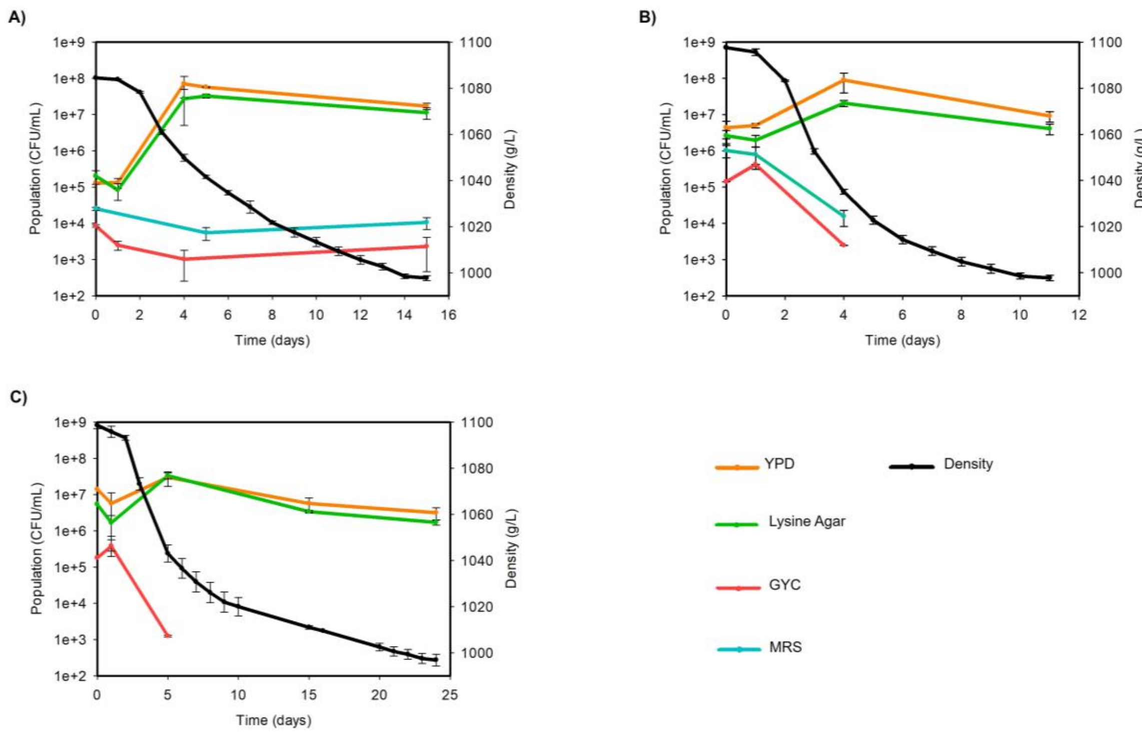

Figure 1. Fermentation density and population dynamics in YPD, Lysine Agar, MRS and GYC medium of (A) healthy, (B) rotten and (C) botrytized grape fermentations.

Table 1. Fermentation kinetics of healthy, rotten and botrytized grapes. The values indicate initial sugar content, sugar (residual sugars), ethanol and acetic acid concentration of the last sampling point of healthy, rotten and botrytized grapes. t_{50} and t_{90} are the time used to consume the 50% and 90% of initial sugars, respectively.

Fermentation	Initial sugar content (g/l)	t_{50} (days)	t_{90} (days)	Residual sugars (g/l)	Ethanol (% v/v)	Acetic acid (g/l)
Healthy	205,26 ± 0,59	5	11	11,40 ± 1,36	11,30 ± 0,09	0,79 ± 0,07
Rotten	225,45 ± 4,12	4	7	2,97 ± 1,51	12,83 ± 0,65	0,52 ± 0,03
Botrytized	226,21 ± 1,12	5	16	5,21 ± 2,63	12,74 ± 0,54	0,79 ± 0,04

Fungal and bacterial taxonomic composition of Healthy, Rotten and Botrytized musts and fermentations

Changes in microbial population were monitored along the alcoholic fermentation [must, beginning, middle fermentation and, finally, late fermentation (density was below 1000 g/l for two consecutive days)] of H, R and B Macabeo grapes.

As we have previously mentioned, culture dependent (plate culturing in specific media) and independent techniques (qPCR, PCR-DGGE and MS) were applied and compared.

Plate culturing

The yeast population quantification was based on the colony growth in YPD (total yeast population) and lysine agar medium (most non-*Saccharomyces* yeasts) while LAB and AAB populations were quantified using MRS and GYC media, respectively. Total yeast, non-*Saccharomyces* yeast, and AAB counts were higher in the musts and the beginning of the fermentation from R and B grapes compared with the same stages from H grapes (Table 2). However, during the mid and late fermentation, yeast populations were comparable for both damaged and healthy grapes (Table 2).

LAB population was also higher in R must than in the H one. Nevertheless, LAB colonies count increased slightly through the end of H must fermentation while decreased to undetectable levels in R samples. In *Botrytis*-affected samples no LAB colonies were detected at any stage of the fermentation (Table 2).

Even if the AAB populations were higher in R and B musts samples, the number of colonies decreased through the fermentation to undetectable levels while it remained low but constant in H samples (Table 2).

Table 2. Abundance and detection of the fungal (upper) and bacterial (lower) groups determined by the different compared techniques. Results are the mean of three different biological replicates evaluated from the must (Must), the beginning of the fermentation (IF), the middle of the fermentation (MF) and the final sampling point (FP) taken when juice density was below 1000 g/L for two consecutive days. Nd for not detected.

	Healthy				**Rotten**				**Botrytized**			
	Must	**IF**	**MF**	**FF**	**Must**	**IF**	**MF**	**FF**	**Must**	**IF**	**MF**	**FF**
Plate culture (CFU/ml)												
Total yeast (YPD)	1.3E+05	1.3E+05	5.7E+07	1.7E+07	4.4E+06	5.0E+06	9.0E+07	9.2E+06	1.4E+07	5.7E+06	3.0E+07	3.2E+06
Non-*Saccharomyces* (Lys)	2.0E+05	8.3E+04	3.3E+07	1.1E+07	2.6E+06	2.0E+06	2.1E+07	4.1E+06	1.3E+07	1.7E+06	3.3E+07	1.7E+06
LAB (MRS)	4.1E+03	2.5E+03	5.5E+03	1.1E+04	1.0E+06	7.9E+05	1.6E+04	Nd	Nd	Nd	Nd	Nd
AAB (GYC-Ca)	7.0E+03	2.5E+03	1.0E+03	4.8E+03	1.4E+05	4.2E+05	2.5E+03	Nd	1.8E+05	2.6E+05	1.3E+03	Nd
PCR-DGGE (-/+/++)												
Kazachstania africana[a] (4)[b]	-	-	-	-	+	+	-	-	+	+	-	-
Rizhopus stolonifer (0)	++	+	-	-	++	++	+	+	+	+	+	-
Botrytis cinerea (2)	-	-	-	-	+	+	-	-	+	+	+	+
Zygosaccharomyces bisporus (5)	-	-	-	-	+	+	+	+	+	+	+	+
Aerobasidium pullulans (0)	-	-	-	-	-	-	-	-	+	+	-	-
Issatchenkia hanoiensis (2)	+	+	-	-	+	+	-	-	+	+	-	-
Candida californica (1)	+	+	-	-	+	+	-	-	+	+	-	-
Starmerella bacillaris (0)	+	+	+	++	+	+	++	++	+	+	++	++
Hanseniaspora uvarum (0)	+	+	++	++	+	-	++	+	+	+	+	+
Saccharomyces cerevisiae (0)	-	-	+	++	-	-	+	++	-	-	-	++
Acetobacter pastorianus (4)					+	+						
Gluconoacetobacter xylinus (0)					+	+						

[a]Closest relative according to BLAST analysis; [b] Sequence difference (number of substitutions/indels) from type strain.

Table 2. Continued.

	Healthy				Rotten				Botrytized			
	Must	**IF**	**MF**	**FF**	**Must**	**IF**	**MF**	**FF**	**Must**	**IF**	**MF**	**FF**
qPCR (cells/ml)												
Total yeast	1.3E+06	1.9E+07	4.8E+08	4.8E+08	2.6E+08	6.4E+07	4.8E+08	1.0E+08	3.7E+07	6.2E+07	2.2E+08	2.0E+07
Hanseniaspora	4.0E+05	1.8E+05	1.5E+08	5.6E+07	2.1E+07	1.1E+07	1.3E+08	1.2E+07	6.6E+06	8.8E+06	3.3E+07	1.4E+06
Sacharomyces cerevisiae	1.6E+04	8.4E+03	2.3E+06	3.5E+07	2.7E+04	1.3E+04	7.3E+05	6.0E+06	2.6E+03	4.6E+03	1.1E+05	2.2E+06
Starmerella bacillaris	8.4E+05	7.2E+05	2.2E+07	1.0E+08	1.8E+07	9.3E+06	8.7E+07	2.5E+07	5.7E+06	6.6E+06	4.5E+07	4.3E+06
Torulaspora delbrueckii	1.2E+03	7.3E+02	5.2E+04	1.3E+05	4.7E+03	2.6E+03	3.7E+04	9.9E+03	1.5E+03	2.8E+03	2.0E+04	7.1E+02
Metschnikovia pulcherrima	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	5.1E+03	8.0E+03	3.2E+03	2.2E+02
Botrytis cinerea	Nd	Nd	Nd	Nd	7.5E+04	2.1E+04	4.9E+03	1.4E+04	1.6E+07	1.8E+07	5.3E+07	1.1E+06
Total LAB	3.9E+03	1.5E+03	7.7E+01	7.5E+05	1.1E+04	2.9E+03	3.5E+02	7.6E+01	6.6E+03	1.1E+04	9.6E+02	1.5E+02
Total AAB	4.7E+04	1.3E+04	2.0E+04	4.2E+03	1.5E+07	5.9E+06	1.5E+06	8.3E+04	1.5E+08	5.3E+07	8.2E+07	1.9E+07
NGS (>1% on average)												
Botrytis cinerea	0	0.3	0	0	0	0	0	0	40.6	36.4	2.3	0
Cladosporium herbarum	4.4	3.3	0	0	0.5	0.8	0	0	3	2.2	0.2	0
Hanseniaspora osmophila	3.8	3.8	1.2	2.5	2.3	5.1	47	25.7	6.4	7.9	53.1	3.1
Hanseniaspora uvarum	10	36.9	95.4	25.8	6.9	12.1	36.7	8.5	14.3	16.4	30.4	1.9
Aspergillus fischeri	14.9	3.9	0	0	23	22.4	1.6	0.8	10.3	9.8	1.4	0
Rhizopus americanus	52.3	21.6	0	0	51.1	39.2	2.1	0.6	4.9	4.5	0.6	0.1
Saccharomyces cerevisiae	1.1	4.7	2.1	59.9	0.5	0.8	1.5	50.2	0.5	0.4	0.6	86.6
Starmerella sp.	1.5	14.2	1	6.2	1.6	2.1	5.4	4.7	0.7	0.8	3	0.5
Penicillium purpurogenus	0	0	0	0	3.3	3.1	0.2	0.1	5.2	4.4	0.5	0
Zygosaccharomyces rouxii	0.2	0.7	0.1	2	6	9.2	5	8.9	6.5	9.8	6.9	7.5
Acetobacter	1.5	2.9	0.3	3.4	3.6	4	4.2	4.7	1.5	1.2	2	2.6
Ameyamaea	0.4	0.3	0	0	4.5	4.8	5.4	5.1	1.3	1.6	1.8	0.5
Gluconacetobacter	9.5	11.9	2.2	0.2	45.1	44.7	48.7	42.3	34.4	36	28.9	14.6
Gluconobacter	67.2	52.7	88.6	5.3	42.9	41.8	34.6	41.4	52.3	49.8	44.5	52.3
Tanticharoenia	0.4	0.8	0	0	3.1	3.3	5.5	0.6	8.1	8.3	8.6	0.2
Oenococcus	15.1	19.2	4.1	90.9	0	0.1	0.2	2.3	0.1	0.1	9.6	17.8

Quantitative PCR (qPCR)

The population levels of total yeast, total LAB, total AAB, *Saccharomyces* spp., *Hanseniaspora* spp., *Torulaspora delbrueckii*, *Metschnikowia* spp., *Starmerella bacillaris* and *Botrytis cinerea* were separately quantified by qPCR with specific primers (Table 2). The total yeast population determined by qPCR was higher in R and B musts than in H one. Apparently, the increase of total yeast in R and B musts was due to an increase in the genera *Hanseniaspora* and *S. bacillaris* while *Saccharomyces* remained at the same level than in H must. Moreover, *Saccharomyces* spp. population in R and B did not increase as much as in H during the fermentation (Table 2). The quantification of *B. cinerea* was only positive for damaged grape samples and it was considerably higher in the B ones that were obtained from grapes visibly affected by this filamentous fungus. However, *B. cinerea* population gradually decreased through the end of fermentation (Table 2). The anaerobic conditions during alcoholic fermentation would explain the sharp decrease of *B. cinerea* in the last fermentation stages and, probably, the quantification could correspond to DNA from dead cells as no viable fungi was recovered on YPD from the mid fermentation stage onwards.

T. delbrueckii was detected in low proportion in the three musts just increasing through the mid and end of alcoholic fermentation of H samples. The last yeast species quantified, *Metschnikowia* spp., was only detected in low proportion in B samples (Table 2).

In the case of bacteria, the quantification of AAB was at least three orders of magnitude higher in musts from damaged grapes than in the healthy ones. Finally, it was remarkable the increase of LAB population on the late fermentation of H samples (Table 2).

PCR-DGGE

Microbial communities from H, R and B grapes were analyzed at different fermentation stages by PCR-DGGE for Eukarya. The excised DGGE bands were re-amplified and identified by sequencing. Occasionally, some bands migrating differently were identified as the same taxon. Though the identification to species level from the short sequences obtained by PCR-DGGE analysis was not reliable, we were able to detect eight different yeast species (closest relatives: *S. cerevisiae*, *Hanseniaspora uvarum*, *S. bacillaris*, *Candida* spp., *Issatchenkia* spp., *Kazachstania* spp., *Zygosaccharomyces* spp. and *Aureobasidium pullulans*) and two filamentous fungi (*Rhizopus* spp. and *B. cinerea*). *Saccharomyces cerevisiae* was not detected with this technique in any grape

must (Table 2). However, it was detected during mid and late fermentation in H, R and B fermentations. Moreover, the highest intensity of *S. cerevisiae* was reached at late fermentations regardless of grape health status. *Hanseniaspora uvarum* and *S. bacillaris* exhibited a similar behaviour being present along all grape fermentations and showing higher band intensity from mid to late fermentation (Table 2).

Kazachstania spp., *Zygosaccharomyces* spp., *B. cinerea* and *A. pullulans* were just identified in damaged grape samples. Concretely, *Kazachstania* was detected just in the must and the beginning of the fermentation while *Zygosaccharomyces* was present in all stages. Besides, *B. cinerea* was observed during all B fermentation phases while it was just detected in the must and the beginning of R fermentations. As previously mentioned, the detection of *B. cinerea* in the last fermentation phases could correspond to DNA from dead cells. In the case of *A. pullulans*, this yeast like fungus was only identified in the first part of B grape fermentation (Table 2).

Apart from *B. cinerea*, we observed another filamentous fungus identified as *Rhizopus* spp. This fungus was present in all grape musts and it was detected until the middle of the R fermentation and late fermentation of B (Table 2).

The PCR for the DGGE analysis with bacterial specific primers did not result in strong amplifications indicating less proportion of bacteria in comparison with yeast population (results not shown). The different DGGE bands from bacterial profiles were excised and amplified for their identification, but most of the resulting sequences did not have a match on the NCBI database probably due to co-migration of bands from similar species and thus, cloning of the excised bands should have been done in order to have single sequences from co-migrating bands. Just two bands recovered from must and the beginning of the fermentation of R grapes were identified as *Gluconoacetobacter* and *Gluconobacter* (Table 2).

Massive sequencing

Barcode amplicon sequencing was used to analyze the bacterial and fungal communities of the different grapes through their fermentation. A total of 382,990 bacterial sequences and 1,954,049 eukaryotic sequences were used to build the OTU tables with an average of 31,916 and 162,837 sequences per sample, respectively (Supplementary Table 2). The massive sequencing analysis detected a higher diversity of fungal and bacterial genera than the other techniques (Table 2 and

Supplementary Figure 1). However, considering those genera more abundant than 1% on average, 9 fungal and 6 bacterial genera were detected (Table 2).

The most abundant yeast on average across all samples was *Hanseniaspora* (38.2%), detected mainly in the beginning and mid fermentation (Figure 2). Interestingly, two different abundant OTUs within *Hanseniaspora* were identified and the closest relatives were *H. uvarum* (23.1%) and *H. osmophila* (15.1%). *Hanseniaspora uvarum* was more abundant in H than in R or B samples, while *H. osmophila* was more abundant in mid fermentations of R and B (Figure 2). Other non-*Saccharomyces* yeast were detected in less proportion on average, for example, *Starmerella* (3.3%), and *Zygosaccharomyces* (5.3%) (Figure 2). *Saccharomyces* (19.8% on average) was detected in all musts and every stage of fermentations, being the predominant yeast (between 50.2 and 59.9% of sequences) during late fermentations samples. Yeast like *Hanseniaspora* or *Saccharomyces* quickly replaced to filamentous fungi or molds detected by this technique in the first stages of the fermentation. Within these molds, *Rhizopus* was abundant (13.6% on average) in H and R, while *B. cinerea*, (6.1% on average) predominated in must and beginning of the fermentation of B grapes (ranging between 36.4-40.6%), *Aspergillus* (6.9% on average) was more abundant in R must at the beginning of the fermentation (23 and 22.4%, respectively) than in the rest of the samples, *Penicillium* was just detected in damaged samples (ranging between 3.1 and 5.2%) and *Cladosporium* (1.1%) slightly more abundant on H and B musts and the beginning of fermentation (ranging between 2.2 and 4.4%) than in the respective R samples (0.5-0.8%). Other fungal genera detected in lower proportion than 1% but higher than 0.1% on average across all samples are indicated on the heat map (Supplementary Figure 1). Some of these low abundance genera were present just in samples from damaged grapes, like *Saccharomycopsis*. On the other hand, *Fusarium* was detected just in H samples. All these taxa, except the fermentative yeast *Saccharomycodes*, disappeared at the late fermentation, indicating a low implication during wine fermentation.

In the case of bacteria detected by MS, the 6 most abundant genera were the AAB genera *Gluconobacter, Gluconoacetobacter, Acetobacter, Tantiocharoenia*, and *Ameyamaea* (accounting for 82.4 % on average across all samples) and the LAB genus *Oenococcus*. The abundance of these bacterial genera varied among the samples with different health states (Figure 2B). *Oenococcus* was predominant during late fermentation of H (90.9%) and also represented an important proportion of the sequences during the rest of H fermentation stages while it was scarcely detected

in damaged grapes samples (Figure 2). R and B samples harbored higher proportion of *Gluconoacetobacter* than H samples and the genus *Gluconobacter* was clearly the most abundant from the must to mid fermentation of H grapes (52.7-88.6%). In addition, sequences related to *Tantiocharoenia* were more abundant in damaged samples than in H ones. Finally, R samples harbored higher proportions of the genera *Acetobacter* and *Ameyamaea* than H or B. Other bacterial genera detected in lower abundance than 1% but higher than 0.1% are listed on Supplementary Figure 1. Within these genera, some LAB like *Aerococcus, Lactococcus* or *Streptococcus* were also identified. All these genera disappeared during late fermentation of H grapes while some of them remained in damaged grapes (Supplementary Figure 1). In addition, some of the genera detected just at late fermentation of R and B grapes increased their abundance with respect to the must and beginning of fermentation samples, for example, *Acinetobacter, Bacillus, Staphylococcus* and *Tatumella*.

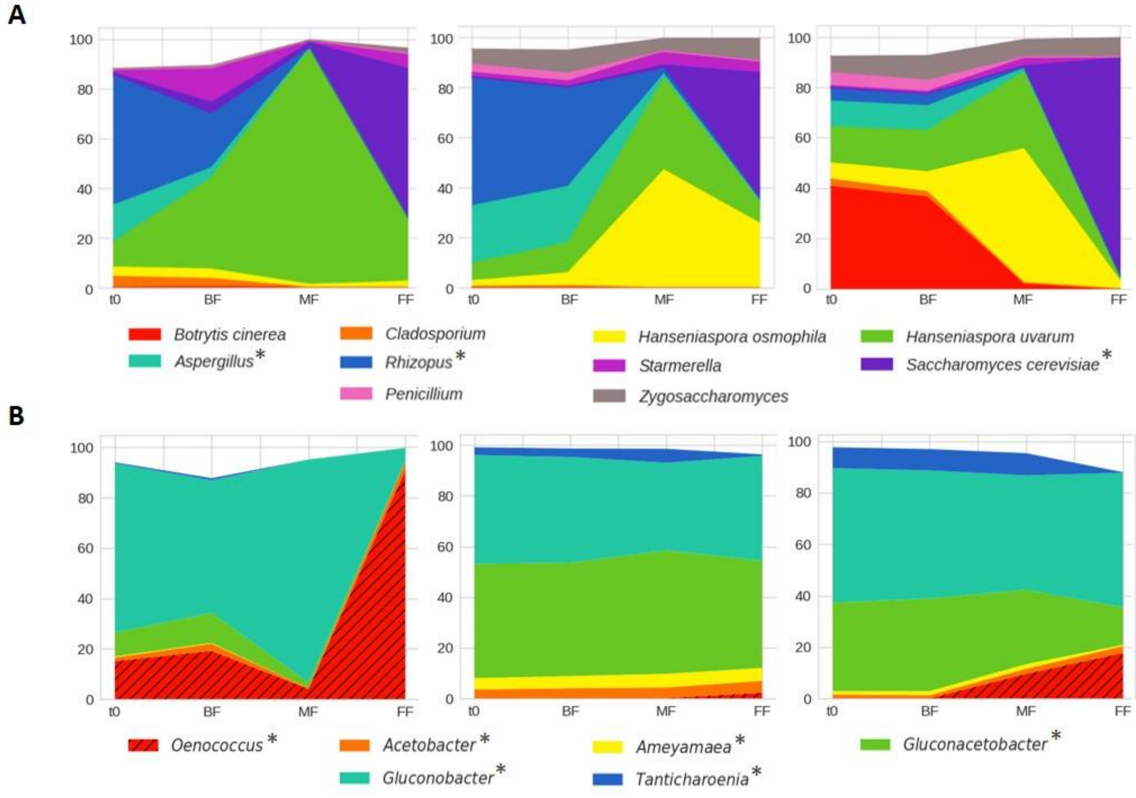

Figure 2. Relative abundance of fungal (A) and bacterial (B) taxa detected at > 1% by MS. Taxa that differed significantly (*P* value <0.05) by fermentation stage (for fungi) or by health status (for bacteria) are indicated by an asterisk.

Bacterial and fungal alpha diversity

The highest microbial diversity as determined by the number of different genera identified by the PCR-DGGE analysis was observed in the must and at the beginning of the fermentation of each health type grape, with higher diversity in the must samples and also more diversity in R and B samples than in H ones (Table 2). The diversity was lower through the end of H fermentation that for the damaged ones (Table 2).

According to MS analysis, fungal diversity ranged from 1 to 1.6 for the PD whole tree index and from 42 to 68 observed OTUs (Figure 3A, Supplementary Table 3). Higher diversities were reached for all samples in the musts and during the first stages of fermentation with similar values for damaged and H samples in those stages. However, diversity decreased sharply for H samples during the second half of the fermentation while, in the case R samples, diversity remained high and relatively constant along the fermentation and in the case of B samples, diversity decreased just a little during late fermentation stage (Figure 3A). The lowest fungal diversity belonged to late fermentation of H grapes. Simpson and Shannon indexes pointed to H samples during the mid-fermentation as the ones with the lowest diversity values (Supplementary Table 3).

This scenario was slightly different for bacterial diversity. The PD whole tree index for bacterial sequences ranged from 1 and 3.1 while observed OTUs ranged from 15 to 49 (Figure 3B, Supplementary Table 4). The bacterial taxonomic diversity increased through the end of fermentations for damaged samples while decreased sharply from the first 24 hours to late fermentation for H samples. The same tendency was observed for the number of OTUs with the exception of R samples harbored a relatively constant number of OTUs through the fermentation (Figure 3B). Other indexes like Simpson or Shannon also revealed that the lower bacterial diversity was observed for Healthy samples from mid to late fermentation while the values for the other samples remained quite constant (Supplementary Table 4).

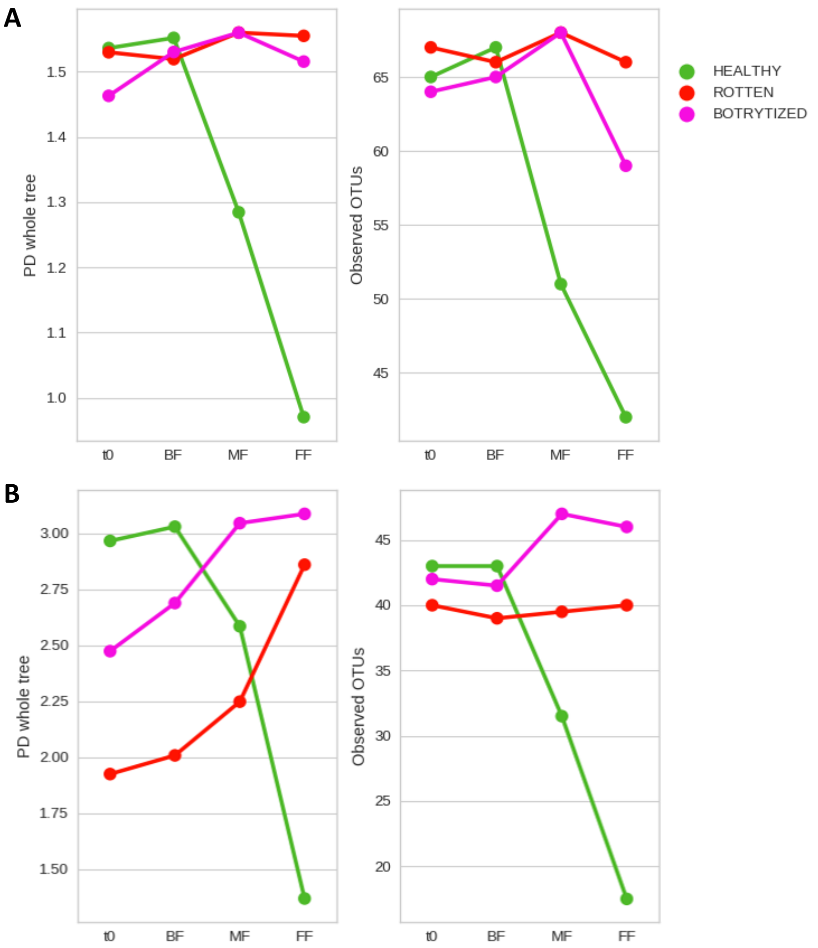

Figure 3. Alpha diversity graphs showing the PD whole tree index (left) and number of different OTUs (right) for the fungal (A) and bacterial (B) communities determined by MS.

Health status of the grapes influences must and fermentation communities

Fungal and bacterial communities changed across the different fermentation stages and between the different health statuses of the grapes used for the alcoholic fermentation (Figure 4). Unifrac distance matrices (Lozupone and Knight, 2005) were calculated with the taxonomic composition and abundance data from samples analyzed by MS in order to be used for the analysis of similarities (ANOSIM) of the microbial communities from the different samples. Fungal communities resulted significantly different and clustered by the different fermentation stages (Table 3, Figure 4A). According to statistical analysis, *Aspergillus, Rhizopus* and *Saccharomyces* were the fungal genera that varied significantly across all fermentation stages, thought other additional genera showed variation in their proportions across the fermentation stages and different health statuses (Figure 2). Bacterial populations from the different samples of H, R and B were significantly different and clustered by health status (Table 3 and Figure 4B). The bacterial

genera that varied significantly in abundance between the H, R and B samples were *Acetobacter*, *Aeyamaea*, *Gluconoacetobacter*, *Gluconobacter*, *Oenococcus* and *Tanticharoenia* (Figure 2).

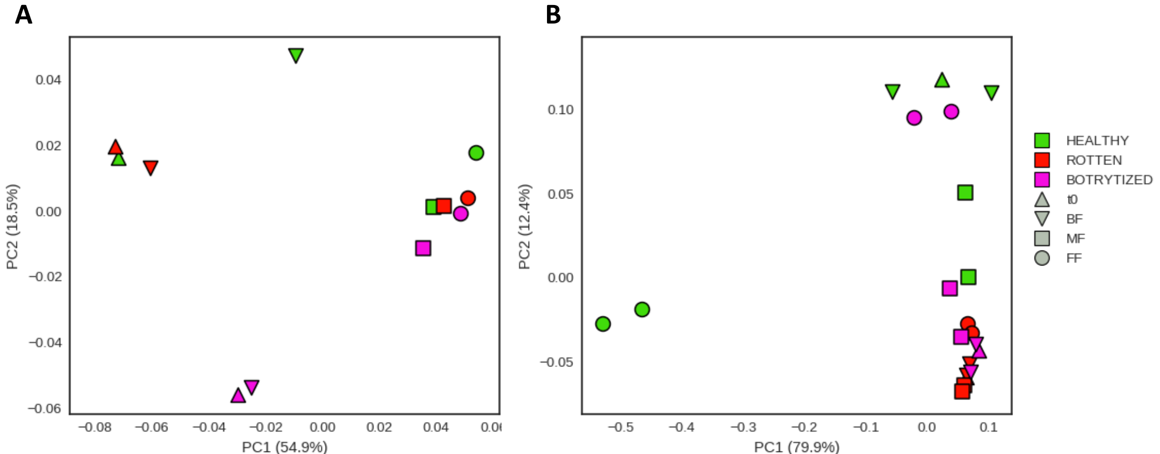

Figure 4. Weighted Unifrac distance PCoA plots for fungal (A) and bacterial (B) communities from Macabeo must and fermentations.

Table 3. ANOSIM results showing the analysis of similarities of the different fungal and bacterial communities calculated from the weighted Unifrac distances matrices for the factors health status of the grape and the fermentation stage.

Factor	ANOSIM Bacterial		ANOSIM Fungal	
	R	*p*	R	*p*
Health	**0.355**	**0.001**	0.013	0.356
Ferm. Stage	0.005	0.455	**0.598**	**0.003**

Discussion

Grape health status is a primordial fact during winemaking and it can negatively impact on the fermentation process and the composition and quality of wine (Ribéreau-Gayon et al., 2006). In the present work, we described the ecological changes along the fermentation of Macabeo grapes with different health status, H, R and B, using various techniques.

Analyzing the influence of grape health state on fermentation kinetics, *Botrytis* infection had the strongest effect on the delay of the fermentation evolution since fermentations affected by this fungus were the slowest to consume 90% of the sugars. Nevertheless, undamaged grape fermentation presented the highest amount of sugar and ethanol in the last sampling point (density below 1000 g/l for two consecutive days). Previous studies have reported higher residual sugar when non-*Saccharomyces* yeasts were abundant during alcoholic fermentation (Ciani et al.,

2006; Magyar and Tóth, 2011), which can occur in spontaneous fermentations (Andorrà et al., 2008; Llauradó et al., 2002).

In the present study, the techniques of plate culturing, qPCR, PCR-DGGE and MS have been used to monitor the changes of microbial community on grapes with three different health statuses. All these techniques allowed for the differentiation of the microbial communities in musts and fermentations of the three types of grapes, but differences in the results were observed depending on the technique.

Most of the studies on sour rot and *Botrytis*-affected grape ecology have been based on plate counts (Barata et al., 2008, 2012b; Mateo et al., 2014; Nisiotou et al., 2007, 2011). However, the inability of some microorganisms to grow in some media and/or under certain conditions (Amann et al., 1995) can give a biased result of the microbial diversity (Rantsiou et al., 2005). Considering these facts, we additionally used molecular methods since they have shown to be more informative about environmental microbial diversity.

One of the most used molecular techniques to quantify microbial populations is the qPCR. Nevertheless, the specific primer design limits the quantification to the targeted groups or species. The PCR-DGGE using general primers is a good molecular technique to obtain a fingerprint of the microbial community in a sample, but hardly detects populations with lower density than 10^3 cfu/ml or two orders of magnitude lower than the most abundant members (Andorrà et al., 2008; Muyzer and Smalla, 1998; Prakitchaiwattana et al., 2004). Recently, high-throughput sequencing or MS techniques can be used to obtain a more detailed image of the microbial communities of various ecosystems, including food processing (Ercolini, 2013). To our knowledge, this is the first study analyzing microbial populations in sour rot or *Botrytis*-affected grape musts and fermentations by qPCR and MS. It is important to consider that the used molecular methods detect both viable and non-viable cells. Thus, it is possible that DNA of dead or non-viable cells lead at some point to the overestimation of a taxonomic group. However, an increase in the proportion of DNA probably will correspond to an increase of the population.

In general, our plate counts agreed with similar studies analyzing sound and damaged grapes with a higher fungal and bacterial population in the affected grapes (Barata et al., 2008; Fleet, 2003; Wang et al., 2015).

Total yeast and AAB bacteria quantification by qPCR was higher than the counts detected by plating probably due to the quantification of viable but non culturable and dead cells by qPCR

(Hierro et al., 2007; Torija et al., 2010). In addition, the primers used to quantify total yeast have been described to also detect many filamentous fungi apart from yeast (Hierro et al., 2006).

Fungal communities varied significantly across the different fermentation stages as shown in this study by the used techniques. As in previous studies, the yeast population number and diversity resulted higher in damaged grape musts than in H one (Barata et al., 2008, 2012a; Barbe et al., 2001; Nisiotou et al., 2011). The higher yeast number might have been induced by physically damaged grapes (Barata et al., 2008; Barbe et al., 2001) together with the release of nutrients from the berry that encourage their growth (Fleet, 2003).

The high proportion of non-*Saccharomyces* in damaged musts, determined by plate counts, qPCR and MS, could interfere with *Saccharomyces* imposition along the fermentation as a consequence of interactions between both populations. Among others, these interactions involve the competition for substrate, yeast-yeast cell contact or the release of antimicrobial compounds (Ciani and Comitini, 2015; Wang et al., 2016). However, R must presented higher difference between total yeast and non-*Saccharomyces*, indicating a higher initial concentration of *Saccharomyces*, which could explain why the R microbial population was the fastest to consume the 90% of the sugars.

Higher populations of *Hanseniaspora* and *Candida* (or *Starmerella*) observed by qPCR in damaged grapes coincided with previous ecological studies on damaged grape berries (Loureiro and Malfeito-Ferreira, 2003; Mills et al., 2002; Prakitchaiwattana et al., 2004). Nevertheless, these species are also predominant worldwide in healthy grapes and during the first stages of fermentation (Constantí et al., 1997; Jolly et al., 2014; Loureiro and Malfeito-Ferreira, 2003; Torija et al., 2001). In our study, independently on the grape status, *H. uvarum* and *S. bacillaris* were detected in high proportions across the alcoholic fermentations by qPCR and PCR-DGGE. A previous study using PCR-DGGE to monitor yeast populations during sound and *Botrytis*-affected fermentations (Nisiotou et al., 2007) found a similar behavior of *H. uvarum* to what we describe by PCR-DGGE but they did not found *Saccharomyce*s. In our study, *Saccharomyces* was not detected in any must sample by PCR-DGGE but was detected from mid fermentation of damaged and H grapes fermentation. MS together with qPCR allowed *Saccharomyces* detection and quantification, respectively, in all samples from the must onwards.

MS also enabled us to identify abundantly *H. uvarum* sequences in all fermentations, but above all, during the middle of H fermentations. Despite the low proportion of *S. bacillaris* identified by

MS, its quantification by qPCR was proportional to the values obtained for *H. uvarum*. Other sequences related to *H. osmophila* were more abundant than *H. uvarum* in damaged grapes but there were not detected or differentiated by PCR-DGGE or qPCR techniques. A previous study on Dolce wine fermentation was able to differentiate *H. osmophila* from *H. uvarum* by PCR-DGGE analysis (Mills et al., 2002), indicating that their bands migrated differently. However, differentiation of different species of *Hanseniaspora* is not possible by qPCR with the used primers.

Some key yeast species previously associated with damaged grapes as *Botrytis, Kazachstania a*nd *Zygosaccharomyces* (Barata et al., 2012a; Nisiotou et al., 2007) were observed by PCR-DGGE just in R and B samples. Barata et al. (2012b) proposed as biomarkers for sour rot the presence of the yeast *Zygoascus hellenicus* and *Issatchenkia*. However, in our case, *Zygoascus* was not detected by PCR-DGGE in any sample while *Issatchenkia* was detected in both damaged and H samples by both PCR-DGGE and MS techniques.

B. cinerea was detected by qPCR and PCR-DGGE in damaged samples, although its quantification in R samples was low and constant. In contrast, MS analysis revealed a very small proportion of *Botrytis* in H must and even lower proportion in R grapes. In B samples though, *Botrytis* represented > 30% of the sequences analyzed by MS in the must and beginning of the fermentation. This proportion lowered close to the end of the fermentation evidencing the sensibility of this fungus to the semi anaerobic conditions and the increasing concentration of ethanol along the fermentation (Steel et al., 2013).

Rhizopus and *A. pullulans* were detected also by both DGGE and MS techniques. *Rhizopus* was present in all must and beginning stages. This fungus has been described as a saprophytic organism that can be a secondary bunch rot invader infecting grape berries (Steel et al., 2013) and lead to organoleptic defects in grapes and wines when is associated with *B. cinerea* (La Guerche et al., 2006). Furthermore, *A. pullulans* was identified in must and initial fermentations of H and damaged grapes by MS analysis. This coincides with previous studies where it was isolated from both sound and damaged grapes (Barata et al., 2012a; Padilla et al., 2016; Prakitchaiwattana et al., 2004).

In general, MS analysis revealed a higher number of yeast genera than PCR-DGGE. The higher proportion of some yeasts could inhibit or impede the detection of other less abundant yeasts or microorganisms by PCR-DGGE (Prakitchaiwattana et al., 2004). However, some of the genera

that PCR-DGGE failed to detect were more abundant than 1% on average as determined by MS (for example, *Aspergillus, Penicillium* and *Cladosporium*). On the other hand, PCR-DGGE analysis detected additional genera that were not abundant or even not detected by MS, like *Kazachstania, Issatchenkia* or *Candida*. These differences in the detection of genera by both techniques might be due to PCR amplification preferences as the primers used for DGGE and MS were not the same. Recent studies using MS technique to analyze the wine fermentation process of different grape varieties have detected the fungal genera *Hanseniaspora, Issatchenkia, Rhodotorula, Penicillium, Cladosporium, Botrytis, Sporobolomyces, Aspergillus, Cryptococcus* and *Pichia* (Bokulich et al., 2014; Pinto et al., 2015; Setati et al., 2012), all of them also detected in the present study and making our fungal community results solid (Table 2, Supplementary Figure 1).

LAB and AAB are the most relevant bacterial groups related to grapes and wine fermentation. In this study, GYC and MRS media were employed to count AAB and LAB populations, respectively. As in previous studies, our plate counts revealed an evident increase of AAB population in musts and beginning of the fermentations of R and B grapes (Barata et al., 2008, 2012a; Barbe et al., 2001; Nisiotou et al., 2007, 2011). As explained above, the reason could be the release of nutrients from the berry that encourages AAB and yeast growth (Fleet, 2003). The evaluation of AAB population by plate culture is usually complicated (Bartowsky and Henschke, 2008; Torija et al., 2010) mainly for its ability to enter in VBNC (viable but non-culturable state) (Millet and Lonvaud-Funel, 2000) or because they die under inappropriate conditions. Thus, the use of specific primers to quantify AAB by qPCR (González et al., 2006) allowed us to detect higher populations of AAB in all fermentations than the plate culturing, indicating the capacity of qPCR to detect VBNC and dead bacteria (Table 2). In order to identify the AAB genera, PCR-DGGE and MS techniques were applied. Unfortunately, PCR-DGGE allowed the identification of just *Gluconoacetobacter* and *Acetobacter* in the must and beginning of R fermentation. This might be due to the limitation of PCR-DGGE to detect populations two orders of magnitude lower than the most abundant members (Prakitchaiwattana et al., 2004), and, as noticed by qPCR results, yeast population was mostly two or even three orders above the bacterial one. On the other hand, MS technique allowed the identification of up to 21 bacterial genera, most of them related to AAB genera. Clear differences in bacterial composition were detected between H and damaged grapes. *Gluconobacter* followed by *Gluconoacetobacter* were the most abundant until the mid-fermentation of H grapes. In R grapes though, *Gluconoacetobacter* represented the most abundant

genus in all fermentation stages and it was also more abundant in Botrytized samples than in H ones. Thus, the abundance ratio between *Gluconobacter* and *Gluconoacetobacter* was higher in H samples than in R and B ones. This fact is really aligned with previous observations where the health status of the grapes indicated that *Gluconobacter* is more abundant in healthy grapes, whereas *Gluconoacetobacter* (or even *Acetobacter*) are more abundant in damaged grapes (Guillamón and Mas, 2017).

In a wine fermentation study in a Grenache variety using MS (Portillo and Mas, 2016), we showed that AAB and LAB were more abundant during fermentation than previously thought, with a dominance of *Gluconobacter* during the mid-fermentation. The latter finding contradicts the previous notion that *Gluconobacter*, being alcohol sensitive, usually declines during the alcoholic fermentation (González et al., 2005; Joyeux et al., 1984; Toit and Lambrechts, 2002). Similar results have also been reported in other studies using MS analysis on low-sulfited or unsulfited wine fermentations (Bokulich et al., 2015). The same authors found *Acetobacter*, *Gluconobacter*, and *Gluconoacetobacter* as dominant bacteria during winemaking processes (Bokulich et al., 2012).

Plate culturing also allowed the quantification of considerable LAB populations in H and R musts, contrasting with previous studies where LAB populations were not detected or detected in low concentrations (Barata et al., 2012a; Nisiotou et al., 2011). In fact, our MRS counts overestimated LAB population respect to those of qPCR analysis using LAB specific primers, probably due to non-LAB species that may grow in MRS media (Barata et al., 2012a). However, both techniques detected a LAB population increase at the H late fermentation.

MS analysis also supported qPCR results, with low percentages of LAB taxa in comparison with those of AAB populations, except during the last sampled point of H fermentation, making solid this tendency. This LAB population increase at late H fermentation suggests that the spontaneous evolution of malolactic fermentation might not occur spontaneously in damaged grapes fermentations. No LAB genus was identified by the PCR-DGGE technique but MS analysis deciphered the LAB community composition in our samples and the main player was *Oenococcus oeni*. Thus, to our knowledge, this is the first study relating the presence of *Oenococcus oeni* to the grape health status.

The diversity of fungal and bacterial communities as determined by the quantification of the identified genera by DGGE gels resulted in higher diversity in the musts and beginning of every

type of fermentations and, in general, higher diversity for damaged grapes. However, according to MS results, similar diversity indexes were observed for the different types of grapes in the musts and the health status of the grapes influenced on fungal and bacterial diversities in a different way. For the fungal communities, H fermentation decreased the diversity through the last fermentation point while R and B samples remain almost constant. In the case of bacterial communities, the diversity declined sharply along H fermentation while increased (PD whole index) or remained relatively constant (number of OTUs) for damaged grapes fermentations. Both DGGE and MS analysis suggest that the microbial diversity of must obtained from H grapes decreased along the alcoholic fermentation while musts from damaged grapes maintain or increase their diversity. The higher diversity during the mid and late damaged fermentations may result from the additional metabolisms present in the infected grapes and musts, making possible the survival of non-conventional yeast for longer time respect to H fermentations but also, compromising the success of the alcoholic fermentation or including off-flavors to the final wine.

Conclusions

The present study is the first to include the molecular techniques qPCR and MS to evaluate the population evolution along spontaneous fermentation of sour rot and *Botrytis* affected grapes in comparison with healthy grapes. Both culture and molecular based analyses showed differences in fungal and bacterial communities of Macabeo grapes depending on its health status. However, MS analysis provided higher diversity at each stage than the other compared techniques and detected *Saccharomyces* and *Oenococcus* even in the initial must samples. The main differences in the fermentations revealed by MS were that *H. osmophila* was predominant during mid-fermentation of damaged samples instead of *H. uvarum*. Besides, *Oenococcus oeni* and *Gluconobacter* were more abundant in healthy samples than in damaged ones, while the later had higher proportion of *Gluconoacetobacter* with respect to the healthy samples. The microbial diversity of healthy fermentations decreased from the middle to the end. Similarly to other studies that used MS to describe the microbial population, in this work MS was the technique that contributed the most in the deciphering of the community microbiome and for the first time, the health status of the grape was related to the relative abundance of *Oenococcus oeni* during the alcoholic fermentation.

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Supplementary material

Supplementary Table 1. Correlation coefficient (R^2), slope, intercept and efficiency of standard curves obtained for the different primer pairs with serial dilutions of the corresponding microorganism's DNA. Efficiency was calculated by the formula E = ((10-1/slope) - 1) x 100.

Target	R^2	Slope	Intercept	Efficiency (%)	Primers	Ribosomal gene region
Total yeast	0,9926	-3,4236	38,751	95,9252	200F/324R	26S rRNA
Saccharomyces spp.	0,9953	-3,4987	37,283	93,1169	CESPF/SCERR	ITS2 and 5.8S rRNA spanning region
Hanseniaspora spp.	0,9959	-3,5347	39,837	91,8269	CESPF/HUVR	ITS2 and 5.8S rRNA spanning region
S. bacillaris	0,9938	-3,7675	43,082	84,2587	AF/200R	D1/D2 (26S rRNA)
T. delbrueckii	0,9974	-3,506	39,282	92,8525	TODSL2/TODSR2	ITS (between 18S rRNA and 26S rRNA)
Metschnikovia spp.	0,9998	-3,5226	35,181	92,2566	MP5FW/MP3BW	26S rRNA
B. cinerea	0,9976	-3,4934	41,912	93,3099	BC3F/BC3R	ITS (between 18S rRNA and 28S rRNA)
LAB	0,9986	-3,6645	41,338	87,4513	WLAB1/WLAB2	16S rRNA
AAB	0,9992	-3,292	46,767	101,2643	AQ1F/AQ2R	16S rRNA

Supplementary Table 2: Number of sequences obtained by Massive sequencing analysis **Before** and **After** quality filtering. The number of sequences used to build the **OTU table** is also indicated.

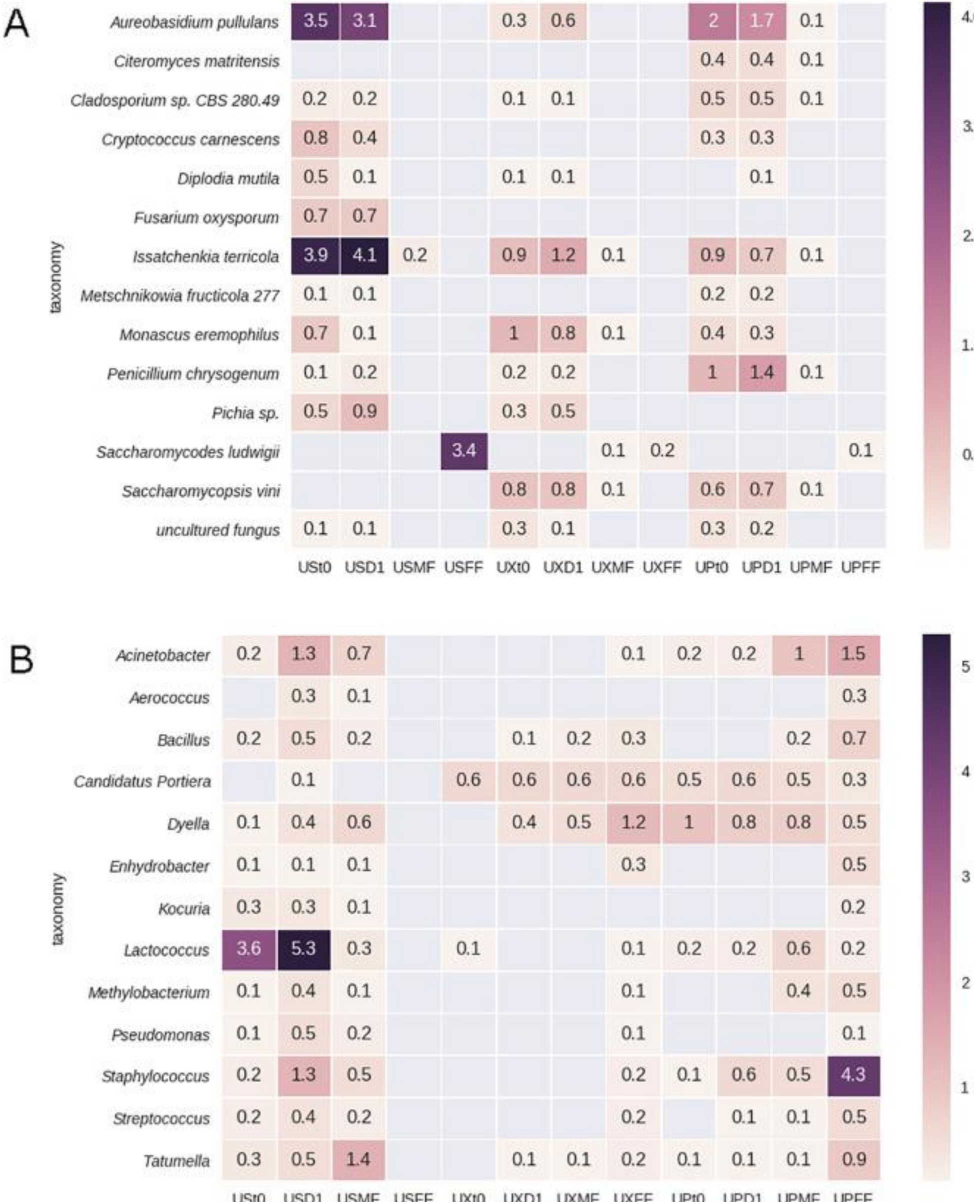
	Bacteria			Eukaryotes			Total		
Sample	Before	After	OTU table	Before	After	OTU table	Before	After	OTU table
Healthyt0	187058	101891	1057	149561	116171	101236	336619	218062	102293
HealthyD1	156795	87314	1630	122932	105324	91316	279727	192638	92946
HealthyMF	115106	87536	39101	155963	128718	113336	271069	216254	152437
HealthyFF	176803	140748	114457	204918	159046	137148	381721	299794	251605
Rottent0	247598	175776	32402	427632	311732	275651	675230	487508	308053
RottenD1	197261	143346	28006	348188	277696	249161	545449	421042	277167
RottenMF	118589	88681	19267	333245	267481	243875	451834	356162	263142
RottenFF	146690	107150	2994	275136	238860	222353	421826	346010	225347
Botrytizedt0	215983	139773	25024	209529	160971	136889	425512	300744	161913
BotrytizedD1	211867	139867	28455	176344	134603	113884	388211	274470	142339
BotrytizedMF	217797	141060	53010	237642	181350	156396	455439	322410	209406
BotrytizedFF	150132	109777	37587	157124	128101	112804	307256	237878	150391
Total	**2141678**	**1462919**	**382990**	**2798214**	**2210053**	**1954049**	**4939892**	**3672972**	**2337039**
% Filtering	**35,23%**			**23,69%**			**29,21%**		
Average/sample	**178473**	**121910**	**31916**	**233185**	**184171**	**162837**	**411658**	**306081**	**194753**

Supplementary Table 3: Diversity indexes of fungal communities calculated from the different OTUs obtained in the MS analysis.

	PD_whole_tree	Observed_OTUs	Simpson	Shannon
Healthyt0	1.5	65	0.83	3.49
HealthyD1	1.6	67	0.92	4.31
HealthyMF	1.3	51	0.76	2.70
HealthyFF	1.0	42	0.90	3.82
Rottent0	1.5	67	0.83	3.51
RottenD1	1.5	66	0.87	3.97
RottenMF	1.6	68	0.86	3.65
RottenFF	1.6	66	0.90	3.90
Botrytizedt0	1.5	64	0.89	4.20
BotrytizedD1	1.5	65	0.91	4.30
BotrytizedMF	1.6	68	0.83	3.41
BotrytizedFF	1.5	59	0.80	2.94

Supplementary Table 4: Diversity indexes of bacterial communities calculated from the different OTUs obtained in the MS analysis.

	PD_whole_tree	Observed_OTUs	Simpson	Shannon
Healthy t0	2.97	43	0.93	4.34
Healthy BF	3.04	43	0.93	4.38
Healthy MF	2.58	32	0.83	3.41
Healthy FF	1.37	18	0.63	1.95
Rotten t0	1.93	40	0.92	4.30
Rotten BF	2.01	39	0.92	4.31
Rotten MF	2.51	40	0.92	4.18
Rotten FF	2.86	40	0.92	4.30
Botrytized t0	2.85	42	0.92	4.15
Botrytized BF	2.42	41	0.93	4.24
Botrytized MF	3.05	47	0.93	4.35
Botrytized FF	3.09	45	0.92	4.26



Supplementary Figure 1. Heatmaps of the relative abundance of the fungal (A) and bacterial (B) taxonomy groups represented by <1% on average across all the samples. "US" represent the healthy samples, "UX" the rotten samples and "UP" the botrytized samples taken during grape must (t0), beginning (D1), middle (MF) or final (FF) fermentation stages.

CHAPTER 2

Saccharomyces and non-*Saccharomyces* competition during microvinification under different sugar and nitrogen conditions

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Abstract

The inoculation of wines with autochthonous yeasts allows obtaining complex wines with a peculiar microbial footprint characteristic from a wine region. Mixed inoculation of non-*Saccharomyces* yeasts and *S. cerevisiae* is of interest for the wine industry for technological and sensory reasons. However, the interactions between these yeasts are not well understood, especially those regarding the availability of nutrients. The aim of the present study was to analyze the effect of nitrogen and sugar concentration on the evolution of mixed yeast populations on controlled laboratory-scale fermentations monitored by density, plate culturing, PCR-DGGE and sugar and nitrogen consumption. Furthermore, the effect of the time of inoculation of *Saccharomyces cerevisiae* respect the initial co-inoculation of three non-*Saccharomyces* yeasts was evaluated over the evolution of fermentation. Our results have shown that *S. cerevisiae* inoculation during the first 48 h conferred a stabilizing effect over the fermentations with non-*Saccharomyces* strains tested and, generally, reduced yeast diversity at the end of the fermentation. On the other hand, nitrogen limitation increased the time of fermentation and also the proportion of non-*Saccharomyces* yeasts at mid and final fermentation. High sugar concentration resulted in different proportions of the inoculated yeast depending on the time of *S. cerevisiae* inoculation. This work emphasizes the importance of the concentration of nutrients on the evolution of mixed fermentations and points to the optimal conditions for a stable fermentation in which the inoculated yeasts survived until the end.

Keywords: *Torulaspora, Hanseniaspora, Starmerella,* fermentation, wine

Introduction

Wine is the result of alcoholic fermentation performed by yeasts during a complex process that transforms the sugars present in the grape must into ethanol and carbon dioxide. During this alcoholic fermentation, a microbiological population evolves as a consequence of the chemical changes produced in the environment (Ribéreau-Gayon et al., 2006). Many studies have established the yeast succession of non-*Saccharomyces* to *Saccharomyces* during spontaneous fermentation of grape juice. These non-*Saccharomyces* yeasts are the predominant microbiota in grapes and the main responsible for starting spontaneous alcoholic fermentation and often, under uncontrolled fermentations, lead to sluggish or stuck fermentations. For that reason, winemakers tend to inoculate grape must with commercial yeasts to ensure the completion of the fermentation, but compromising the complexity or the particular microbial footprint of wines of a certain region. In recent years, good properties and contribution of the non-*Saccharomyces* yeasts to wine and fermentation process have been described (Ciani and Comitini, 2011; Fleet, 2008; Jolly et al., 2014; Padilla et al., 2016b; Pretorius, 2000). With the aim to obtain wines that reflect a certain *terroir*, a previous study part of the WILDWINE project (Mas et al., 2016) accomplished the isolation and the characterization of multiple yeast strains from Priorat region to better understand the winemaking process and also to determine the source of microorganisms that produce a particular microbial footprint (Padilla et al., 2016a). The contribution of non-*Saccharomyces* takes part mostly during beginning and mid fermentation (Fleet, 2008). Non-*Saccharomyces* yeasts are able to produce metabolites or hydrolyze aromatic precursors providing new wine styles and enhancing their complexity (Andorrà et al., 2012; Ciani et al., 2010; Jolly et al., 2014; Viana et al., 2011).

The possibility to obtain wines with differential characteristics due to the role of non-*Saccharomyces* yeasts explains the increasing interest of using mixed cultures. As we have mentioned, one of the objectives of the WILDWINE project is to mimic the natural microbiota of a vineyard by the use of mixed inocula to perform fermentations to fight the wine uniformity derived from the widespread use of commercial *S. cerevisiae* starter cultures (Mas et al., 2016). Besides, interaction between non-*Saccharomyces* and *S. cerevisiae* has not been extensively studied, however some positive metabolic interactions have been described (Ciani et al., 2010; Ciani and Comitini, 2015). In the present study, the most characteristic non-*Saccharomyces* yeast

isolated during the WILDWINE project were subjected to mixed alcoholic fermentation under different nutrient conditions (Mas et al., 2016; Padilla et al., 2016a).

The main problems during mixed fermentations are related to the nutrient composition of the must and the competition between the different yeast strains involved (Andorrà et al., 2010; Wang et al., 2015, 2016). It has been demonstrated that the consumption of nitrogen at the beginning of the fermentation by non-*Saccharomyces* yeast can prevent the correct development of *S. cerevisiae*.

Sugar and nitrogen composition of the grape must are key factors for the evolution of the alcoholic fermentation and the development of the yeasts (Bell and Henschke, 2005; Beltran et al., 2005; Martínez-Moreno et al., 2012).

During the last few years, sugar content in grape must has become an important aspect since its concentration is increasing as a consequence of climate change and some viticultural practices (Mira de Orduña, 2010; Webb et al., 2012). The higher sugar content in grapes and, consequently, in musts is a problem for yeast physiology and it creates an osmotic stress that can produce, among others, stuck fermentations or wines with higher alcohol content.

In case of nitrogen, a higher or lower content can be harmful on fermentation kinetics and it has been demonstrated that a nitrogen concentration of 140 mg/l is the minimum required for yeasts to complete alcoholic fermentation (Bell and Henschke, 2005), although this value is dependent on the sugar concentration (Martínez-Moreno et al., 2012). The same as sugar concentration, many factors can influence the nitrogen content on grapes and, consequently, on must such as environmental conditions and cultural practices (Bell and Henschke, 2005).

The aim of this study was to determine the yeast dynamics and nutrient consumption during mixed fermentations of *Saccharomyces* and non-*Saccharomyces* yeast under four different nutrient conditions and with sequential addition of *S. cerevisiae* at four different time points. The fermentations were followed by density, plate culturing, PCR-DGGE and sugar consumption. According to our results, we propose the most suitable inoculation strategy for mixed fermentations using four strains isolated from Priorat region under the different nutrient concentrations.

Materials and methods

Yeast strains and starter cultures preparation

Four different yeast strains frequently isolated from natural must from Priorat Appellation of Origin (Catalonia, Spain) were employed (Padilla et al., 2016a). These yeasts were identified by ITS sequencing and identified and deposited in the Spanish Type Culture Collection (CECT) as *Saccharomyces cerevisiae* CECT 13132, *Hanseniaspora uvarum* CECT 13130, *Candida zemplinina* CECT 13129 (synonym: *Starmerella bacillaris*, Duarte et al., 2012) and *Toluraspora delbrueckii* CECT 13135. The starter cultures were prepared by growing the yeasts strains separately in liquid YPD medium (2% glucose, 2% peptone, 1% yeast extract, 2% agar, w/v; Cultimed, Barcelona, Spain) at 28 °C with a stirring rate of 150 rpm in an orbital shaker.

Mixed inoculum conditions

Fermentations were carried out in 250 ml of synthetic grape must (pH 3.3) as described by Riou et al. (1997), but with some modifications. The final concentration of sugars was either 200 or 240 g/l (denominated 200S or 240S, respectively) with a combination of glucose and fructose of 100 or 120 g/l each. The available nitrogen was either 100 or 300 mg/l (denominated 100N or 300N, respectively). Another variable was the time of the inoculation of *S. cerevisiae*: co-inoculation (0D), at 24 h (1D), at 48 h (2D) and at the 5th day (5D) after the inoculation of the non-*Saccharomyces*. Also, control fermentations were conducted for each nutrient condition with the sole inoculation of *S. cerevisiae*. Fermentations were considered finished when density was below 1000 g/l, or without variation for three consecutive days.

All the fermentations were performed in duplicate and inoculated at a concentration of $1.2 \cdot 10^6$ cells/m of *H. uvarum*, $5 \cdot 10^5$ cells/ml of *S. bacillaris*, $1 \cdot 10^5$ cells/ml of *T. delbrueckii* and $2 \cdot 10^6$ cells/ml of *S. cerevisiae*. These concentrations resemble yeast populations of natural musts from Priorat, where the non-*Saccharomyces* yeasts were isolated (Wang et al., 2015) and the practice of inoculating commercial *Saccharomyces* presentations.

Density, acetic acid and sugar measurements

The fermentations were monitored daily by density with Densito 30PX Portable Density Meter (Mettler Toledo, Spain). Once the fermentations were finished (the density was under 1000 g/l or

stable for three days), concentrations of glucose and fructose and the acetic acid concentration in the final fermentation samples were analyzed by Miura One Multianalyzer (TDI, Barcelona, Spain) using the enzymatic kit from Biosystems S. A. (Barcelona, Spain). Samples for plating, qPCR and PCR-DGGE were taken at the beginning (24 h after incubation started), in the middle (density approximately 1020-1030 g/l) and at the end of fermentation (density below 1000 g/l or stable for three days). Maximum fermentation rate (R) was calculated as maximum slope of the density measurements respect the time. Also, time to reach the 10, 50 and 75% of the final density (referred as t_{10}, t_{50} and t_{75}, respectively) were calculated as additional parameters of the fermentation kinetics (Supplementary Table 1). Successful fermentations were considered when density was below 1000 and residual sugar was below 3 g/l.

Plate culturing

Fresh samples were directly analyzed by culture-dependent techniques at each fermentation stage (beginning, middle and end of fermentation). The total yeast populations were enumerated on plates with YPD medium. The Wallerstein Laboratory nutrient agar (WL; Oxoid, England) is useful to quantify and identify wine microorganisms and it was used to discriminate between the used yeast species by colony morphology and color (Pallmann et al., 2001).

DNA extraction

Cell pellets from 1 ml of samples at each fermentation stage (beginning, middle and end of fermentation) were collected by centrifugation after washing with sterile water and kept at -80°C for further culture-independent analysis and PCR-DGGE. DNA cell pellets were extracted according to Hierro et al. (2007). The concentration and purity of DNA was determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, U.S.A.).

PCR-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. A marker prepared with the PCR products of each individual yeast species was included in the DGGE gels for migration comparison and yeast identifications.

Statistical analysis

Fermentation kinetics variables (residual sugar, acetic acid concentration, R, t_{10}, t_{50} and t_{75}) have been used to construct a dissimilarity matrix based on Euclidean distance between their values. All these variables have been used to construct a dissimilarity matrix based on the Euclidean distance between their values. ANOSIM (an analogue of univariate ANOVA which tests for differences between groups of samples) was run in PRIMER v6 (Clarke and Gorley, 2006) to determine significant differences between the different fermentations among the main experimental factors (sugar and nitrogen content, residual sugar, *S. cerevisiae* inoculation time). Principal coordinate analysis (PCoA) was used to summarize and visualize the different fermentations under each Nitrogen condition respect the final residual sugar (as an estimator of fermentation success). Pearson correlation analysis were performed between the residual sugar and the rest of parameters.

Results and Discussion

Effect of nutrients concentration on fermentation kinetics

Fermentations with optimal nitrogen concentration (300N-240S and 300N-200S) were all completed in 5-13 days, being the fermentations under excess of sugar (300N-240S) the slower ones (Table 1) (Supplementary Figure 1). On the other hand, most of the fermentations performed under limiting nitrogen concentration (100N-240S and 100N-200S) got stuck (Table 1). From these results we observed that the nitrogen content had a stronger effect than the sugar concentration in yeast metabolism and affected the fermentation kinetics. Also, ANOSIM results showed that the fermentations under different nitrogen concentration (100N and 300N) were significantly different (Table 2), i.e., their kinetics parameters (R, t_{10}, t_{50}, t_{75}, residual sugar and acetic acid) were different for each nitrogen condition. However, sugar concentration (200S and 240S) did not result in significant differences (Table 2).

It has been previously described that nitrogen concentration below 140 mg/l are limiting for growth and result in a decrease of the fermentation rate by *S. cerevisiae*, an increase risk of sluggish and stuck fermentation together with an increase in residual sugars (Bell and Henschke, 2005; Martínez-Moreno et al., 2012; Tesnière et al., 2015). However, according to our results, both 100N control fermentations inoculated just with *S. cerevisiae* were able to be completed in 7-8 days (Table 1). This could be explained by the different nitrogen requirements of the selected *S.*

cerevisiae strain, autochthonous yeast that was grown in YPD before its inoculation in the synthetic must, thus allowing inner nitrogen accumulation. Mixed fermentations with the four yeast species, with expected different nitrogen and sugar requirements, got generally stuck under 100N and it would be interesting to investigate the required addition of nitrogen to complete those fermentations (Table 1) (Figure 1). This could be due to the known higher nitrogen requirements of non-*Saccharomyces* yeasts (Andorrà et al., 2010, 2012). The consumption of the available nitrogen by the non-*Saccharomyces* yeasts and the delay in *S. cerevisiae* inoculation could increase the risk of stuck and sluggish fermentations (Medina et al., 2012).

High-sugar must (240S) was indeed expected to result in longer fermentations since it has been previously described that high sugar concentration slows down yeasts growth and the progress of fermentation (Ribéreau-Gayon et al., 2006). It has been suggested that the main stress factor under high sugar conditions would be the ethanol content and not the sugar osmotic pressure (Mauricio and Salmon, 1992; Nishino et al., 1985). Bisson and Butzke (2000) observed that a nitrogen supplementation could be appropriate in fermentations with *S. cerevisiae* under 240 g/l of sugar to complete the fermentation and Martínez-Moreno et al. (2012) suggested that 160 mg/l of nitrogen would be the minimum requirement at this sugar concentration. Conversely, other authors demonstrated in *S. cerevisiae* that the addition of nitrogen in high-sugar musts did not necessarily lead to complete fermentations even taking into account the nitrogen utilization requirements by different strains of *S. cerevisiae* (Childs et al., 2015; Martínez-Moreno et al., 2012). According to our results, a supplementation of 300 mg/l of nitrogen was enough to finish all the 240S fermentations.

Table 1. Evolution of the different fermentations (0D, co-inoculated fermentation; 1D, inoculation of *S. cerevisiae* at 24 hours; 2D, inoculation of *S. cerevisiae* at 48 hours; 5D, inoculation of *S. cerevisiae* at 5 days; and Control, only *S. cerevisiae*) under four nutrient conditions (300N-200S, 300N-240S, 100N-200S and 100N-240S). Results expressed as days spent to reach the middle (MF) and the end of the fermentation (EF), population growth in YPD at the beginning (BF), middle (MF) and end of the fermentation (EF) and the residual sugar (glucose+fructose) measured at the end of the fermentation or, when density was stable for three consecutive days, the last point was considered.

Nutrient condition	Inoculation time	MF (days)	EF (days)	BF (CFU/ml)	MF (CFU/ml)	EF (CFU/ml)	Residual sugar (g/l)
300N 200S	0D	3	5	4,0±0,04E+06	6,7±0,08E+07	3,9±0,05E+07	4,87±0,21
	1D	5	7	3,2±0,08E+06	2,7±0,09E+07	2,0±0,04E+07	0,01±0,01
	2D	5	8	3,2±0,05E+06	4,1±0,01E+07	4,8±0,03E+07	Nd
	5D	4	6	7,1±0,02E+06	3,9±0,03E+06	3,0±0,03E+06	10,18±0,37
	Control	3	5	5,6±0,09E+06	7,5±0,07E+07	2,5±0,07E+07	0,01±0,01
300N 240S	0D	5	7	8,4±0,02E+06	2,9±0,04E+08	1,9±0,06E+08	5,52±0,37
	1D	5	9	5,3±0,05E+06	5,0±0,01E+07	3,2±0,04E+07	2,80±0,14
	2D	7	13	3,0±0,03E+06	4,0±0,06E+07	2,3±0,05E+07	Nd
	5D	7	12	8,5±0,08E+06	2,3±0,06E+08	1,2±0,08E+08	30,90±0,71
	Control	3	5	2,0±0,05E+06	1,0±0,09E+07	2,5±0,03E+08	0,19±0,01
100N 200S	0D	5	8	8,0±0,05E+06	7,4±0,07E+07	5,4±0,05E+07	0,32±0,01
	1D	6	-	4,8±0,07E+06	1,8±0,04E+07	1,4±0,05E+07	43,80±3,68
	2D	6	-	4,2±0,08E+06	2,7±0,05E+07	7,6±0,04E+06	53,80±4,38
	5D	6	-	3,1±0,03E+06	7,2±0,06E+06	3,8±0,04E+06	57,50±2,62
	Control	5	8	3,0±0,08E+06	1,1±0,06E+07	7,4±0,03E+06	Nd
100N 240S	0D	7	-	3,9±0,05E+06	2,4±0,04E+07	2,6±0,02E+07	13,20±0,57
	1D	7	-	3,4±0,04E+06	3,0±0,05E+07	9,9±0,04E+06	51,10±0,49
	2D	11	-	2,1±0,04E+06	9,6±0,02E+06	9,8±0,02E+06	40,40±3,25
	5D	11	-	2,4±0,08E+06	1,7±0,06E+07	2,0±0,04E+07	64,40±2,76
	Control	5	7	3,3±0,03E+06	1,1±0,04E+07	8,8±0,05E+06	19,30±0,92

Table 2. ANOSIM of the different factors effect on the fermentations based on a dissimilarity matrix calculated by the Euclidian distance of the kinetic parameters. Values of statistical significance (*P*) below 0.05 (bold values) indicate significantly different fermentations considering a certain factor. Successful fermentation was considered when the residual sugar was below 3 g/l.

Samples	Factor	R	*P*
All	Nitrogen	**0.402**	**0.001**
All	Sugar	0.036	0.15
All	Inoculation Time	**0.243**	**0.001**
All	Residual sugar	**0.864**	**0.001**
All	Succ. Fermentation	**0.561**	**0.001**

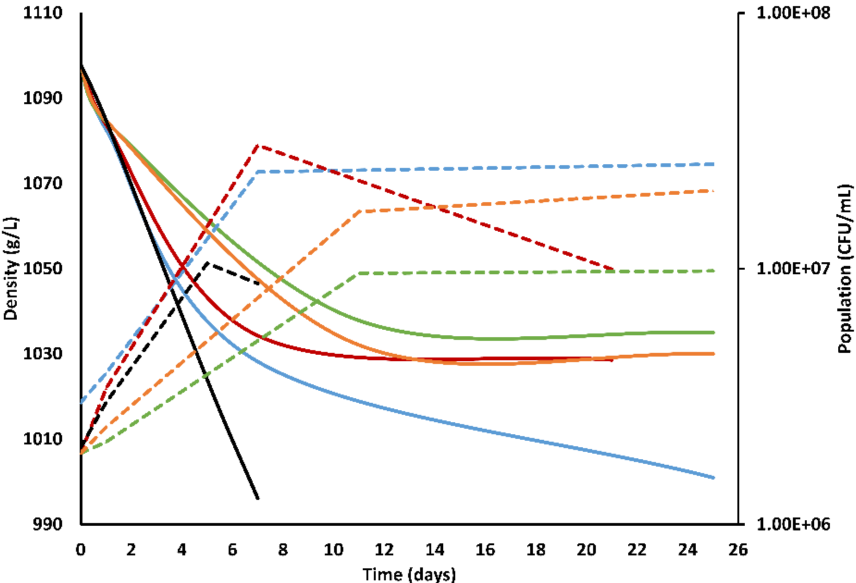

Figure 1. Fermentation kinetics of the different inoculation strategies performed under 100N-240S nutrient conditions. The solid line shows the evolution of the fermentation measured by density (g/l) and the dotted line assessed by plate culturing in YPD (CFU/ml). The line color corresponds to each fermentation strategy: blue, co-inoculated fermentation; red, inoculation of *S. cerevisiae* at 24 hours; green, inoculation of *S. cerevisiae* at 48 hours; orange, inoculation of *S. cerevisiae* at 5 days, and black; control fermentation with only *S. cerevisiae.* Standard deviations were always lower than 10% and have been avoided in the figure for clarity.

Effect of sequential inoculation of *S. cerevisiae* over fermentation kinetics

The inoculation time of *S. cerevisiae* have a significant impact over the fermentation kinetics parameters (Table 2), especially within each nitrogen concentration (Figure 2A and 2B). Control fermentations performed just with *S. cerevisiae* were the fastest to complete (5-8 days) under any of the nutrient conditions and only matched by co-inoculation (0D) under optimal sugar concentrations (300N-200S and 100N-200S).

Under optimal nitrogen concentration (300N), the sequential inoculation *of S. cerevisiae* from 24 h onwards had different effect over the fermentation kinetics depending on the sugar concentration. However, the earlier inoculation of *S. cerevisiae* did not imply that fermentation finished faster (Table 1). For example, it is interesting to observe that fermentations where *S. cerevisiae* was inoculated at 24-48 h (1D, 2D) under a nitrogen concentration of 300 mg/l took longer to finish than those where *S. cerevisiae* was added five days after the beginning of the fermentation (Table 1; Supplementary Figure 1). This result was also reflected in the separation of these samples from the rest of the 300N samples as a consequence of the differences in the fermentation kinetics parameters (Table 2, Figure 2B). A possible explanation could be that at day

5, when *S. cerevisiae* was inoculated, half of the fermentation had already been spent and the viable non-*Saccharomyces* yeast were decreasing (Table 1) (Figure 3) which meant less competition for nutrients by *S. cerevisiae.* Additionally, the death and the autolysis of non-*Saccharomyces* yeast could result in an extra nitrogen source for *S. cerevisiae* (Hernawan and Fleet, 1995).

Under limiting nitrogen concentration (100N), as stated in the previous section, most of the fermentations got stuck and have a high residual sugar (Table 2, Figure 2A and 2C). However, control fermentations were able to finish and, under optimal sugar conditions, the co-inoculation of *S. cerevisiae* and the three non-*Saccharomyces* allowed the fermentation to complete as well (Table 1). These results allowed the separation of these fermentations from the rest fermentations on the PCA analysis taking into account all the kinetics parameters (Figure 2C). Some authors have proved that co-inoculated fermentations with one or two non-*Saccharomyces* yeast species are a good strategy to ensure *S. cerevisiae* development and the fermentation process (Andorrà et al., 2010; Medina et al., 2012). According to our results, the time of *S. cerevisiae* inoculation acquired more importance under limiting nitrogen content as a consequence of nutrient consumption by the different yeast species. Medina et al. (2012) demonstrated that an increase of the inoculum size of non-*Saccharomyces* yeasts or the inoculation of *S. cerevisia*e after 24 h decreases the growth of the latter and slowed the fermentation rate of the mixed fermentation as a consequence of the nutrient consumption by non-*Saccharomyces* yeasts.

Thus, a limiting nitrogen concentration together with a sequential inoculation of *S. cerevisiae* later than 48 h involves nitrogen consumption by non-*Saccharomyces* yeasts that limits *S. cerevisiae* development and the fermentation progress.

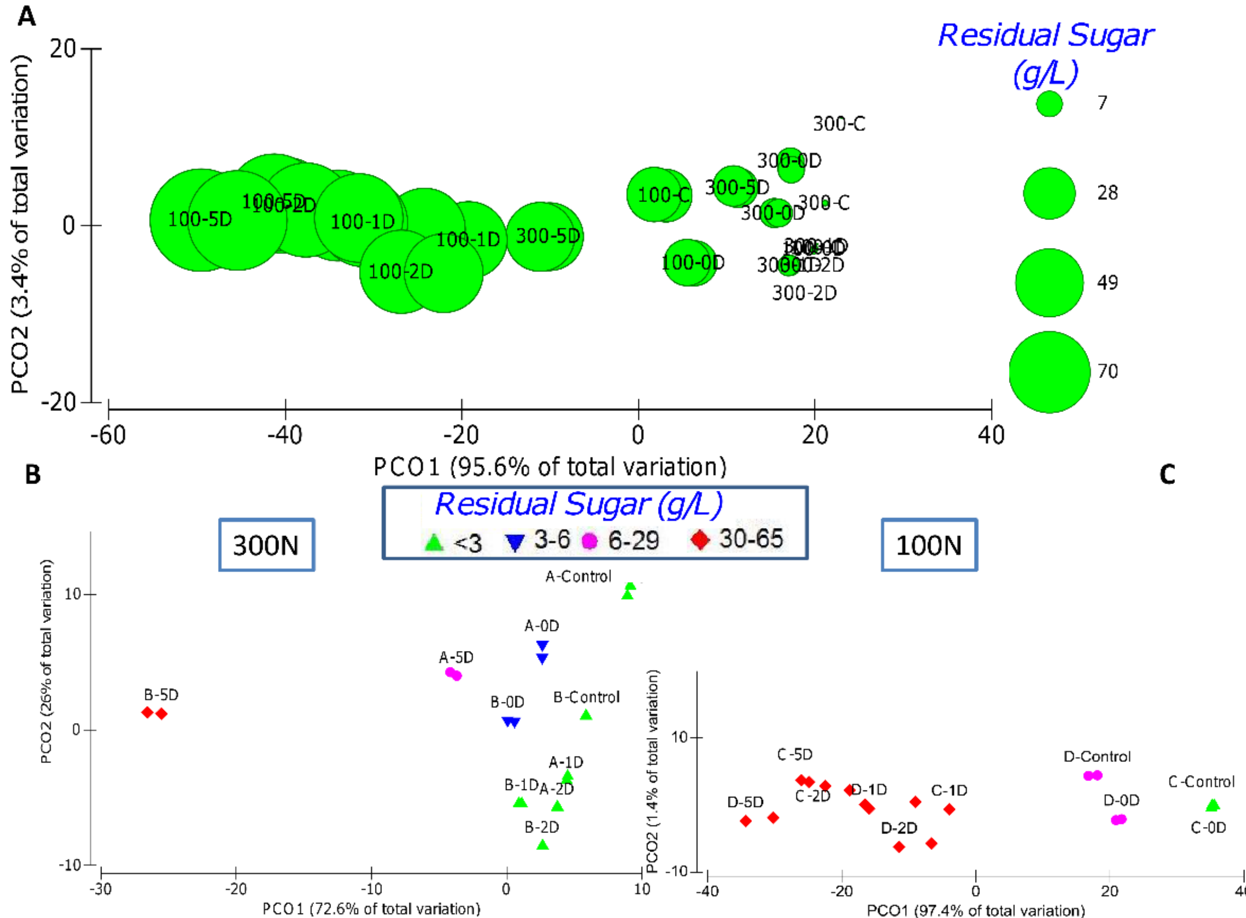

Figure 2. PCA graphs displaying the dissimilarity between the different fermentations taking into account the kinetics parameters R, t_{10}, t_{50}, t_{75}, acetic acid and residual sugar. (A) PCA representing all the fermentations respect the residual sugar content (proportional to the bubbles size) with the clustering of most of 100N fermentations at the left and most of the 300N fermentations at the right. PCA of the 300N (B) and 100N (C) fermentations respect the residual sugar where the initial sugar concentration is indicated by A or C (200S) and B or D (240S), Control represent the inoculations with only *S. cerevisiae* and the inoculation time of S. cerevisiae is indicated by 0D, 1D, 2D and 5D.

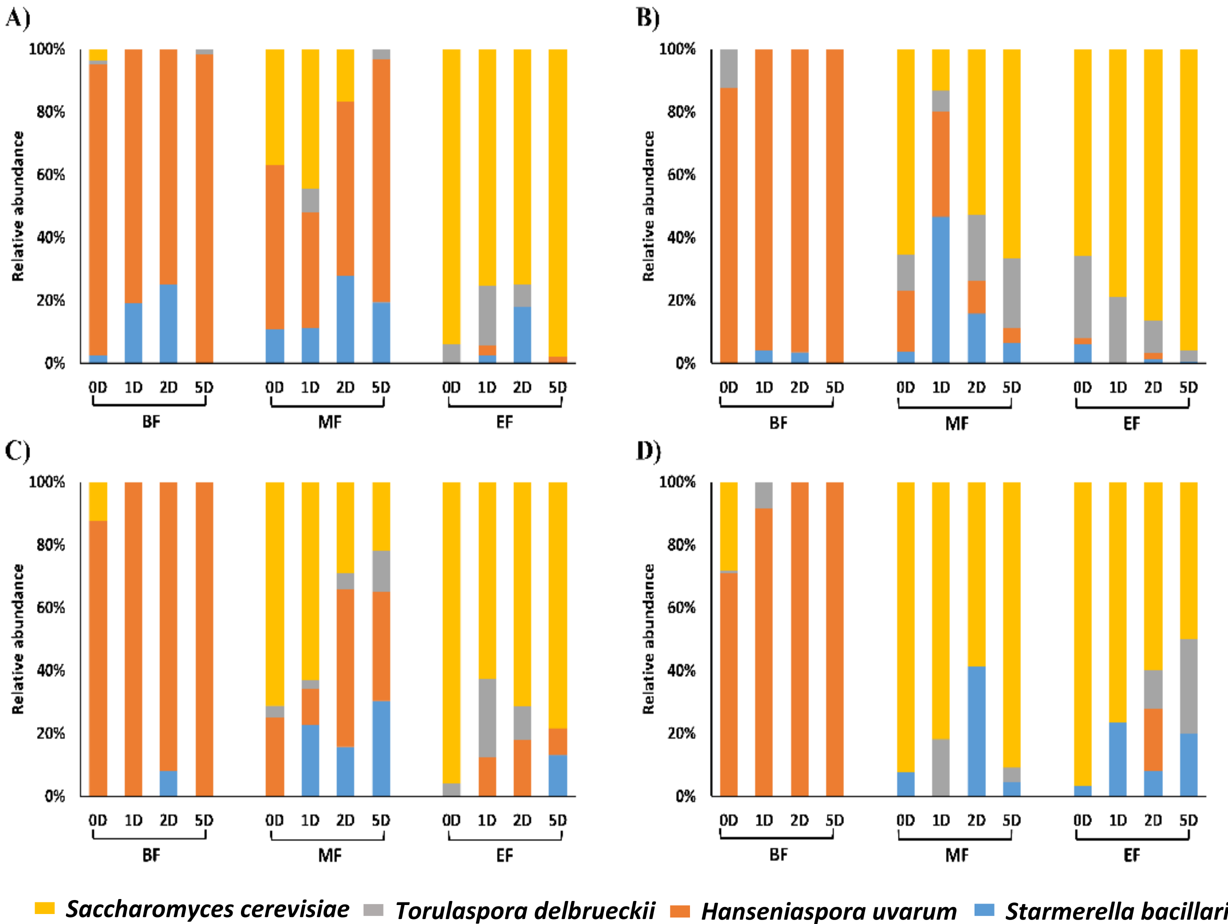

Figure 3. Yeast population dynamics at the beginning (BF), middle (MF) and end of the fermentation (EF) under four different nutrient conditions, (A) 300N-200S, (B) 300N-240S, (C) 100N-200S and (D) 100N-240S. The fermentations strategies were: 0D, co-inoculated fermentation; 1D, inoculation of *S. cerevisiae* at 24 h; 2D, inoculation of *S. cerevisiae* at 48 h; and 5D, inoculation of *S. cerevisiae* at 5 days.

Yeast dynamics by plate culturing and PCR-DGGE

Both culture dependent and independent techniques (plate culturing and PCR-DGGE) were used to follow yeast dynamics at each fermentation stage (beginning, the middle and the end of the fermentation). The differential morphology of the colonies on WL medium of the four selected yeast species allowed us to calculate the proportion of each cultivable yeast species at each fermentation stage (Figure 3). Moreover, to compare with molecular analysis results thus avoiding underestimation by the presence of viable but non cultivable (VBNC) yeast, we performed PCR-DGGE analysis of the extracted DNA at each fermentation stage using general yeast primers (Meroth et al., 2003).

Figure 3 and Table 3 show that the results obtained by these two techniques were usually comparable. However, as previous studies have reported (Andorrà et al., 2008, 2010) plate culturing proved to be more sensitive than using PCR-DGGE when the proportion of a specific species was very low at some fermentation stages. For example, by DGGE we could not detect *S. bacillaris* and *T. delbrueckii* in most of the fermentation stages while a little proportion of these species was recovered by plate-culturing technique in almost all fermentation stages and conditions. However, under nutrient limiting and sugar excess conditions (100N-240S) the DGGE technique was more efficient and we were able to detect higher yeast diversity maybe as a consequence of the loss of yeast cultivability under these extreme conditions (Table 3).

The main yeast species at the beginning of the fermentation (24 h) in all cases *was H. uvarum* while, at the end of the fermentation *S. cerevisiae* took over. We used a higher inoculum of *H. uvarum* compared to the other non-*Saccharomyces*, as occurs on natural must from the Priorat DOQ region (Wang et al., 2016), and this would explain the *H. uvarum* high proportion at the beginning of the fermentation respect to *S. bacillaris* and *T. delbrueckii*. In this sense, our results are similar to those obtained in spontaneous grape fermentations where *H. uvarum* was in great proportion at the first stages of the fermentation in Priorat area (Constantí et al., 1998; Torija et al., 2001; Wang et al., 2016).

It is interesting that a low proportion of *S. cerevisiae* was recovered at the beginning of all the fermentations even when it was co-inoculated with the non-*Saccharomyces* even taking into account that its inoculum size was similar to that of *H. uvarum*. Previous studies have reported that the initial growth of *H. uvarum* retarded the growth of *S. cerevisiae* (Herraiz et al., 1990) which could be an explanation of this effect.

In the middle of the fermentation the yeast species proportion deeply varies depending on the nutrients and the time of inoculation of *S. cerevisiae* (Figure 3). For example, under optimal nutrient conditions (300N-200S) at the mid fermentation, the non-*Saccharomyces* yeasts overgrew *S. cerevisiae* that was just more abundant at inoculation 0D or 1D (37% and 44.4%, respectively). Medina et al. (2012) noticed a negative effect of non-*Saccharomyces* yeast on nutrient availability for *S. cerevisiae* reducing its ability for grow especially when it was sequentially inoculated. Interestingly, when they added nitrogen supplementation, the fermentation rate and the proportion of *S. cerevisiae* increased. This effect was more prominent when they added a supplement of YAN and vitamin. This YAN consumption by non-*Saccharomyces* yeasts would explain the low imposition of *S. cerevisiae* over the different fermentations at the middle of the fermentation, specifically when *S. cerevisiae* was inoculated 24 h and after. However, under excess of sugar (300N-240S), *S. cerevisiae* was the most frequently recovered at 0D, 2D and 5D (52.6-66.6%) being in low proportion at 1D when the non-*Saccharomyces* yeasts (mainly *S. bacilaris*) represented more than 80%. Thus, at 300N-240S, *S. cerevisiae* was able to overtake non-*Saccharomyces* yeasts at the middle of the fermentation except when it was inoculated at 24 h although the presence of non-*Saccharomyces* yeasts in the mid fermentation under any of the conditions contemplated in the present study. We also observed that the excess of sugar (240S) affected negatively to *H. uvarum* respect the 200S conditions. Under nitrogen limitation (100N-200S/240S), we recovered higher proportion of *S. cerevisiae* at the middle of the fermentation than under the respective 300N fermentations.

At the end of the fermentation, *S. cerevisiae* was the most abundant yeast under any of the analyzed conditions, though *S. bacillaris* and *T. delbrueckii* were also present and generally in higher proportion than *H. uvarum*. In a previous study, Ciani et al. (2006) proved the high persistence of *H. uvarum* in mixed fermentations with *S. cerevisiae* under excess of sugar (270 g/l) and low temperature (15 °C), which is in accordance with our results. Wang et al. (2016) demonstrated that *T. delbrueckii* and *S. bacillaris* were able to maintain its cultivability longer than *H. uvarum* when they were inoculated with *S. cerevisiae*. Furthermore, many interactions between non-*Saccharomyces* yeasts and *S. cerevisiae* can occur in the mixed fermentations under the studied conditions: yeast-yeast cell contact, antimicrobial compounds release or competition for substrate (Ciani and Comitini, 2015). It has been described that *S. cerevisiae* produce metabolites that negatively affect non-*Saccharomyces* yeasts (Pérez-Nevado et al., 2006; Pretorius,

2000; Wang et al., 2016). So, the effect of these metabolites together with the chemical changes on the medium could provide an explanation for the decrease of *H. uvarum* and the persistence and increase of *T. delbrueckii* and *S. bacillaris* along the fermentation, because the sensibility to these antimicrobial compounds is species and strain specific (Wang et al., 2016).

Table 3. Results of the DGGE-PCR for *H. uvarum* (Hu), *S. bacillaris* (Sb), *T. delbrueckii* (Td) and *S. cerevisiae* (Sc) expressed as "++" (the intensity of the band detected by DGGE gel was high), "+" (the intensity of the band detected by DGGE gel was weak) and "-" (no band was detected by DGGE gel).

Nutrient condition	Inoculation time	Beginning Fermentation				Middle Fermentation				End Fermentation			
		Hu	Sb	Td	Sc	Hu	Sb	Td	Sc	Hu	Sb	Td	Sc
	0D	++	-	-	-	+	-	-	-	-	+	+	++
300 N	1D	++	-	-	-	++	+	+	++	-	+	+	++
200 S	2D	++	-	-	-	++	-	-	+	-	-	-	+
	5D	++	-	-	-	+	-	-	-	-	-	-	+
	0D	+	-	-	-	-	+	+	++	-	+	+	++
300N	1D	++	-	+	-	++	-	+	+	-	+	-	++
240S	2D	++	-	+	-	-	+	-	++	-	-	-	++
	5D	++	-	-	-	++	-	+	++	-	-	+	+
	0D	+	-	-	+	+	-	-	++	-	-	-	++
100N	1D	+	+	-	-	++	-	+	+	-	-	-	+
200S	2D	++	+	-	-	++	-	+	+	+	-	-	++
	5D	+	-	+	-	++	-	-	-	-	-	-	+
	0D	++	-	-	+	+	+	+	++	-	-	-	++
100N	1D	++	-	-	-	++	-	-	+	+	-	-	++
240S	2D	++	-	-	-	-	+	+	++	-	+	+	++
	5D	++	-	-	-	+	+	+	++	-	-	-	++

Fermentation products

Total residual sugars were evaluated at the end of the fermentation or, in the case of stuck fermentations, at the last considered point with stable density for three consecutive days, using an enzymatic kit as described at materials and methods section. Residual sugars were significantly correlated with all the kinetic parameters considered except with the initial sugar concentration (Supplementary Table 2).

Successful fermentations with residual sugar below 3 g/l where just those performed under optimal nitrogen concentration inoculated with *S. cerevisiae* at 48 h or before and under limiting nitrogen concentration when *S. cerevisiae* was the only yeast inoculated or when the non-

Saccharomyces yeasts where co-inoculated (Figure 2B and 2C). These successful fermentations had kinetics parameters statistically different form the rest of fermentations tested (Table 2).

Fermentations performed under suitable nitrogen content (300N-200S/240S) presented the lowest residual sugars when they were sequentially inoculated at 24 or 48h (Table 1). Unexpectedly, co-inoculated fermentations had a final sugar content between 4 and 6 g/l which could be explained by the high persistence of non-*Saccharomyces* yeasts (Figure 3) that have been described as low fermentative yeasts (Pretorius, 2000). Besides, when *S. cerevisiae* was added after 5 days, sugar content was quite high as a consequence of the *S. cerevisiae* nutrient deprivation by non-*Saccharomyces* yeasts, which compromised its development and metabolic capacities (Andorrà et al., 2010; Medina et al., 2012).

On the other hand, under nitrogen limiting conditions (100N-200S/240S) the residual sugar concentration was very high at all fermentation stages as a consequence of the stuck fermentations resulting from the nutrient limitation (Bell and Henschke, 2005) and just the co-inoculated fermentations (100N 200S) that completed the fermentation showed a lower residual sugar (Table 1).

Conclusions

Nowadays, the use of mixed fermentations represents a powerful tool as a consequence of the combination of the positive abilities of non-*Saccharomyces* yeasts with *S. cerevisiae*. Despite this fact, nutrient must conditions and the time of the inoculation of *S. cerevisiae* can determine an adequate fermentation performance. We have demonstrated the negative impact of limiting nitrogen musts on mixed fermentation resulting in stuck fermentations with higher significance than sugar concentration. However, an excess of sugar must slowed down the fermentation rate. Furthermore, the best inoculation time of *S. cerevisiae*, under adequate nitrogen concentration would be before 48 h to ensure the completion of the fermentation due to the nitrogen consumption by non-*Saccharomyces*. However, inoculations before 24 h low the proportion of non-*Saccharomyces* yeasts that could contribute to the complexity of the wines. On the other hand, under nitrogen-limiting conditions, *S. cerevisiae* should be co-inoculated to ensure the fermentation process and the nitrogen availability for this yeast.

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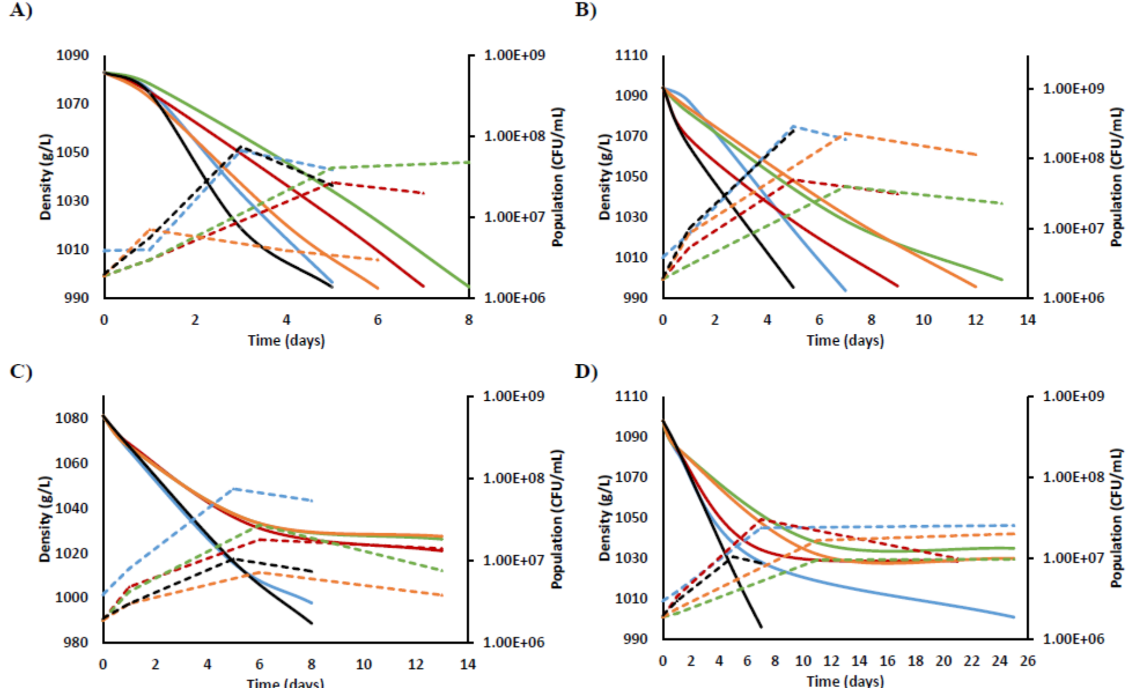
Supplementary material

Supplementary Table 1. Kinetic parameters of the different fermentations performed by duplicate. Maximum fermentation rate (R), time to reach the 10, 50 and 75% of the final density (referred as t_{10}, t_{50} and t_{75}, respectively) and residual sugar and acetic acid concentrations in g/l.

Samples	R	t_{10}	t_{50}	t_{75}	Acetic acid	Residual Sugar
300N200S-C	27.25	0.58	2.33	3.41	1.35	0.0
300N200S-C	28.05	1.58	2.33	3.42	1.35	0.0
300N200S-0D	21.70	0.74	2.58	3.72	1.02	4.9
300N200S-0D	20.75	1.74	2.55	3.71	1.02	4.6
300N200S-1D	12.85	0.86	3.45	5.07	1.10	0.0
300N200S-1D	13.20	1.86	3.47	5.11	1.10	0.0
300N200S-2D	10.98	1.08	4.04	5.89	0.63	0.0
300N200S-2D	11.03	2.08	4.05	5.92	0.63	0.0
300N200S-5D	17.47	0.65	2.82	4.17	0.96	10.2
300N200S-5D	17.57	1.65	2.81	4.16	0.96	10.7
300N240S-C	17.45	0.27	2.24	3.47	0.72	0.2
300N240S-C	17.45	1.27	2.24	3.47	0.72	0.2
300N240S-0D	15.78	0.88	3.44	5.04	1.70	5.5
300N240S-0D	15.88	1.88	3.43	5.02	1.70	5.0
300N240S-1D	10.33	0.13	3.75	6.01	0.94	2.6
300N240S-1D	10.25	1.13	3.72	5.98	0.94	2.8
300N240S-2D	8.87	0.67	5.80	9.00	1.04	0.0
300N240S-2D	8.87	1.67	5.75	8.96	1.04	0.0
300N240S-5D	8.92	0.99	5.54	8.38	1.24	29.9
300N240S-5D	8.65	1.99	5.50	8.37	1.24	30.9
100N200S-C	12.90	0.49	3.55	5.46	0.20	0.0
100N200S-C	12.75	1.49	3.29	5.02	0.20	0.0
100N200S-0D	12.65	0.41	3.46	5.37	0.16	0.3
100N200S-0D	12.43	1.41	3.45	5.36	0.16	0.3
100N200S-1D	7.54	0.47	4.77	12.02	1.10	38.6
100N200S-1D	7.50	1.47	4.72	11.37	1.10	43.8
100N200S-2D	6.80	0.18	4.95	11.05	1.30	60.0
100N200S-2D	6.84	1.18	4.89	10.85	1.30	53.8
100N200S-5D	6.88	0.24	4.95	11.05	1.30	61.2
100N200S-5D	6.70	1.24	4.91	10.85	1.30	57.5
100N240S-C	15.30	0.95	3.64	5.31	0.15	18.0
100N240S-C	15.13	1.95	3.62	5.28	0.15	19.3
100N240S-0D	9.13	0.83	5.16	7.84	0.40	13.2
100N240S-0D	9.02	1.83	5.12	7.83	0.40	14.0
100N240S-1D	8.22	0.95	5.76	15.02	1.50	50.4
100N240S-1D	8.30	1.95	5.63	14.03	1.50	51.1
100N240S-2D	4.65	1.08	9.58	16.51	1.40	45.0
100N240S-2D	4.67	2.08	9.35	14.06	1.40	40.4
100N240S-5D	5.22	8.65	8.65	15.32	1.60	68.3
100N240S-5D	5.32	9.65	8.45	13.16	1.60	64.4

Supplementary Table 2: Pearson correlation values between the residual sugar and the rest of fermentation parameters. The values in bold are different from 0 with a significance level alpha = 0.05.

Variables	Residual sugar
R	**-0.654**
t10	0.441
t50	**0.691**
t75	**0.863**
Acetic acid	**0.518**
N	**-0.639**
S	0.124



Supplementary Figure 1. Fermentation kinetics of the different inoculation strategies performed under four different nutrient conditions, (A) 300N-200S, (B) 300N-240S, (C) 100N-200S and (D) 100N-240S. The solid line shows the evolution of the fermentation measured by density (g/l) and the dotted line assessed by plate culturing in YPD (CFU/ml). The line color corresponds to each fermentation strategy: blue, co-inoculated fermentation; red, inoculation of *S. cerevisiae* at 24 h; green, inoculation of *S. cerevisiae* at 48 h; orange, inoculation of *S. cerevisiae* at 5 days, and black; control fermentation with only *S. cerevisiae*. Standard deviations were always lower than 10% and have been avoided in the figure for clarity.

CHAPTER 3

Comparison of fermentation and wines produced by inoculation of *Hanseniaspora vineae* and *Saccharomyces cerevisiae*

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Abstract

Interest in the use of non-*Saccharomyces* yeasts in winemaking has been increasing due to their positive contributions to wine quality. The non-*Saccharomyces* yeast *Hanseniaspora vineae* is an apiculate yeast that has been associated with the production of wine with good aromatic properties. However, little is known about the fermentation dynamics of *H. vineae* in natural must and its interaction with autochthonous yeasts.

In the present study, we performed semi industrial fermentations of Macabeo and Merlot musts inoculated with either *H. vineae* or *S. cerevisiae*. The yeast population dynamics were monitored by plate culturing, PCR-DGGE and massive sequencing techniques. The results obtained with these techniques show that *H. vineae* was able dominate the autochthonous microbiota in Macabeo must but not in Merlot must, which exhibited a larger, more diverse yeast population. The presence of *H. vineae* throughout most of the Macabeo fermentation resulted in more fruity and flowery wine, as indicated by the chemical analysis of the final wines, which demonstrated a strong presence of phenyl ethyl acetate at concentrations higher than the threshold of perception and approximately 50 times more than that produced in wines fermented with *S. cerevisiae*. This compound is associated with fruity, floral and honey aromas.

Keywords: non-*Saccharomyces*, *Hanseniaspora*, alcoholic fermentation, PCR-DGGE, massive sequencing

Introduction

Wine is the result of the alcoholic fermentation of grape must. Alcoholic fermentation is driven by yeasts, and it consists of the transformation of sugars present in the must, glucose and fructose, into ethanol and carbon dioxide. The yeast species *Saccharomyces cerevisiae* is considered to be primarily responsible for this process. *S. cerevisiae* is known for only metabolizing sugars via the fermentative pathway when the sugar concentration is high, even in the presence of oxygen. This phenomenon is known as the Cabtree effect (Crabtree, 1929).

Winemaking is currently changing because of an emerging interest in the use of non-*Saccharomyces* yeasts during alcoholic fermentation to increase wine complexity and differentiation. Non-*Saccharomyces* yeasts are commonly found on the grape surfaces, and these yeasts have been associated with spontaneous and unpredictable fermentation, which can result in arrested or sluggish fermentation and wine spoilage. Nevertheless, several recent studies have shown that these yeasts positively affect wine fermentation and the final wine. The positive role of non-*Saccharomyces* ranges from a better fermentation performance to improve wine quality and complexity (Fleet, 2008; Jolly et al., 2014).

Non-*Saccharomyces* yeasts can contribute to the sensorial profile of wine as a result of the production of various metabolites and the activity of certain enzymes that interact with the precursors of aromatic compounds, such as β-glucosidases, which are present in many non-*Saccharomyces* yeasts but not in *S. cerevisiae*. β-glucosidases hydrolyze aromatic glycosylated precursors into free volatile compounds to improve the final wine flavor (Jolly et al., 2014; Swangkeaw et al., 2011). Many other enzymes of technological relevance are also secreted by non-*Saccharomyces* yeasts, such as pectinases. Enzymes with proteolytic activity are of key interest in oenological fields because they facilitate the clarification process in wine and improve protein stability (Maturano et al., 2012; Strauss et al., 2001).

These yeasts have garnered interest in winemaking due to their beneficial effects and because consumers are demanding new wine styles. Many commercial yeast companies have also begun to promote mixed and sequential wine fermentations in order to satisfy consumer and producer demands. Therefore, companies have begun to thoroughly study and commercialize non-*Saccharomyces* strains, like *Torulaspora delbrueckii* or *Metschnikowia pulcherrima* (Jolly et al., 2014). Moreover, some of the yeast species that are being evaluated belong to *Hanseniaspora* spp., the main non-*Saccharomyces* yeasts in grape must that are considered apiculate yeasts due their

cell morphology. Specifically, the yeast *Hanseniaspora vineae* (anamorph *Kloeckera africana*) of this genus has been of great interest because it produces several key aromatic compounds (Medina et al., 2013; Viana et al., 2011).

The strain of *H. vineae* used in this study was isolated from Uruguayan vineyards and selected due to its positive effect on wine fermentation and good contribution to the aroma profile of the final wine. *H. vineae* has been demonstrated to increase fruity aromas and produce a high amount of acetate esters, such as 2-phenylethyl acetate and ethyl acetate, in wines elaborated by sequential fermentation with *S. cerevisiae* (Medina et al., 2013; Viana et al., 2011).

In summary, the use of non-*Saccharomyces* yeasts to produce new wine styles has been increasing due to the different aromatic profiles obtained. The aim of this work was to compare the fermentation dynamics of *H. vineae* and *S. cerevisiae* and the different obtained wines after the inoculation of these two species. To this end, we used natural must from two grape varieties, Macabeo and Merlot, inoculated either with *H. vineae* or *S. cerevisiae* fermented in semi-industrial conditions. The yeast population dynamics were monitored by plate culturing, PCR-DGGE and 18S rRNA gene massive sequencing techniques. To confirm the differences between the two species, the final wines underwent a sensory evaluation, and the aromatic profile was determined.

Materials and methods

Yeast strains

The commercial wine yeast strain used in this study was *Saccharomyces cerevisiae* QA23 (Lallemand). The apiculate yeast strain used in this work, *H. vineae* T02/5AF, was isolated from Uruguayan vineyards. Strain QA23 of *S. cerevisiae* was obtained in active dry yeast (ADY) form and rehydrated according to the manufacturer's instructions (Lallemand). The *H. vineae* strain T02/5AF was obtained in fresh paste form and rehydrated in the same manner as QA23 using warm water. The inoculation was in both cases $2 \cdot 10^6$ cells/ml of must.

Fermentation conditions

The Macabeo and Merlot grape varieties were fermented at the experimental cellar of the Faculty of Oenology (Mas dels Frares, Tarragona Spain). The Macabeo musts were fermented in triplicate in 100 l tanks at 18 °C, and 6 kg of Merlot grapes were fermented in 8 l submerged cap

fermentation tanks at 26 °C. The Macabeo must was submitted to a vacuum filtration process, whereas the Merlot grapes were selectively handpicked in the vineyard.

Fermentation activity was followed by daily density monitoring using a portable densimeter (Mettler Toledo). Samples were taken once a day from each fermenter and studied as described in the following sections.

Cell growth measurements

Samples were taken once a day, diluted in sterile MilliQ water (Millipore Q-POD™ Advantage A10), plated on YPD medium (Glucose 2%, Peptone 2%, Yeast Extract 1%, Agar 1.7%) and lysine agar medium (Oxoid, England) plates using an automated spiral platter WASP II (Don Whitley. Scientific Limited, England), and incubated at 28 °C for 48 h. The YPD medium provided the total yeast counts, whereas the lysine agar medium only provided the non-*Saccharomyces* cell counts because *S. cerevisiae* cannot grow using lysine as a unique nitrogen source. Appropriate dilution plates were counted, and 20 colonies from the must before the inoculation and the beginning (density 1070 for Macabeo and 1090 for Merlot, both of them at day 1), middle (density between 1050 and 1040) and end (density below 1000, and residual sugars below 5 g/l) of the fermentation were randomly selected and purified on YPD plates for yeast identification.

Yeast identification

The yeasts were identified based on the RFLPs of the PCR-amplified ITS-5.8S rDNA region from the isolated colonies as described by Esteve-Zarzoso et al. (1999). The RFLP patterns of the yeast isolates were compared with those of the www.yeast-id.com (http://www.yeast-id.org/english/index.html) based on the method described by Esteve-Zarzoso et al. (1999) and grouped to a known yeast species. Yeast identification was confirmed by sequencing the amplified D1/D2 domain of the 26S rDNA of representative colonies of each identified group as described by Kurtzman and Robnett (1998) and comparing this sequence with those of the type strains included in GenBank. Identification was considered appropriate with similarities higher than 99%. The sequencing was performed by Macrogen.

Saccharomyces cerevisiae cells from the isolated colonies identified as *S. cerevisiae* were further characterized by Interdelta PCR analysis as described by Legras and Karst (2003).

Massive sequencing analysis

DNA (5-100 ng) was extracted from 1 ml samples taken at the beginning, middle and end of the fermentation using the recommended procedure for the DNeasy Plant Mini kit (Qiagen, Hilden, Germany), including three bead-beating steps for 3 min in a FastPrep-24 bead beater (MP Bio, Solon, OH) to homogenize the samples. The extracted DNA was stored at -20 °C until further processing. A 350 bp (on average) 18S rRNA gene fragment was amplified in triplicate from each DNA sample with the universal primers FR1 (5-ANCCATTCAATCGGTANT-3) and FF390 (5-CGATAACGAACGAGACCT-3) (Prévost-Bouré et al., 2011). All primers had an Iontorrent tag, and the universal primer included a 10-bp barcode unique to each amplified sample. The PCR reactions contained 5-100 ng DNA template, 1× GoTaq Green Master Mix (Promega), 1 mM $MgCl_2$, and 2 pmol of each primer. The reaction conditions consisted of 94 °C for 3 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 52 °C and 1 min at 72 °C, and a final extension phase for 10 min at 72 °C. The PCR products were pooled by sample and cleaned using a GeneRead Size Selection kit (Qiagen, Hilden, Germany). The cleaned PCR products were submitted to the Centre for Omic Sciences (Reus, Spain), where their quality was assessed with a Bionalyzer and their quantity was adjusted for sequencing. The raw sequences were demultiplexed and quality filtered using QIIME v1.8.0 (Caporaso et al., 2010b). Reads were discarded if the average quality score of the read was <25, if the length of the read was <200 or >400 and they contained one or more ambiguous base calls. Operational taxonomic units (OTUs) were assigned using QIIME's uclust-based (Edgar, 2010) open-reference OTU-picking workflow with a threshold of 97% pairwise identity. The OTU sequences were aligned using PYNAST (Caporaso et al., 2010a) against the SILVA 119 reference database (Pruesse et al., 2007). Taxonomic assignments were made in QIIME against the SILVA 119 database using the naive Bayesian classifier rdp (Wang et al., 2007). The template alignment of the Greengenes core set filtered at 97% similarity. The OTU taxonomy was determined using the RDP classifier retrained toward the GreenGenes bacterial 16S rRNA database (13_8 release) (DeSantis et al., 2006) at 97% similarity. Chimeric sequences were identified and removed using ChimeraSlayer (Haas et al., 2011), and a phylogenic tree was generated from the filtered alignment using FastTree (Price et al., 2009). A final OTU table was created excluding unaligned sequences and singletons (sequences observed just once). To avoid biases generated by differences in sequencing depth, the OTU table was rarified to an even depth of 20,000 sequences per sample in comparisons of all sample types in this study.

PCR-DGGE

The $U1^{GC}$/U2 primers were used to amplify the specific U1/U2 domain of the 28S ribosomal region of yeast (Meroth et al., 2003). The PCR amplifications were performed on a Gene Amp PCR System 2700 (Applied Biosystems, Fosters City, USA) using EcoTaq DNA Polymerase (Ecogen, Spain). The Dcode universal mutation detection system (Bio-Rad, Hercules, Calif.) was used to run the DGGE analysis. The amplification of the fragments and denaturing electrophoresis were performed according to Meroth et al. (2003). The bands were excised from the gels, and the DNA was eluted overnight in 40 μl of 10 mM Tris pH 8 and 1 mM EDTA (TE) at 4 °C. The DNA was re-amplified with the same pair of primers without the GC-clamp and sequenced by Macrogen. The BLASTN algorithm was applied to the GenBank database to identify sequences (http://www.ncbi.nlm.nih.gov/BLAST/). We considered appropriate the identification of the sequences with the corresponding type strain sequences when the sequence identity was higher than 98%.

Analysis of volatile compounds

The aromatic compounds were extracted using adsorption and separate elution from an isolute ENV+ cartridge packed with 1 g of highly crosslinked styrene-divinyl benzene (SDVB) polymer (40–140 mm, cod. no. 915- 0100-C), as previously reported by Boido et al. (2003). The cartridges were sequentially equilibrated with methanol (15 ml) and distilled water (20 ml). A sample of 50 ml of wine diluted with 50 ml of distilled water and containing 0.1 ml of internal standard (1-heptanol at 230 mg/l in a 50% hydroalcoholic solution) was applied with an appropriate syringe (4–5 ml/min), and the residue was washed with 15 ml of distilled water. The aromatic compounds were eluted with 30 ml of dichloromethane. The solution was dried with Na_2SO_4, concentrated to 1.5 ml on a Vigreux column, stored at 10 °C, and, immediately prior to GC–MS analysis, further concentrated to 150 μl under a gentle nitrogen stream. The GC/MS analyses were conducted using a Shimadzu-QP 2010 ULTRA (Tokyo, Japan) mass spectrometer equipped with a Stabilwax (30 m x 0.25 mm i.d., 0.25 μm film thickness) (Restek) capillary column. The components of the wine aromatic compounds were identified comparing their linear retention indices with those of pure standards. (Aldrich, Milwaukee, 194 WI). The mass spectral fragmentation patterns were also compared with those stored in databases. GC-FID and GC-MS instrumental procedures using an internal standard (1-heptanol) were applied for quantitative

purposes, as described previously by Boido et al. (2003). Ethanol and residual sugars were quantified using Winescan FT 120 (WineScan FT120 Type 77110, Foss Analytical, Denmark).

Sensory Analysis

A specialized panel (13 panellists) analyzed the sensorial attributes of Macabeo and Merlot wines fermented with *H. vineae* and *S. cerevisiae*. The wines were analyzed by means of a triangle test and descriptive analysis. The aim of the triangle test was to distinguish the wine fermented with *H. vineae* from the wine fermented with *S. cerevisiae*. The descriptive test emphasized the aroma and flavor attributes: Reduction, fresh fruit, candied fruit, flowery, aromatic plant, yeast, toasted (phenolic), herbaceous, aroma, sourness, structure, bitterness, volume and global impression.

Statistical analysis

The variance the aromatic compounds was analyzed using the Statistica 7.1 software (StatSoft, Tulsa, OK, 1984-2005). The sensory analysis results were submitted to Student's *t-test*. The results were considered significant when the associated *p*-value was below 0.05.

Results

H. vineae and *S. cerevisiae* fermentations

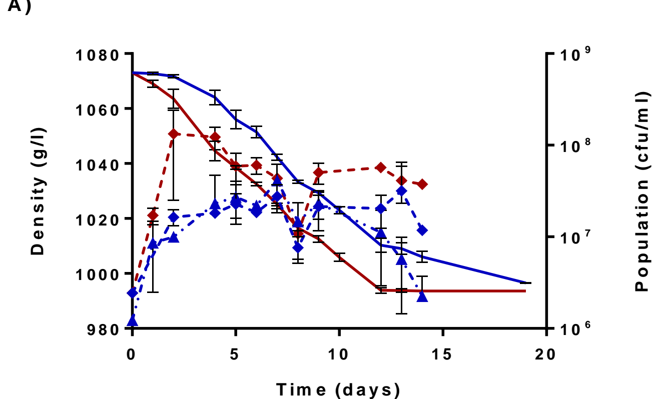
The changes in the density and yeast populations during the alcoholic fermentations of both Macabeo and Merlot grapes are presented in Figure 1. The Macabeo must (Figure 1A) inoculated with *H. vineae* required a longer fermentation process (19 days) than those inoculated with *S. cerevisiae* (14 days) due to slower fermentation kinetics and a longer latency phase. However, Merlot grapes (Figure 1B) inoculated with *H. vineae* and with *S. cerevisiae* showed a similar fermentative progress, completing the fermentation in 9 days. This fact could be explained by the early presence of non-inoculated *S. cerevisiae* in the first stages of the fermentation.

No significant differences were observed in the ethanol concentration obtained at the end of the fermentation of both varieties (10.75 ± 0.20 for Macabeo and 12.75 ± 0.10 for Merlot wines). Although all the wines were considered as "dry" (sugar concentration bellow 2 g residual sugars/l), a small difference was observed in the residual sugars in the Macabeo fermentation because the

musts fermented with *H. vineae* left 1.7 ± 0.3 g fructose/l, while all the other wines each of the residual sugars (glucose or fructose) were below 1 g/l.

The yeast population was quantified based on the colony growth on YPD and lysine agar medium. The total yeast population (YPD) was similar for the Macabeo and Merlot fermentations. The non-*Saccharomyces* yeasts counts (lysine agar) were slightly lower than the total yeast population counts in tanks inoculated with *H. vineae* for both grape musts. The Macabeo must was submitted to a vacuum filtration, which reduced the initial yeast population and resulted in yeast counts of 8.8·10⁴ cfu/ml on YPD and 5.8·10⁴ cfu/ml on lysine agar in must before inoculation.

A)



B)

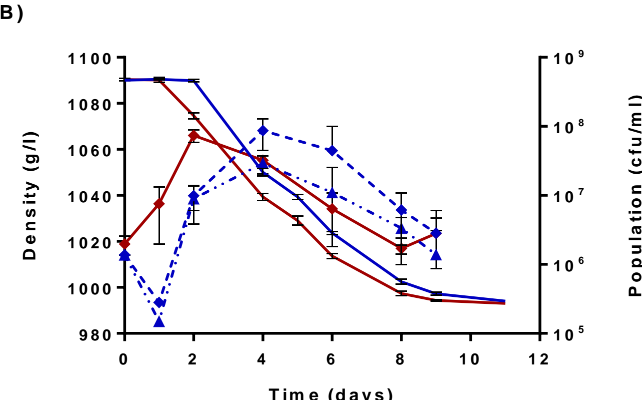


Figure 1. Density measures of fermentations kinetics (—) and yeast population (cfu/ml) growth in YPD (♦) and lysine agar (▲) medium of Macabeo (A) and Merlot (B) tanks inoculated with *H. vineae* (Blue) or *S. cerevisiae* (Red).

Yeast biodiversity in Merlot and Macabeo musts

We identified only three yeast species in Macabeo must (Figure 2A), with *Candida zemplinina* (synonym *Starmerella bacillaris*) being the main yeast species representing more than 80% of the

yeast population. The other two yeast species identified were *Hanseniaspora uvarum* and *Torulaspora delbrueckii*. Of these, *H. uvarum* represented 12.50% of the total yeast population, whereas *T. delbrueckii* represented only 3.13% of the population. This distribution significantly differed in the yeast population recovered from Merlot must (Figure 2B). We identified up to eleven yeast species, with *C. zemplinina* and *H. uvarum* being the main species representing a percentage of 41 and 39% of the total yeast population, respectively. The low yeast diversity in Macabeo must may be due to the prefermentative filtration protocol, which reduces the yeast population. Moreover, during Merlot fermentation the must maintains contact with grape skins, which releases yeasts during the whole process. In both musts, only non-*Saccharomyces* yeasts were detected.

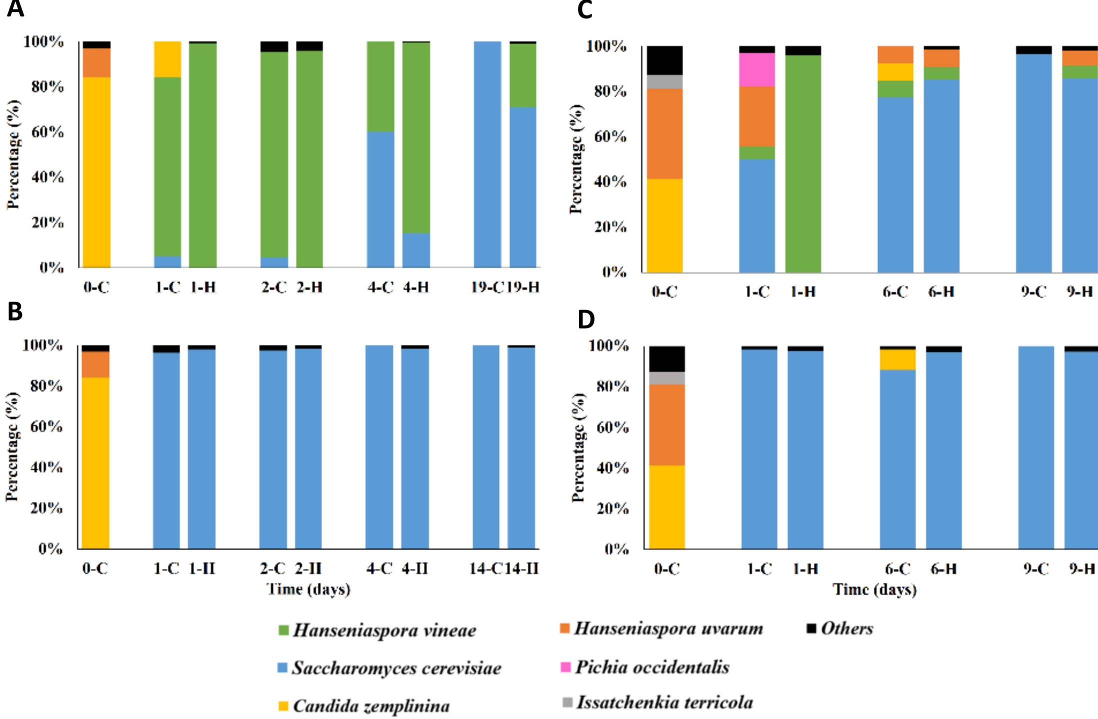


Figure 2. Comparison of the percentage of yeast species grown in YPD medium and the main genera detected by HTS along the fermentation from Macabeo tanks inoculated with *H. vineae* (A) or *S. cerevisiae* (B) and Merlot tanks inoculated with *H. vineae* (C) or *S. cerevisiae* (D). The letters C (Culture) and H (HTS) correspond to the different techniques used. The results included as others refer to the yeast species that represent less than 5% of the total yeast population.

Yeast population dynamics during fermentation

The yeast population dynamics during fermentation after the inoculation of *S. cerevisiae* or *H. vineae* was followed in yeast colonies grown on YPD plates and based on PCR-DGGE, and the massive sequencing of the 18S rRNA gene at the beginning, middle and end of the fermentation.

For the Macabeo fermentation, the inoculated strain accounted for 80-90% of the yeast population recovered from the plates one and two days after inoculation with *H. vineae*, whereas *C. zemplinina* had completely disappeared by the second day. *S. cerevisiae* was present at the beginning of fermentation, but at a very low proportion (Figure 2A). Nevertheless, the *S. cerevisiae* population began to increase from the middle to the end of the fermentation, accounting for 60% of the population at the middle of the fermentation and 100% at the end of the fermentation. From this population, up to three different *S. cerevisiae* strains could be identified by interdelta analysis, although QA23 was the most abundant at the end of the fermentation (more than 90%, results not shown). Cross contamination between cellar vats is common in commercial cellars during vintage. Nevertheless, *H. vineae* represented 40% of the yeast population at the middle of the fermentation, which demonstrated the capacity of this yeast to dominate the native microbiota, and a high proportion of its population remained active after the middle of the fermentation. However, this yeast was not able to overcome the initial microbiota in Merlot fermentations inoculated with *H. vineae* and exhibited very low recovery on plates at the beginning and middle of the fermentation (≈ 5 -7%). Other non-*Saccharomyces* yeasts (*H. uvarum, C. zemplinina* and *Pichia occidentalis*) outgrew *H. vineae* at these stages. The predominant yeast throughout the fermentation was a non-inoculated *S. cerevisiae*, which was recovered from plates and represented 50% of the total population at the beginning of the fermentation.

Macabeo and Merlot fermentations inoculated with *S. cerevisiae* showed similar yeast population patterns (Figure 2B and 2D). In both cases, the inoculated *S. cerevisiae* was able to rapidly dominate the fermentation because it was the only cultivable yeast recovered throughout the fermentation.

The PCR-DGGE profiles obtained for the DNA extracted directly from the wine during alcoholic fermentation identified the detected yeasts as *S. cerevisiae*, *H. uvarum*, *H. vineae* and *C. zemplinina* (Table 1). *S. cerevisiae* was detected in all fermentations after the first day of inoculation, including in fermentations not inoculated with the commercial *S. cerevisiae*. Nevertheless, the *S. cerevisiae* in these latter fermentations appeared to be a different strain, as evidenced different migration patterns on DGGE gels. *S. cerevisiae* QA23 shows a particularity in PCR-DGGE because it produces a double band, which is not observed in other *S. cerevisiae* strains. All the bands excised from the gel migrating to the same height resulted in at least 99.9% sequence similarity to *S. cerevisiae* type strain. Merlot and Macabeo musts inoculated with *H.*

vineae exhibited more yeast diversity at the beginning of the fermentations than musts inoculated with *S. cerevisiae*, and *H. vineae* was detected until the end of these fermentations.

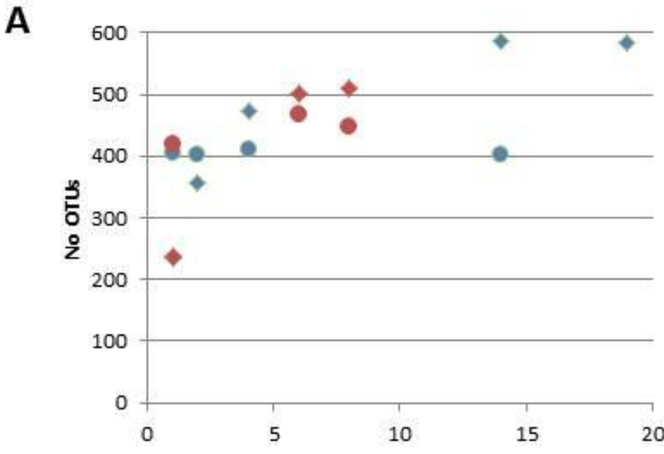
A high-throughput sequencing (HTS) approach was also used to assess the fermented wine yeast biodiversity. After the removal of low quality sequences and those failing alignment, 642,105 18S rRNA amplicon sequences were generated from 9 Macabeo and 6 Merlot wine samples. The average number of sequences per sample was 42,807, with an average length of 299 bp, and these sequences clustered into 16,302 operational taxonomic units (OTUs; 97% nucleotide identity). To avoid diversity overestimation, singletons (sequences observed only once) were eliminated, and each sample was rarified to an even depth of 20,000 sequences to avoid biases generated by differences in sequencing depth. The number of different OTUs was then reduced to 634, and 34 genera were identified. Good's coverage index was 99.7% on average, indicating that the global yeast diversity was mostly covered. The numbers of observed OTUs did not differ between Macabeo or Merlot wine samples inoculated with *S. cerevisiae* or *H. vineae* (Figure 3A). However, the number of genera was significantly higher at the beginning of the Merlot fermentation and tended to decrease towards the end of the fermentation, whereas the number of genera in Macabeo fermentation samples was lower than that in Merlot fermentations and relatively constant throughout the fermentation (Figure 3B). Most of the yeast population in all fermentations (97.7% on average) was represented by the inoculated *S. cerevisiae* and *H. vineae* strains (Table 1 and 2), whereas other non-*Saccharomyces*, such as *H. uvarum* and *Zygosaccharomyces,* accounted for only 1.9% of the sequences, and the remaining genera represented less than 0.5% of the sequences (Table 2). Some of the detected fungi were not related to alcoholic fermentation (p.e. *Aerobasidium, Aspergillus, Sporobolomyces*); however, they were mainly detected at the beginning of the fermentation, and their populations quickly decreased or disappeared (Table 2). Interestingly, *Dekkera* was only detected in Merlot samples, and we were able to observe a small but distinct increase during the fermentations with both inocula.

Table 1. The most abundant yeast genera detected by each technique in the different fermentations. The YPD-Culture and HTS results are expressed as percentages of the selected yeast specie from the total yeast population. The symbol “<” indicates that the correspondent yeast specie is present but represents less than 5% of the total yeast population. The symbol “–” indicates that the correspondent yeast specie was not detected with this technique. In case of DGGE-PCR, “++” indicates that the band is very strong, “+” indicates that the band is weak and “–” indicates that the band is nonexistent.

	Yeast species	***H. vineae***				***S. cerevisiae***			
		MACABEO							
		Day 1	Day 2	Day 4	Day 19	Day 1	Day 2	Day 4	Day 14
YPD Culture (%)	*S. cerevisiae*	5,26	<	60,00	100,00	96,30	97,50	100,00	100,00
	H. vineae	78,95	90,70	40,00	-	-	-	-	-
	H. uvarum	-	-	-	-	-	-	-	-
	C. zemplinina	15,79	-	-	-	<	<	-	-
	P. occidentalis	-	-	-	-	-	-	-	-
HTS (%)	*S. cerevisiae*	<	<	15,20	70,91	97,97	98,23	98,40	98,87
	H. vineae	99,28	95,81	84,39	28,00	<	<	<	<
	H. uvarum	<	<	<	<	<	<	<	-
	Candida	<	<	<	<	<	<	<	<
DGGE- PCR	*S. cerevisiae*	-	+	++	++	+	++	++	++
	H. vineae	++	++	+	+	-	-	-	++
	H. uvarum	-	-	-	-	-	-	-	-
	C. zemplinina	+	+	+	+	+	++	++	+
		MERLOT							
		Day 1	Day 6	Day 9			Day 1	Day 6	Day 9
YPD Culture (%)	*S. cerevisiae*	50,00	77,36	96,61			98,33	88,14	100,00
	H. vineae	5,88	7,55	<			-	<	-
	H. uvarum	26,47	7,55	-			<	-	-
	C. zemplinina	-	7,55	<			-	10,17	-
	P.occidentalis	14,71	-	-			-	-	-
HTS (%)	*S. cerevisiae*	<	85,25	85,93			97,67	96,78	97,20
	H. vineae	96,09	5,51	5,53			<	<	<
	H. uvarum	<	7,94	6,77			<	<	<
	Candida	<	<	<			<	<	<
DGGE- PCR	*S. cerevisiae*	+	++	++			++	++	++
	H. vineae	++	+	+			-	-	-
	H. uvarum	+	+	+			-	-	-
	C. zemplinina	-	-	-			-	-	-

Table 2. Percentage of main genera and species detected by HTS after the inoculation of *H. vineae* or *S. cerevisiae* on Merlot and Macabeo wines. The symbol “<” indicates percentages values lower than 0.01 and bigger than 0. The symbol “-” indicates not detected by HTS.

	Macabeo *H.vineae*					**Macabeo *S. cerevisiae***				**Merlot *H. vineae***			**Merlot *S. cerevisiae***		
Days from inoculation	**1**	**2**	**4**	**14**	**19**	**1**	**2**	**4**	**14**	**1**	**6**	**8**	**1**	**6**	**8**
Saccharomyces cerevisiae	0.47	3.68	15.20	60.32	70.91	97.97	98.23	98.40	98.87	0.44	85.25	85.93	97.67	96.78	97.20
Hanseniaspora vineae	99.28	95.81	84.39	38.75	28.00	0.64	0.48	0.57	0.24	96.09	5.51	5.53	0.53	1.69	1.60
Hanseniaspora uvarum	0.07	0.07	0.01	<	0.01	0.05	0.02	0.01	<	0.33	7.94	6.77	0.24	0.40	0.15
Zygosaccharomyces	0.01	0.05	0.23	0.47	0.60	0.67	0.66	0.52	0.39	0.41	0.49	0.49	0.53	0.51	0.47
Saccharomyces (others)	0.01	0.03	0.08	0.31	0.39	0.55	0.54	0.43	0.44	<	0.33	0.36	0.36	0.37	0.36
Aureobasidium	<	<	-	0.01	-	-	-	-	-	1.83	0.03	0.02	0.33	0.05	0.05
Candida	0.09	0.14	0.03	0.06	0.07	0.11	0.07	0.06	0.05	0.31	0.18	0.46	0.19	0.18	0.12
Pichia	0.05	0.17	0.05	<	0.01	-	-	<	-	0.03	0.26	0.26	0.01	0.01	-
Dekkera	-	-	-	-	-	-	-	-	-	-	<	0.17	-	-	0.03
Aspergillus	-	0.01	-	-	-	-	-	-	-	0.11	<	0.01	0.03	<	-
Sporobolomyces	-	-	-	-	-	-	-	-	-	0.08	<	-	0.02	-	-
Issatchenkia	0.01	0.02	0.01	0.01	0.01	0.01	-	<	-	0.02	0.01	-	0.01	-	-
Cryptococcus	-	<	-	<	-	-	-	-	-	0.07	-	-	0.01	-	-
Diplodia	-	-	-	-	-	-	-	-	-	0.07	-	-	<	-	-
Zygoascus	-	-	-	-	-	<	-	-	-	0.03	-	-	0.02	0.01	0.01
Rhizina	-	-	-	-	-	-	-	-	-	0.04	-	-	0.01	-	-
Catenulostroma	-	-	-	-	-	-	-	-	-	0.05	-	-	-	-	-
Bensingtonia	-	-	-	0.04	-	-	-	-	-	<	-	-	-	-	-
Saccharomycodes	-	-	<	0.02	0.01	-	-	-	<	-	-	-	-	-	0.01
Scheffersomyces	-	-	-	-	-	-	-	-	-	0.02	<	<	0.01	-	-
Wickerhamomyces	0.01	0.01	<	-	-	-	-	-	-	0.01	-	-	-	-	-
Cladosporium	-	-	-	-	-	-	-	-	-	0.01	-	-	0.01	-	-
Sugiyamaella	0.01	0.01	<	-	-	-	-	-	-	-	-	-	-	-	-
Trigonopsis	0.01	0.01	-	-	<	-	-	-	-	-	-	-	-	-	-
Lipomyces	-	-	-	-	-	-	-	-	-	-	0.01	0.01	-	-	-
Phillipsia	-	-	-	-	-	-	-	-	-	0.01	-	-	-	-	-
Wallemia	0.01	-	-	-	-	<	-	-	-	<	-	-	-	-	-
Vanderwaltozyma	-	-	-	-	-	-	-	-	<	-	<	-	-	-	<
Cochliobolus	-	-	-	-	-	-	-	-	-	0.01	-	-	-	-	-
Malassezia	-	-	-	-	-	<	-	-	-	<	-	<	-	-	-
Bispora	-	-	-	-	-	-	-	-	-	<	-	-	<	-	-
Rhodotorula	-	-	-	-	-	-	-	-	-	<	-	-	<	-	-
Metschnikowia	-	-	-	-	-	-	-	-	-	<	<	-	-	-	-
Phoma	-	-	-	-	-	-	-	-	-	<	-	<	-	-	-
Agaricostilbum	-	-	-	-	-	-	-	-	-	<	-	<	-	-	-
Baudoinia	-	-	-	-	-	-	-	-	-	<	-	-	-	-	-



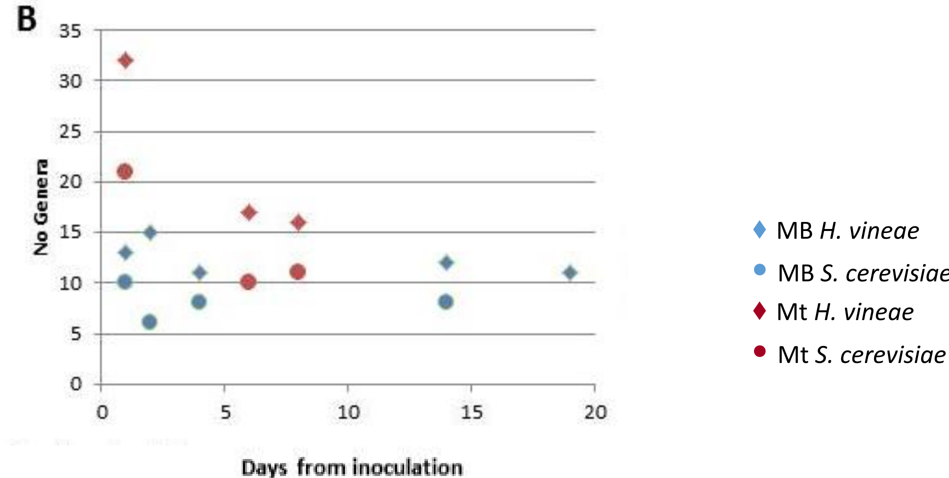


Figure 3. Number of OTUs (A) and genera (B) detected by HTS after inoculation of *H. vineae* (♦) or *S. cerevisiae* (•) on Merlot wine (red symbols) or Macabeo wine (blue symbols).

Volatile compound composition

Fifty volatile compounds produced during alcoholic fermentations of natural Macabeo musts inoculated with *H. vineae* and *S. cerevisiae* were identified and quantified in the Macabeo wines. These compounds were classified into ten groups [acetates, acids, alcohols, C6 compounds, carbonyl compounds, esters, phenols, lactones, unusual compounds (named here as "rares") and terpenes]. Table 3 shows the mean concentration of the identified volatile compounds. To assess the possible contribution of the different components to the wine aroma, the detection threshold and aroma descriptor reported in the literature are included for each compound.

Significant differences between yeasts were only observed in three of the ten groups of compounds (Acetates and rares in Figure 4A and alcohols in Figure 4B).

Both yeasts primarily produced alcohols and esters, and three (isobutanol, isoamyl alcohol, and phenyl ethanol) of the eleven identified alcohols reached the threshold of perception reported in the literature. Of these three alcohols, phenyl ethanol provides good aromas that are described as rose and honey-like. Among the identified esters, ethyl hexanoate reached the threshold of perception and contributes a green apple aroma. The compounds constituting the next most abundant group produced by *H. vineae* are classified as rare and included N-acetyl tyramine and 1H-indole-3-ethanol acetate ester. These compounds were not found in the wine fermented with *S. cerevisiae*.

As shown in Table 3, a total of 7 acids were identified; hexanoic, decanoic and octanoic acid showed the highest concentration, and octanoic acid exceed the odor threshold reported in the literature.

Four acetates were identified, and phenethyl acetate was the most interesting. Specifically, this compound was 50 times more abundant in wines fermented with *H. vineae* than in those fermented by *S. cerevisiae*. This compound endows wine with floral, fruity and honey-like aromas.

Six phenolic compounds were identified, as shown in Table 3. These compounds did not reach the threshold of detection, and their contribution to wine aroma is consequently expected to be insignificant. One of these compounds, 4-ethylguaiacol, is generally attributed to the presence of *Brettanomyces*, although it was identified in wines fermented with *H. vineae*.

Six terpenes were identified, as shown in Table 3. The concentrations of these compounds were lower than the threshold, and they are consequently not expected to contribute to the wine flavor profiles.

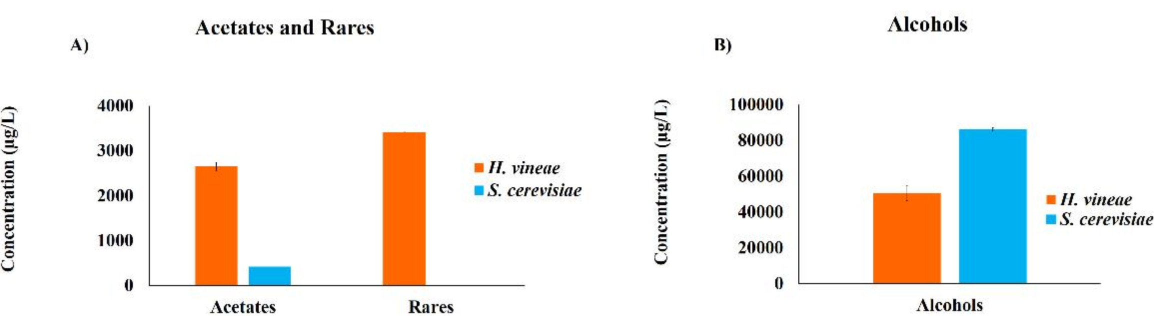


Figure 4. Sum of compounds with significant differences produced by *H. vineae* and *S. cerevisiae* (A) acetates and compounds listed as rare (N-acetyltyramine and 1H-indole-3-ethanol acetate ester) (B) Alcohols. Code: *, **, *** indicate significance at $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively.

Table 3. Average concentrations of the two fermentations (± Standard Deviation) in µg/l. Odor descriptor and odor thresholds reported in the literature are included.

	H. vineae Average SD	*S. cerevisiae* Average SD	Odor descriptor	Odor threshold (µg/l)
ACETATES				
Isobutyl acetate	11 ± 1	0 ± 0*	N/A	N/A
Isoamyl acetate	222 ± 20	218 ± 93	Banana[a]	30
1,3-Propanediol, diacetate	99 ± 18	160 ± 7	N/A	N/A
Phenethyl acetate	2322 ± 50	47 ± 13**	Fruity, honeyed, floral[a]	250
Acetate sum	2653 ± 89	425 ± 100**		
ACIDS				
Isobutyric acid	74 ± 40	0 ± 0	Acid, fatty[b]	230
Heptanoic acid	231 ± 28	304 ± 35*	N/A	N/A
Hexanoic acid	330 ± 35	777 ± 70*	Fatty, cheese[a]	420
Octanoic acid	734 ± 12	1757 ± 335	Fatty[a]	500
Decanoic acid	979 ± 31	389 ± 212	Rancid, fat[a]	1000
9-Hexadecenoic acid	479 ± 11	72 ± 57	N/A	N/A
Acids sum	2825 ± 48	3299 ± 708		
ALCOHOLS				
Isobutyl alcohol	2388 ± 277	1895 ± 165	Fusel oil, chemical[b]	0,5
1-Butanol	58 ± 9	84 ± 38	Like wine, medicine[a]	150.000
Isoamyl alcohol	36361 ± 4127	61355 ± 5063*	Alcoholic, fruity at low concentration[b]	0,3
3-Methyl-1-pentanol	36 ± 1	69 ± 7	Like wine, nail polish[a]	40.000
3-Ethoxy-1-propanol	28 ± 0	108 ± 12	Fruity[b]	
Furfuryl alcohol	12 ± 2	0 ± 0	N/A	N/A
3-(Methylthio), 1-Propanol	321 ± 35	599 ± 281	Sweet, potato[a]	1000
Benzyl alcohol	37 ± 7	0 ± 0	Floral, rose, phenolic, balsamic[a]	200.000
Phenyl ethanol	8099 ± 158	16830 ± 957	Rose, honey[a]	10000
Tyrosol	1855 ± 156	5274 ± 3149	N/A	N/A
Tryptophol	1365 ± 95	0 ± 0**	N/A	N/A
Alcohols sum	50557 ± 4276	86214 ± 897*		
C6 COMPOUNDS				
1-Hexanol	386 ± 7	328 ± 50	Grass just cut[a]	2500
Trans 3-Hexen-1-ol	7 ± 1	129 ± 19	Green[a]	1000
Cis3-Hexen-1-ol	120 ± 1	0 ± 0**	Green, kiwi[a]	400
C6 compounds sum	513 ± 9	457 ± 31		
CARBONYL COMPOUNDS				
Acetoin	15 ± 13	56 ± 59	Creamy, butter, fat[b]	0,15
Furfural	9 ± 2	0 ± 0	Fusel alcohol, toasted bread[a]	770
Carbonyl compounds sum	23 ± 16	56 ± 59		

Table 3. Continued.

	H. vineae Average SD	*S. cerevisiae* Average SD	Odor descriptor	Odor threshold (µg/l)
ESTERS				
Methyl butyrate	9 ± 4	14 ± 7	N/A	N/A
Ethyl butyrate	62 ± 15	158 ± 38	N/A	N/A
Ethyl hexanoate	81 ± 4	241 ± 24	Green apple[a]	14
Ethyl lactate	8285 ± 378	3071 ± 1915	Strawberry, raspberry[a]	60.000
Ethyl octanoate	79 ± 33	225 ± 9	Sweet, banana, pineapple[a]	500
Ethyl 3-hydroxybutyrate	119 ± 8	52 ± 16	N/A	N/A
Ethyl decanoate	143 ± 46	76 ± 6	Sweet, hazelnut oil[a]	200
Ethyl succinate	1240 ± 47	1775 ± 836	Toffee, coffee[a]	1.000.000
Diethyl malate	88 ± 6	428 ± 165	Green[a]	760.000
Diethyl 2 hydroxy glutarate	233 ± 6	268 ± 67	Grape, green apple, marshmallow[a]	20.000
Diethyl succinate	4012 ± 255	15671 ± 6792	Overripe melon, lavender[a]	100000
Ester sum	14348 ± 509	21979 ± 9334		
PHENOLS				
Guaiacol	6 ± 1	0 ± 0	Smoky, hospital[a]	9,5
4-ethylguaiacol	73 ± 66	0 ± 0	Bretty flavors[a]	110
4-vinylguaiacol	33 ± 21	28 ± 14	Clove, curry[a]	40
Phenyl lactate	53 ± 10	128 ± 32	N/A	N/A
Ethyl vanillate	5 ± 0	17 ± 20	N/A	N/A
Acetovainillone	14 ± 5	15 ± 13	N/A	N/A
Phenol sum	183 ± 41	188 ± 11		
LACTONES				
Butyrolactone	223 ± 1	251 ± 6	Toasted burned[a]	1000
5-carboethoxy-gamma-butyrolactone	127 ± 7	176 ± 11	N/A	N/A
Lactone sum	350 ± 8	327 ± 17		
RARES				
N-acetyl tyramine	2040 ± 11	0 ± 0**	N/A	N/A
1H-Indole-3-ethanol, acetate(ester)	1377 ± 8	0 ± 0**	N/A	N/A
Rare sum	3417 ± 4	0 ± 0***		
TERPENES				
Linalool	12 ± 2	28 ± 13	Rose[a]	50
Alpha-terpineol	112 ± 31	0 ± 0	Floral, pine[a]	400
Citronellol	27 ± 6	39 ± 5	Sweet, floral[b]	18
Terpene sum	150 ± 23	67 ± 18		

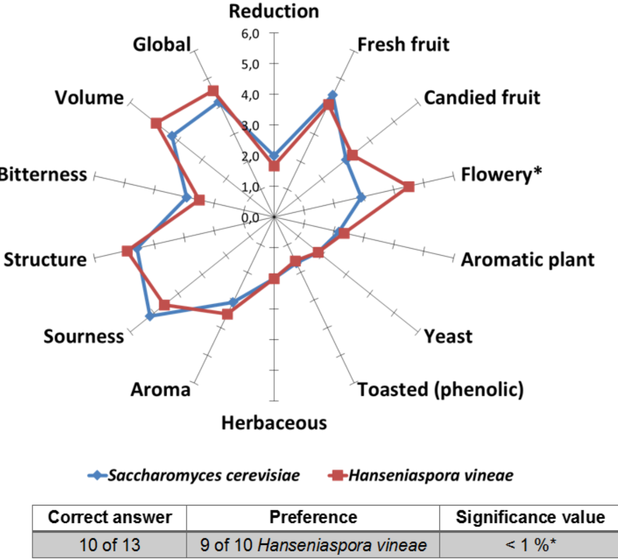
* ,**, *** indicate significance at $p < 0,05$, $p < 0,01$, $p < 0,001$ respectively.
[a] Fariña et al. (2015).
[b] Boido (2002).

Sensory analysis

To evaluate the ability of *H. vineae* to produce a wine with attributes that differ from those of a wine fermented with *S. cerevisiae*, the produced wines were analyzed with triangle and descriptive tests.

In the triangle test of Macabeo wine (Figure 5), wine-tasters easily distinguished the wine fermented with *H. vineae* from that fermented with *S. cerevisiae*, and the majority selected the wine fermented with *H. vineae* as their preference. In the descriptive test, the wine fermented with *H. vineae* received the best rating. Notably, wine fermented by *H. vineae* showed a significantly stronger flowery aroma profile ($p = 0.037$) than wine produced with *S. cerevisiae*.

The Merlot must could not be evaluated as a consequence of a powerful reduction note that could not be corrected for the tasting.



Correct answer	Preference	Significance value
10 of 13	9 of 10 *Hanseniaspora vineae*	< 1 %*

Figure 5. Results of triangle (table) and descriptive (graphic) test of Macabeo wine fermented with *H. vineae* and *S. cerevisiae*.

Discussion

In this work, we performed semi-industrial fermentations with *H. vineae* and S. *cerevisiae* using Macabeo and Merlot musts in order to evaluate the differences in yeast populations during fermentation and the wines produced. We followed the yeast population dynamics in both grape musts inoculated with *H. vineae* and *S. cerevisiae* by plate culturing on YPD medium, PCR-DGGE with yeast general primers, as well as yeast general primers and the HTS of 18S rRNA gene.

As expected, the fermentation of musts inoculated with *H. vineae* required more time than fermentations inoculated with *S. cerevisiae*, demonstrating the high fermentative ability of this wine yeast with respect to *H. vineae*. However, rapid fermentation might not be a desired characteristic in the production of quality wines, due to flavor lost or high energetic demand for refrigeration (Medina et al., 2013).

The initial yeast diversity of the must was only analyzed after plate culturing. Before the inoculation, Merlot must presented a higher yeast diversity than Macabeo must, as evidenced by up to eleven different yeast species that were identified in the red variety, whereas the Macabeo contained only three species (*C. zemplinina, H. uvarum* and *T. delbrueckii*). As expected, only non-*Saccharomyces* yeasts were recovered by cultivation from both musts before the inoculation because *S. cerevisiae* is not present in relevant amounts in grapes and is mostly associated with cellar equipment (Beltran et al., 2002; Pretorius, 2000; Torija et al., 2001).

The PCR-DGGE analysis identified *S. cerevisiae* and *H. vineae* as the main yeasts in both the Macabeo and Merlot fermentations. *C. zemplinina* was found only in Merlot, and these results corroborated those observed after the plate culture. Other minor yeast species were not detected by PCR-DGGE, especially if their population densities were below 10^3-10^4 cfu/ml or if their abundance was two orders of magnitude lower than that of the main species, as reported in previous studies (Andorrà et al., 2008; Mills et al., 2002; Prakitchaiwattana et al., 2004).

Even if must samples were not included in the HTS approach, this technique clearly detected higher levels of fungal diversity than the other techniques. Specifically, a total of 32 genera with a great diversity of OTUs were identified within each genus. The HTS technique was also able to detect yeast genera not related with fermentation, and some of these yeasts are associated with spoilage (like *Dekkera/Brettanomyces*). Although the proportion of these yeasts was very low, the changes in their proportion throughout the fermentation suggested that they were active and represented a potential risk for the spoilage of the final wine. Thus, the HTS technique confirmed the general trend obtained for the most abundant yeast populations by plate culturing and PCR-DGGE, but it also facilitated the detection and tracking of some minor yeast genera that may significantly impact the quality of the wine.

The culturing, PCR-DGGE and HTS analysis confirmed a decrease in the yeast genera diversity from the beginning to the end of fermentations, and these techniques also consistently indicated that the yeast diversity was higher in Merlot fermentations than in Macabeo

fermentations. The low diversity exhibited by Macabeo must before inoculation may be a consequence of its treatment with a vacuum filter. The objective of this treatment was to clean the must and remove solid and colloidal particles, but it also reduced autochthonous yeasts and nutrients in the must. We used this protocol for two reasons: to clean the Macabeo must and to remove colloidal and solid particles and also it was affected by rain and exhibited some spoilage. Thus, we wanted to reduce the autochthonous yeast population because we planned to inoculate the must with *H. vineae*. We achieved these objectives. Furthermore, the Merlot was selectively handpicked in order to obtain the healthiest bunches of grapes. The results from plate culturing, PCR-DGGE and HTS indicate that *H. vineae* was able to overcome the autochthonous microbiota in the Macabeo must, constituting a high proportion of the yeast population until the middle of the fermentation and showing good fermentative capacity. However, *H. vineae* represented a very low proportion of the yeast population in Merlot must after the inoculation. However, after the inoculation (day 1), the percentages of the identified yeasts were different based on the method of estimation, being the population of *H. vineae* hardly recovered on plates. *S. cerevisiae* was the most abundant yeast recovered from plates, whereas it was present at much lower levels in all culture-independent methods (HTS and DGGE). This observation could be related to the well-reported interaction between *S. cerevisiae* and non-*Saccharomyces* yeasts during wine fermentation: non-*Saccharomyces* yeasts are quickly displaced by *S. cerevisiae*, which might kill or at least result in viable but not cultivable (VBNC) statuses, as indicated in several recent reports (Andorrà et al., 2010, 2011; Millet and Lonvaud-Funel, 2000; Pérez-Nevado et al., 2006; Wang et al., 2015). However, we should emphasize that these culture-independent techniques also detect DNA from dead cells, which could also be the case. At later fermentation time points, all methods again produced coincident results and identified *S. cerevisiae* as the main population. Interestingly, the dominant *S. cerevisiae* was not the inoculated strain, suggesting that a cellar-resident strain took over. Furthermore, Merlot grapes are among the latest in the harvest in this cellar, and, thus, the environmental contamination of the cellar is already high. The *S. cerevisiae* population began to increase and became the dominant specie according to HTS that produced most intense in the band profile of DGGE, and this unique yeast was recovered at the end of the fermentation.

The final wine obtained by fermenting Macabeo must with *H. vineae* was preferred over the wine fermented with *S. cerevisiae* and notable for its fruity and flowery aroma. This result

corroborates those of studies that performed mixed fermentations *with H. vineae* and obtained high amounts of an acetate ester, phenethyl acetate, which is responsible of the fruity and flowery aroma of wine (Viana et al., 2009, 2011). The chemical analysis revealed that wines inoculated with *H. vineae* contained 50 times more phenethyl acetate than wines inoculated with *S. cerevisiae*, which explains the results of our sensory analysis and agrees with previous observations (Medina et al., 2013).

The production of N-acetyltyramine and 1H-indole-3ethanol acetate ester also differed. These compounds were abundant in wines inoculated with *H. vineae* and could not be detected in wines fermented with *S. cerevisiae*. These compounds could be derived from tyrosol, and this hypothesis is supported by the high concentrations of tyrosol in wines inoculated with *S. cerevisiae*. This difference could be explained by the production of unusual compounds from tyrosol in wines inoculated with *H. vineae*. However, aromatic descriptors associated with these compounds have not yet been reported.

Conclusion

Interest in the use of non-*Saccharomyces* yeasts in winemaking has been increasing. *H. vineae* is an apiculate non-*Saccharomyces* yeast that has demonstrated a good fermentative rate in Macabeo must and resulted in more flowery wines, which is likely related to the higher production of phenylethyl acetate. However, the need for inoculation with *S. cerevisiae* must be emphasized because *H. vineae* is unable to finish the alcoholic fermentation. We did not use a *S. cerevisiae* strain in the inoculations with *H. vineae*, and the end of fermentation was consequently improperly controlled. Furthermore, the use of this yeast requires very healthy grape musts and it is not recommended to use with grapes with a high and diverse yeast population or red musts, in which maceration with skins may be a significant source of yeast. In addition, the present study shows that the HTS technique detected not only the most abundant yeast populations obtained by plate culturing and PCR-DGGE but also some minor yeast genera that may significantly affect the quality of the wine.

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CHAPTER 4

Analysis of the NCR mechanisms in *Hanseniaspora vineae* and *Saccharomyces cerevisiae* during winemaking

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UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF NON-SACCHAROMYCES YEAST ON WINEMAKING AND QUALITY
Jéssica Lleixà Daga

Abstract

There is increasing interest in the use of non-*Saccharomyces* yeasts in winemaking due to their positive attributes. The non-*Saccharomyces* yeast *Hanseniaspora vineae* is an apiculate yeast that has been associated with the production of wine with good fermentation capacity and an increase in aromatic properties. However, this yeast represents a concern in mixed culture fermentation because of its nutrient consumption, especially nitrogen, as its mechanisms of regulation and consumption are still unknown.

In this study, we analyzed the nitrogen consumption, as well as the nitrogen catabolism repression (NCR) mechanism, in two genome-sequenced *H. vineae* strains, using synthetic must fermentations. The use of synthetic must with an established nitrogen content allowed us to study the NCR mechanism in *H. vineae*, following the amino acid and ammonia consumption, and the expression of genes known to be regulated by the NCR mechanism in *S. cerevisiae*, *AGP1, GAP1, MEP2* and *PUT2*. *H. vineae* exhibited a similar amino acid consumption and gene expression profile to *S. cerevisiae*. However, the wine strain of *S. cerevisiae* QA23 consumed ammonia and valine more quickly and, in contrast, tyrosine and tryptophan more slowly, than the *H. vineae* strains. Our results showed a similar behaviour of nitrogen regulation in *H. vineae* and *S. cerevisiae*, indicating the presence of the NCR mechanism in this *Hanseniaspora* yeast differentiated before the whole genome duplication event of the *Saccharomyces* complex. Future study will elucidate if the NCR mechanism is the only strategy used by *H. vineae* to optimize nitrogen consumption.

Keywords: non-*Saccharomyces*, Yeast Assimilable Nitrogen (YAN), nitrogen consumption, alcoholic fermentation, amino acids

Introduction

For many years, the microbiological process of winemaking has been focused on the use of starter cultures of *Saccharomyces cerevisiae*. The inoculation of commercial strains of *S. cerevisiae* is a common practice in wineries to ensure the completion of the fermentation and the quality of the final product. However, the elaboration of uniformed wines is not always desired, and winemakers are becoming more interested in obtaining characteristic and differential wines. Considering this fact, in recent years, much effort has been focused on the use of non-*Saccharomyces* yeasts to obtain wine with new organoleptic characteristics (Carrau et al., 2015; Fleet, 2008; Jolly et al., 2014). Non-*Saccharomyces* yeasts are naturally present on grape surfaces, and they can start spontaneous fermentations that can lead in incomplete fermentations or result in wines with unpleasant properties. Despite this fact, many of these non-*Saccharomyces* yeasts have proven to produce enzymatic activities and release metabolites that improve some oenological processes and the wine flavor (Jolly et al., 2014; Padilla et al., 2016; Varela, 2016). For this reason, the interest in the use of co-fermented or sequential mixed cultures of non-*Saccharomyces* and *S. cerevisiae* has increased to take advantage of both trends during the winemaking process.

Hanseniaspora vineae is one species of yeast that belongs to the non-*Saccharomyces* yeasts of oenological interest (Martin et al., 2018). The primary positive contributions of this yeast during the winemaking process are basically related to the aroma profile in the final wine. *H. vineae* has been demonstrated to increase fruity aromas and produce high amounts of acetate esters, primarily 2-phenylethyl acetate and benzenoids, in wines elaborated in either synthetic (Martin et al., 2016) or natural musts inoculated with *H. vineae* (Lleixà et al., 2016) or by sequential fermentation with *S. cerevisiae* (Medina et al., 2013; Viana et al., 2011). The higher ester content produced by this non-*Saccharomyces* yeast can be explained by its prominent β-glucosidase activity that enables it to release these compounds into the media (Barquet et al., 2012; López et al., 2015).

The development of non-*Saccharomyces* yeasts can affect the growth of the primary wine yeast *S. cerevisiae* and the fermentation progress as a consequence of the consumption of important nutrients, such as nitrogen and vitamins (Medina et al., 2012). Some studies confirmed the effect of non-*Saccharomyces* yeasts on nutrient availability in mixed cultures. Andorrà et al. (2010) observed that mixed cultures with *Candida zemplinina* and *Hanseniaspora uvarum* had a higher

amino acid consumption than pure cultures of these yeasts. Indeed, pure and mixed cultures showed a preferential uptake of some amino acid groups related with the synthesis of aroma compounds that might be strain-dependent as was shown for *S. cerevisiae* (Bisson, 1991). This higher nitrogen consumption also happened in mixed cultures with *H. vineae* (Medina et al., 2012). The moment of inoculation, simultaneous or sequential, and the inoculum size in mixed cultures determine the progress of the fermentation because of the nutrient competition between the *Saccharomyces* and non-*Saccharomyces* yeasts. Some researchers have demonstrated that a sequential fermentation resulted in sluggish or stuck fermentations as a consequence of the nutrient consumption of the non-*Saccharomyces* strain, which reduced the nutrient availability to the *Saccharomyces* strain (Medina et al., 2012; Taillandier et al., 2014). In *S. cerevisiae* has been observed to activate the genes responsible for nitrogen and glucose metabolism to prevent this situation when it was co-cultivated with different non-*Saccharomyces* yeast to decrease the nutrients available to the non-*Saccharomyces* yeast (Curiel et al., 2017). In addition, recent studies focused on the specific use of ammonia and amino acids by the different non-*Saccharomyces* species and its implication on *S. cerevisiae* performance in sequential fermentations. Non-*Saccharomyces* yeast have been shown to exhibit a specific amino acid consumption profile depending on the yeast species, which interferes with *S. cerevisiae* development and generates changes in the volatile profile during sequential fermentations (Gobert et al., 2017; Rollero et al., 2018). In summary, the specific nutrient addition of amino acids, ammonia or vitamins has to be evaluated to ensure a good fermentation performance under sequential yeast inoculation (Gobert et al., 2017; Medina et al., 2012; Rollero et al., 2018).

Specifically, in grape must, we can find different nitrogen compounds, but only some of them can be consumed by *S. cerevisiae* to produce biomass and encourage the fermentation process. These compounds, known as Yeast Assimilable Nitrogen (YAN), are comprised of the ammonia and the amino acids present in the grape juice (Bell and Henschke, 2005). From this YAN, we can differentiate the preferred nitrogen sources, such as ammonia, asparagine and glutamine, which promote *S. cerevisiae* growth, and the non-preferred nitrogen sources, such as urea, that result in a low growth rate when it grows only with those nitrogen sources (Magasanik and Kaiser, 2002; Ter Schure et al., 2000).

Therefore, *S. cerevisiae* has developed a mechanism called Nitrogen Catabolism Repression (NCR) that selects the best nitrogen sources for growth. The NCR mechanism consists in the

reduction of proteins responsible for utilization and uptake of non-preferred nitrogen sources in the presence of preferred nitrogen sources. This mechanism acts at two levels to assure the consumption of preferred nitrogen sources. The first consists in the inactivation and degradation of the existing non-preferred nitrogen source permeases, and the second consists in the repression of genes encoding for non-preferred nitrogen source permeases (Magasanik and Kaiser, 2002; Ter Schure et al., 2000).

From the 19 amino acid permeases that *S. cerevisiae* contains, there are three high-capacity permeases that are nitrogen-regulated, including *AGP1* (high-Affinity Glutamine Permease), *GAP1* (General Amino acid Permease) and *PUT4* (Proline UTilization). In addition, other non-permeases proteins like *PUT2* (Delta-1-pyrroline-5-carboxylate dehydrogenase), which is a key enzyme for the conversion of proline into glutamate in the mitochondria once it has entered the cell through *PUT4*, are also nitrogen-regulated (Hofman-Bang, 1999). The amino acid permesases *GAP1* and *PUT4* together with the dehydrogenase *PUT2* are active during growth in non-preferred nitrogen content and repressed in the presence of a preferred nitrogen source, such as ammonium (Forsberg and Ljungdahl, 2001). Alternatively, *AGP1* is active in the presence of a preferred nitrogen source and repressed when this nitrogen is consumed (Regenberg et al., 1999).

In the case of ammonium, three permeases are responsible of its uptake, namely *MEP1, MEP2* and *MEP3.* When the concentration of ammonia in the medium is low, these permeases become active. However, in a non-preferred nitrogen source, the expression of *MEP2* is much higher than those of *MEP1* and *MEP3*, since it is the one with the highest affinity for ammonium (Ter Schure et al., 2000). Previous studies have reported that the expression of those nitrogen-regulated proteins can be used as a biomarker for nitrogen deficiency in wine fermentations (Beltran et al., 2005; Deed et al., 2011; Gutiérrez et al., 2013). The study of the expression of these proteins can be also an indirect evidence of the existence of NCR mechanism. In fact, NCR genes are regulated by several transcription factors, amongst others Gln3 and Nil1, and also by their regulator Ure2p. Under nitrogen limitation, Gln3 dissociates from Ure2p, the dephosphorylated Gln3 goes to the nucleus and increases the transcription of genes containing UAS_{NTR} sequence (Upstream activating sequence), like *GAP1, PUT4, PUT2* and *MEP2* genes (Ter Schure et al., 2000; Tesnière et al., 2015).

Nitrogen metabolism and the NCR mechanism have been deeply studied in *S. cerevisiae*, both in laboratory and wild strains, showing the multiple mechanisms used by this species under

nitrogen-limited conditions (Beltran et al., 2004; Godard et al., 2007; Gutiérrez et al., 2013; Tesnière et al., 2015). However, very little is known about nitrogen preferences and regulation in non-*Saccharomyces* species. A better understanding of nitrogen utilization among the different yeast species is important to increase the efficiency, predictability and quality of wine production, as well as of other biotechnological uses of yeast. The great variability on respiro-fermentative metabolism observed in non-*Saccharomyces* yeasts (Gonzalez et al., 2013) is an example of the possible divergences in nitrogen metabolism between *Saccharomyces* and non-*Saccharomyces* species. One of the limitations for performing molecular studies on non-*Saccharomyces* yeasts has been the lack of genomic data. Fortunately, in the last decade, the genomes of a large number of wine yeast species have been sequenced (Masneuf-Pomarede et al., 2016), and these sequences are available for molecular or genetic studies, such as those of the wine yeast *H. vineae* (Giorello et al., 2014).

In summary, the use of non-*Saccharomyces* yeasts is increasing to produce new wine styles taking advantage of their potential abilities. The nitrogen availability is important for yeast for its growth, as well as for the production of volatile compounds during the fermentation process. The mechanism used by *S. cerevisiae* to select the best nitrogen source is well known and documented, while it has not been studied in non-*Saccharomyces* yeasts.

The aim of this study was to evaluate the presence of the nitrogen catabolite repression (NCR) mechanism in *H. vineae*. We performed laboratory-scale fermentations of *H. vineae* and *S. cerevisiae* using a synthetic must with a defined nitrogen content. We followed the expression of the ortholog NCR-sensitive genes in *H. vineae* and the amino acid and ammonium consumption during the fermentation. Finally, we compared the results of *H. vineae* fermentations with the fermentations performed using a commercial *S. cerevisiae* strain.

Materials and methods

Yeast strains

The commercial wine yeast strain used in this study was *Saccharomyces cerevisiae* QA23 (Lallemand, Canada). The apiculate yeast strains used, *Hanseniaspora vineae* T02/5AF and *Hanseniaspora vineae* T02/19AF, were both isolated from Uruguayan vineyards (Barquet et al., 2012). The use of two strains of *H. vineae* responds to the need of validating the results in this

specie since the strains chosen have shown differences in aroma production which could be related with nitrogen metabolism (Martin et al., 2016).

Yeast strain *S. cerevisiae* QA23 was in active dry yeast (ADY) form. The rehydration process was performed according to the manufacturer's instructions (Lallemand, Canada). Both strains of *H. vineae*, T02/5AF and T02/19AF, were in fresh paste form, and both were prepared in the same way as QA23 using warm water.

Fermentation conditions

To determine the uptake and metabolism of nitrogen, yeast strains were grown at 28°C during 24 h in a solid yeast extract-peptone dextrose (YPD) medium (1% yeast extract, 2% peptone, 2% glucose, and 1.7% agar). A colony from the yeast culture was inoculated in 50 ml liquid YPD media for 24 h in Erlenmeyer flasks at 120 rpm and 28°C. A population of $1{\cdot}10^6$ cells/ml of the yeast strain was inoculated into an Erlenmeyer flask with 100 ml of yeast nitrogen base (YNB) media without amino acids (Difco™) with 150 mg/l of $(NH_4)SO_4$ and 20 g/l of glucose (AppliChem Panreac) for 24 h at 120 rpm and 28°C. The YNB medium was used to exhaust the yeast nitrogen reserves.

After a microscopic counting of the cells using a Neubauer chamber, 1,500 ml of synthetic must was inoculated to a final concentration of $2{\cdot}10^6$ cells/ml. The cells were washed and resuspended with synthetic must before inoculation to remove the nitrogen residues.

The fermentations were performed in synthetic must (Supplementary Table 1) with a nitrogen content of 140 mg YAN/l (Supplementary Table 2) since this concentration has been established as the ideal one to achieve a complete fermentation without residual or excess nitrogen (Bely et al., 1990).

The fermentations were conducted in triplicate in laboratory-scale fermenters, i.e., 500 ml bottles filled with 440 ml of synthetic must and covered with a cap with two tubes that allowed sampling and the exit of carbon dioxide. The fermenters were maintained on a rotating shaker at 120 rpm at room temperature (22-23 °C). The fermentation activity was assayed by the juice density every day using a portable density meter (Mettler Toledo).

Cell growth measurements

In the laboratory-scale fermentations, cell population monitoring was established by measuring the absorbance at 600 nm. The samples were measured every 4 h during the first 48 h after inoculation and once a day from 48 h to the end of the fermentation.

Determination of relative gene expression

The evaluation of the gene expression affected by the nitrogen catabolite repression (NCR) was performed during the first hours on the synthetic must fermentation. Sampling every 4 h during the first 24 h and every 6 h from 24 to 36 h was followed by centrifugation (16,000 rpm, 5 min and 4 °C) and removal of the supernatant. The pellet was washed with cold sterile MilliQ water (Millipore Q-POD™ Advantage A10), centrifuged (16,000 rpm, 5 min and 4°C) and after removal of the supernatant, it was frozen in liquid nitrogen and stored at -80 °C.

The RNA was extracted from these samples using an RNeasy Mini kit (QIAGEN) and RNase-Free DNAse Set (QIAGEN) according to the manufacturer's instructions. The RNA obtained was then measured using a Nano Drop (NanoDrop 1000 Thermo Scientific) and diluted to a final concentration of 320 ng/µl in a total volume of 11 µl. The cDNA synthesis of each sample was performed using the corresponding RNA, 1 µl of oligo-dT primer (Invitrogen™), 1 µl of dNTPs (10 mM) and 1 µl of transcriptase (SuperScript II Reverse Transcriptase-Invitrogen™) and amplified using a 2720 Thermal Cycler (Applied Biosystems) according to the manufacturer's instructions.

The genes evaluated in this experiment considering their role in the NCR mechanism were *AGP1, GAP1, MEP2* and *PUT2* and their orthologous in *H. vineae*. Annotation of putative orthologous was based on BLASTx searches using *H. vineae* predicted CDS and the proteome of *S. cerevisiae*. A hit was considered significant if: (i) e-value threshold was less than 1e-10 (ii) the alignment length covered more than 90% of the length of both sequences and (iii) both sequences presented the same pfam domain. In case of multiple hits we selected the *H. vineae* prediction with higher percentage of amino acid identity (Supplementary Table 3). Primer design for each gene was performed using Primer Express software (Primer Express 3.0 Applied Biosystems) (Table 1). The housekeeping genes encoding Actin (*ACT1*) and Inorganic PyroPhosphatase 1 (*IPP1*) from *S. cerevisiae* and *H. vineae* were used to normalize the amplification curves of the

selected genes considering their stability (Ståhlberg et al., 2008). All samples from each fermentation replicate were analyzed in duplicate.

Table 1. Primers used for the analysis of the expression of NCR-related genes in *H. vineae*. The primer design for the *H. vineae* genes was performed using Primer Express software (Primer Express 3.0 Applied Biosystems). The primers used for *S. cerevisiae* have been previously described by Beltran et al. (2004) and Gutiérrez et al. (2013).

Gene	Name	Oligonucleotide sequence (5'-3' end)
ACT1	ACT-F	GGCTTCTTTGACCACTTTCCAA
	ACT-R	GATGGACCACTTTCGTCGTATTC
AGP1	AGP1-F	ATTGCTGGGTGACGGTTCTT
	AGP1-R	TGACATTGGTAGCGGCAATAAC
GAP1	GAP1-F	CAAAGGTTTGCCATCTGTCATC
	GAP1-R	TGCAGAGTTACCGACAGACAACA
MEP2	MEP2-F	TCGATGACGGGTTGGATGTT
	MEP2-R	CATAACGGCACCACTTAAACCA
PUT2	PUT2-F	GATACGACATGTTGGCAGCAA
	PUT2-R	TTTCGCCTTGGAAAACGTTT

In all the samples, the Real-Time Quantitative PCR reaction was performed using 10 µl of SYBR Green [SYBR Premix Ex Taq II (Tli RNaseH Plus)], 0.4 µl of ROX Reference Dye (SYBR Premix Ex Taq II), 0.8 µl of each specific primer (10 µM) and 6 µl of sterile MilliQ water (Millipore Q-POD™ Advantage A10). The amplification process was conducted using a 7300 Real Time PCR System (Applied Biosystems) as follows: 50 °C for 2 min, 95 °C for 10 min and 40 cycles at 95 °C for 15 s, 60 °C for 2 min and 72 °C for 30 s.

The relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method (Beltran et al., 2005), where the Ct value corresponds to the number of cycles needed to achieve the background fluorescence. This method is used to compare the Ct values of the gene of interest, and the Ct values of the reference genes (*ACT1* and *IPP1*) (ΔCt); and -$\Delta\Delta$Ct consists of the difference of ΔCt from the samples of each time point, and the ΔCt of the reference time (4 h after inoculation). Results were expressed as the mean Log10 relative gene expression. All samples were analyzed in triplicate, and the resulting Log10 $2^{-\Delta\Delta Ct}$ values were statistically analyzed using ANOVA and Tukey's post-test.

Nitrogen content analysis of laboratory fermentation

The individual amino acid and ammonium contents of each sample were determined using high-performance liquid chromatography (HPLC) (Agilent 1100 Series HPLC) (Gómez-Alonso

et al., 2007). The sample (400 µl) was mixed with borate buffer (700 µl), methanol (300 µl), diethyl ethoxymethylenemalonate (DEEM) (15 µl) and L-aminoadipic acid (internal control) (10 µl). After 2 h at 80 °C, 50 µl of each sample was directly injected into the HPLC, which consists of a low pressure gradient quaternary pump, a thermostatted autosampler, a DAD ultraviolet detector and a fluorescence detector (Agilent Technologies, Germany). The separation process of the sample was performed using a 4.6 x 250 mm x 5 µm Hypersil ODS column (Agilent Technologies, Germany).

The solvent system was as follows: A solvent (mobile phase) [4.1 g of sodium acetate anhydrous diluted in 250 ml of MilliQ water, adjusted to pH 5.8 with glacial acetic acid and 0.4 g of sodium azide brought to a final volume of 2 l with MilliQ water (Millipore Q-POD™ Advantage A10)] and B solvent (stationary phase) [80% acetonitrile and 20% methanol]. The analytical temperature was 20 °C, and the flow rate was 0.9 ml/min. The concentration of each amino acid and ammonia was calculated using an external calibration curve of each component and expressed as mg N/l. The software used for the integration was Agilent ChemStation Plus (Agilent Technologies, Germany).

Statistical analysis

Statistical analysis of the gene expression data was performed using an ANOVA and indicated by the Tukey's post-test (all pair comparisons) using XLSTAT Software. The results were considered statistically significant at a *p*-value less than 0.05.

Results

Fermentation kinetics and YAN consumption

The fermentations were performed using a synthetic must with a nitrogen content of 140 mg YAN/l (corresponding to 190 mg N/l). Two strains of *H. vineae*, T02/5AF and T02/19AF, were evaluated, and *S. cerevisiae* strain QA23 was used as a control. Media density, cell growth and nitrogen content were assessed along with alcoholic fermentation. Both *H. vineae* strains showed a similar behaviour in fermentation kinetics, cell growth and YAN consumption (Figure 1). These strains achieved a must density below 1000 g/l in approximately 13 days (324 h), while the *S. cerevisiae* strain was faster and reached this point in 8 days (192 h). The YAN was completely consumed by all the strains during the exponential growth phase that coincides with the initial

stages of the fermentation (Figure 1). Ammonia and amino acids were consumed in 36 h by the *H. vineae* strains. Even though *S. cerevisiae* also consumed all the amino acids in 36 h, it exhausted the ammonia earlier, specifically before 30 h.

The consumption of each amino acid and ammonia was measured during the first 36 h of the different strain fermentations. Figure 2 and Table 2 show the evolution of their consumption at different time points. In general, a similar consumption pattern of amino acids occurred in both *H. vineae* strains and *S. cerevisiae*. In all cases, lysine, glutamic acid, cysteine, isoleucine, leucine and phenylalanine were completely assimilated during the first 24 h. As for the previous amino acids, histidine was also exhausted during this period solely by the *H. vineae* strain T02/5AF. The slowest consumed amino acids, arginine and valine, were still available in very small amounts after 30 h in both *S. cerevisiae* and *H. vineae*. The remaining amino acids were consumed between 24 and 36 h in every case.

Table 2. Time (h) required for each yeast strain to exhaust the different nitrogen compounds of the synthetic must.

	Consumed in 16-20 h	**Consumed in 20-24 h**	**Consumed in 24-30 h**	**Consumed in 30-36 h**
H. vineae T02/5AF	Lys	Glu, His, Cys, Ile, Leu, Phe,	Asp, Ser, Gln, Gly, Thr, Ala, Met, Trp, Tyr	Arg, Val, NH_4^+
H. vineae T02/19AF	Lys	Glu, Cys, Ile, Leu, Phe	Asp, Ser, Gln, His, Gly, Thr, Ala, Trp, Tyr, Met	Arg, Val, NH_4^+
S. cerevisiae QA23	Lys, Cys, Ile, Leu	Glu, Phe	Asp, Ser, Gln, His, Gly, Thr, Ala, Val, Met Trp, NH_4^+	Arg, Tyr

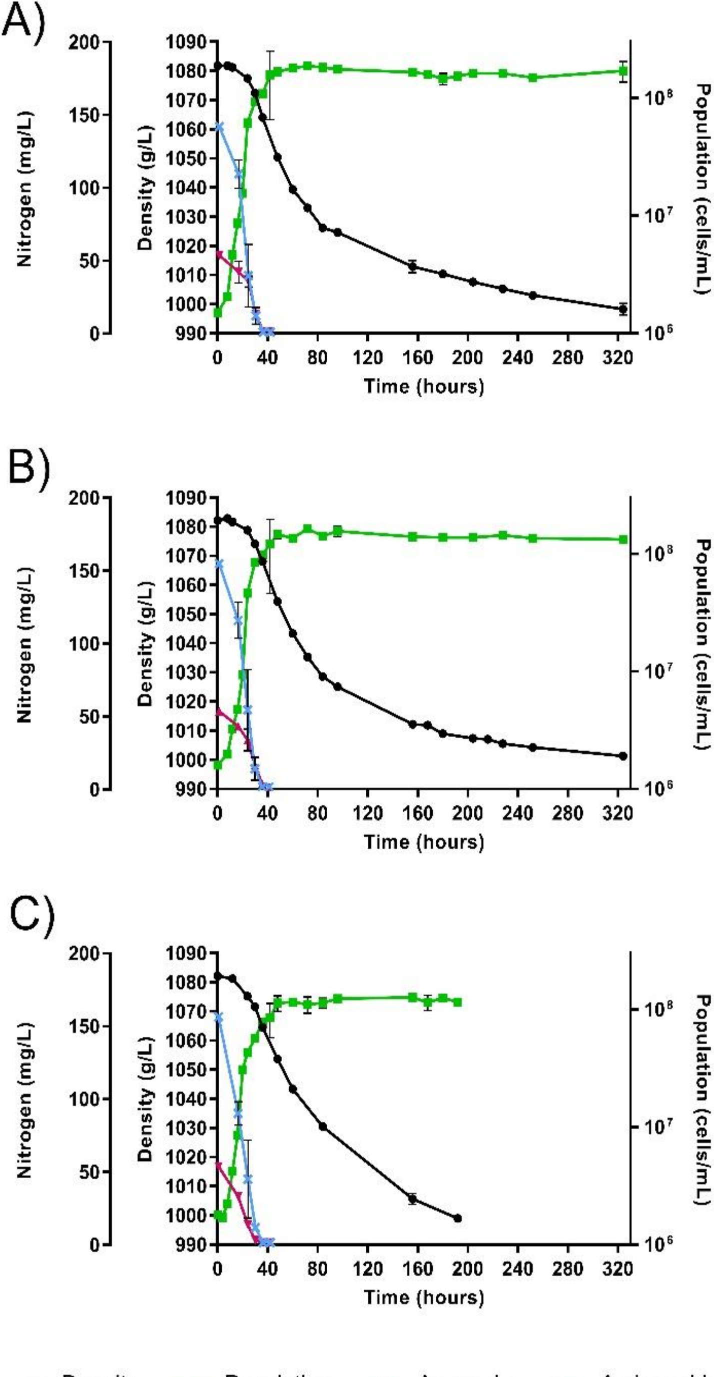

Figure 1. Fermentation kinetics, cell growth and YAN consumption expressed separately as amino acids and ammonia of (A) *H. vineae* T02/5AF, (B) *H. vineae* T02/19AF and (C) *S. cerevisiae* QA23 during alcoholic fermentation. The figure shows the mean of the three fermentation replicates for each strain and its standard deviation.

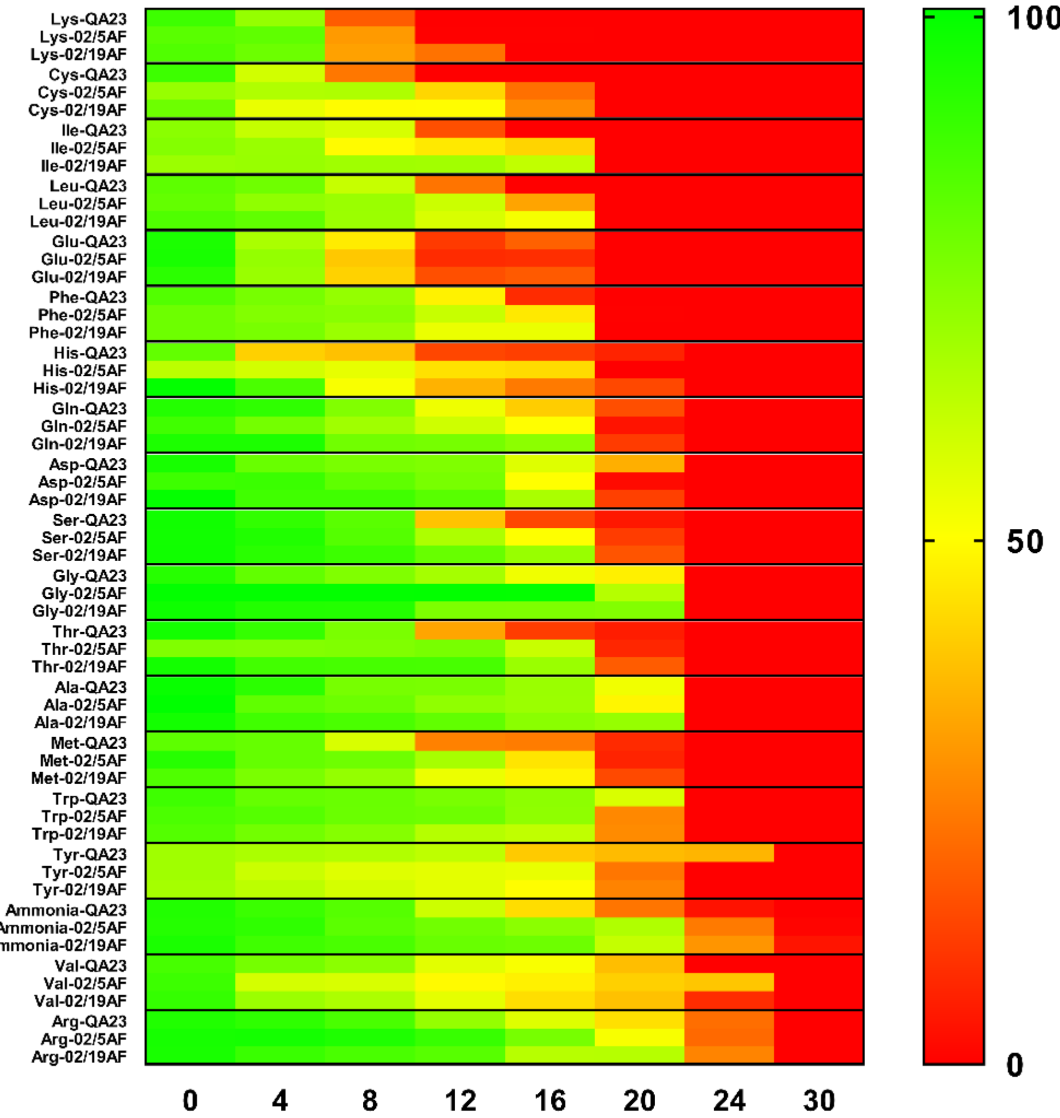

Figure 2. Heat map of the available percentage of each amino acid in the must during the first 30 h of *H. vineae* T02/5AF, *H. vineae* T02/19AF and *S. cerevisiae* QA23 fermentations. Green color corresponds to 100% of the total amino acid content available, and red corresponds to 0% of amino acid content available in the media. Standard deviations were always lower than 10% and have been avoided in the figure for clarity.

Expression of NCR-regulated genes

Different genes related to NCR mechanism were evaluated for their homology in *H. vineae* including three permeases (*AGP1, GAP1, MEP2*), one dehydrogenase (*PUT2*) and four transcriptional factors (*GAT1, GLN3, GZF3, DAL80*). Except from *DAL80*, all the other genes had their homologous in *H. vineae* suggesting the presence of this nitrogen regulation mechanism in this yeast (Supplementary Table 3). To further check that this species displays this regulation, four genes related to nitrogen transport into the cell (*AGP1, GAP1, MEP2*) and one gene related to proline utilization (*PUT2*) were selected to analyze their expression pattern during the first fermentation hours. These genes have been described and used in *S. cerevisiae* as markers for nitrogen limitation (Beltran et al., 2004, 2007; Gutiérrez et al., 2013) and also as an indirect marker of transcriptional factors activity.

Figure 3 and Supplementary Table 4 show the expression evolution of the different genes during the first 48 h for each strain. Gene expression at 4 h was considered to be a reference, since the expression at 0 h corresponds to the inoculum that was nitrogen-depleted. The pattern of gene expression was similar for all the strains. *AGP1* was the only gene that was down-regulated during the fermentation, compared to the expression obtained at 4 h. Thus, the expression of *AGP1* is higher at the beginning of the fermentation (e.g., 4 h, our reference time), when the amino acid concentration is also higher, and decreases as the amino acids are consumed. The other three genes started to be up-regulated at different time points along the fermentation depending on the yeast species. Therefore, both *H. vineae* strains activated the *GAP1* and *MEP2* expression after 24 h, and *PUT2* after 16 h of fermentation. Finally, *S. cerevisiae* QA23 expressed *GAP1, MEP2* and *PUT2* after 16, 20 and 24 h of fermentation, respectively. Despite the differences between the yeast strains, the gradual activation or repression of the different genes coincided with the progressive consumption of the amino acids and ammonia during fermentation.

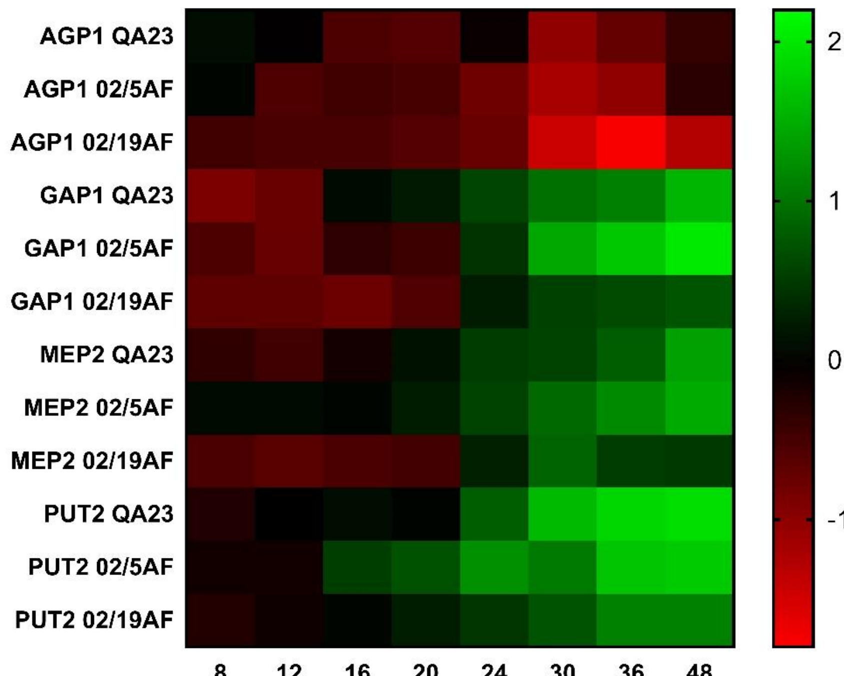

Figure 3. Heat map of the expression of *AGP1, GAP1, MEP2* and *PUT2* at different time points during the first 48 h of the fermentation for each yeast strain. Green color indicates an activation of gene expression, while red color indicates the repression of gene expression. Standard deviations have been avoided in the figure for clarity.

Discussion

In this study, we aimed to determine if *H. vineae*, a non-*Saccharomyces* yeast of oenological interest, displays the NCR mechanism under fermentation conditions. This metabolism has been thoroughly studied in *S. cerevisiae* during alcoholic fermentation (Beltran et al., 2004; Tesnière et al., 2015), and it was considered to be a reference in this study. Our results suggest that *H. vineae* exhibits an NCR mechanism similar to that of *S. cerevisiae*.

Fermentations using synthetic must with 140 mg YAN/l allowed us to evaluate ammonia and amino acid consumption together with the analysis of the expression of NCR-regulated genes during the first hours of fermentation. First, the nitrogen content of the synthetic must used in this study was not limiting, and it is considered to be the minimum concentration needed for yeasts to complete the alcoholic fermentation (Ribéreau-Gayon et al., 2006). In fact, all the strains tested in this work were able to complete the fermentation process (Figure 1) which agrees on previous reports (Ribéreau-Gayon et al., 2006). However, as we expected, *S. cerevisiae* finished the fermentation more quickly, because of its oenological abilities to resist the fermentation conditions. In addition, *H. vineae*, as well as *S. cerevisiae*, exhausted all the available YAN in 36 h

even though *S. cerevisiae* consumed all the ammonia in 30 h, 6 h sooner than *H. vineae* (Figure 2 and Table 2). Medina et al. (2012) showed a competition for nutrients, especially nitrogen, in mixed fermentations of *S. cerevisiae* and *H. vineae*. The similar consumption of the nitrogen of these two yeasts observed in this study would explain the competition for this nutrient noted by Medina et al. (2012) in mixed fermentations.

In the same way as previous studies performed in *S. cerevisiae*, the strains evaluated exhausted all the YAN during the growth phase demonstrating how nitrogen availability plays a role as a limiting fermentation factor (Beltran et al., 2004; Crépin et al., 2012). In addition, the kinetic consumption of different nitrogen compounds has been evaluated simulating oenological conditions in different *S. cerevisiae* strains and conditions in different studies. Beltran et al. (2007) demonstrated how temperature affects the amino acid intake, which affects yeast growth and metabolism. In our case, the fermentations proceeded at 22-23 °C, and the consumption pattern of the nitrogen compounds was similar to that reported by Crépin et al. (2012). In fact, Crépin et al. (2012) classified nitrogen compounds in three groups according to their order of use by different *S. cerevisiae* strains: prematurely consumed, early consumed and late consumed. We classified the nitrogen compounds considering the time it took for them to be completely exhausted by each yeast strain (Table 2). However, we can observe that lysine is the fastest to be consumed by all the strains, and it is the one classified as prematurely consumed or that arginine, valine, tyrosine and NH_4^+ are the later ones to be completely exhausted, which belong to the late consumed compounds group established by Crépin et al. (2012). Considering these aspects, we observed that *H. vineae* has a similar behavior to *S. cerevisiae* in nitrogen uptake, and the variability of nitrogen compound preferences in *H. vineae* appear to also depend on the strain coinciding with previous studies on different *S. cerevisiae* strains (Crépin et al., 2014).

Interestingly, arginine was the slowest amino acid to be consumed in all cases. This amino acid is known as a non-preferred nitrogen source, since its support to yeast growth is very poor (Cooper, 1982), and it is the most stored amino acid in the vacuole during the growth phase (Crépin et al., 2014). In addition, the evaluation of arginase activity was proposed to be an indicator of the available nitrogen in fermentation (Carrasco et al., 2003), because as nitrogen becomes limiting, yeasts start to metabolize the stored nitrogen for additional growth (Crépin et al., 2014). In addition, Beltran et al. (2004) observed that the activation of arginase activity coincides with the mobilization of arginine, the ammonium depletion and the activation of *GAP1*.

In this study, the highest arginine consumption coincided with ammonia depletion in *S. cerevisiae*. However, in *H. vineae*, arginine intake is simultaneous to that of ammonium, which may indicate that this yeast species uses a different way to store or consume this amino acid. The lower preference of *H. vineae* for ammonium is consistent with the reported poor effect of ammonium addition to agave juice fermentations compared to other nitrogen sources (Díaz-Montaño et al., 2010). In addition, *H. vineae* strains produced significantly lower levels of isobutyl alcohol derived from valine (Martín, 2016), which could be related to the slower consumption of this amino acid exhibited by this yeast species in this study.

The gene expression of *GAP1, MEP2* and *PUT2* evolved from nitrogen-repressed to nitrogen-activated conditions as nitrogen was consumed in all cases. Between 16 and 30 h after inoculation with the different yeast strains, the gene expression of *GAP1, MEP2* and *PUT2* began to be significantly activated. On the other hand, *AGP1* began to be repressed after 8 and 12 h of fermentations. As described before, *AGP1* acts as a sensor for amino acids, and its expression is induced by extracellular amino acids via SPS system, and down-regulated when the amino acids are consumed (Godard et al., 2007; Regenberg et al., 1999), which is consistent with our results. In the case of *GAP1* and *PUT2*, the transcription of these genes is known to be activated under limiting nitrogen conditions (Forsberg and Ljungdahl, 2001; Gutiérrez et al., 2013), and this fact would explain their up-regulation once the most preferred nitrogen compounds are consumed. Finally, we analyzed the ammonium permease *MEP2* expression, which is notably higher than other ammonium permeases (Ter Schure et al., 2000). Previous studies in *S. cerevisiae* have observed the activation of both *GAP1* and *MEP2* when ammonium is depleted (Beltran et al., 2004, 2005). However, in our study, the three strains tested showed a gradual activation of these two genes as ammonium and preferred amino acids were being consumed. From these results, we can deduce the activation of the transcriptional factors responsible for the expression of NCR genes.

The homology found on the NCR related proteins between *H. vineae* and *S. cerevisiae*, as well as the similarity in nitrogen consumption and the regulation of NCR genes, suggested the presence of the NCR mechanism in this non-*Saccharomyces* yeast. In addition, similarly to what has been described in *S. cerevisiae* (Beltran et al., 2004; Tesnière et al., 2015), the *H. vineae* wine yeasts evaluated entered the stationary phase coinciding with the exhaustion of nitrogen and consequently, the up-regulation of the NCR genes. However, further research would be necessary

to fully understand nitrogen metabolism in *H. vineae* and to elucidate if other mechanisms not regulated by NCR are responsible for nitrogen transport in this yeast.

Finally, the aim of this study was to determine if *H. vineae*, a non-*Saccharomyces* yeast of oenological interest, exhibits the NCR mechanism. Since nitrogen is one of the most limiting factors during alcoholic fermentation, knowing how it is metabolized gains importance. For that reason, we performed fermentations using synthetic must with an established nitrogen content, and we analyzed the nitrogen consumption and the expression of the NCR-regulated genes. The observed pattern of gene expression and nitrogen intake for the *H. vineae* strains and *S. cerevisiae* was similar, suggesting the presence of this regulatory mechanism in *H. vineae*. This study contributes to a better understanding of nitrogen metabolism in the most active species in terms of the fermentation capacity of the genus *Hanseniaspora*, yeasts differentiated before the whole genome duplication event of the *Saccharomyces* group. In addition to our results, more studies are needed to completely understand nitrogen metabolism in this species.

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Supplementary material

Supplementary Table 1. Synthetic must composition used in the present work.

Synthetic must composition (140 mg N/l)

	1 L
Glucose	100 g
Fructose	100 g
Citric acid	5 g
Malic acid	0.500 g
Tartaric acid	3 g
KH_2PO_4	0.750 g
K_2SO_4	0.500 g
$MgSO_2$ 7 H_2O	0.250 g
$CaCl_2$ 2 H_2O	0.155 g
NaCl	0.200 g
Nitrogen (140 mg N/L)	
NH_4Cl (56 mg N/L)	0.214 g
Amino acid solution (84 mg N/L)	4.78 ml
Oligo-elements solution	1 ml
Vitamins solution	10 ml
Anaerobic factors	1 ml

Vitamins stock solution

	1 L
Myo-inositol	2 g
Pantothenate calcium	0.150 g
Thiamine hydrochloride	0.025 g
Nicotinic acid	0.200 g
Pyridoxine	0.025 g
Biotine	3 ml (from a stock of 100 mg/l)

Oligo-elements stock solution

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

Anaerobic factors stock solution

	0.100 L
Ergosterol	1.5 g
Oleic acid	0.5 ml
Tween 80	50 ml
Ethanol (absolute)	until 100 ml

Supplementary Table 2. Ammonium content and amino acid stock solution content expressed as g l^{-1} and the corresponding nitrogen and YAN concentration in synthetic must in mg N l^{-1}.

Amino acid	g l^{-1}*	mg N l^{-1}	mg YAN/l
Asp	4.42	2.22	2.22
Glu	11.96	5.44	5.44
Ser	7.80	4.97	4.97
Gln	49.92	45.76	22.88
His	3.38	1.46	1.46
Gly	1.82	1.62	1.62
Thr	7.54	4.24	4.24
Arg	36.79	42.45	14.15
Ala	14.56	10.95	10.95
Tyr	1.95	0.72	0.72
Cis	2.08	1.15	1.15
Val	4.42	2.53	2.53
Met	3.12	1.40	1.40
Trp	17.42	5.71	5.71
Phe	3.77	1.53	1.53
Ile	3.25	1.66	1.66
Leu	4.81	2.46	2.46
Lys	1.69	1.55	0.77
Pro	59.93	0,00	0.00
Total aas		137.83	85.88
Ammonia (NH_4Cl)	0.214	56.00	56.00
Total YAN			141.88
Total N		193.83	

* To achieve a final concentration of 140 mg YAN/l (190 mg N/l), 4.78 ml of amino acid stock solution is added to 1 l of synthetic must.

Supplementary Table 3. NCR related genes of *H. vineae* and *S. cerevisiae* EC1118. Genes highlighted in bold are the ones which expression has been analyzed in this study.

Gene name	Systematic name	Pfam domain	Putative orthologous in *H. vineae*	Aminoacidic similarity	Single copy gene in *H. vineae*
AGP1	YCL025C	AA_permease	g1661.t1;g1666.t1	50.43 %; 50.77 %	no
GAP1	YKR039W	AA_permease	g4653.t1	67.06 %	no
MEP2	YNL142W	Ammonium transp; Ammonium transporter AmtB-like domain	g3765.t1	60.82 %	yes
PUT2	YHR037W	Aldehyde dehydrogenase domain	g905.t1	67.50 %	yes
GAT1	YFL021W	GATA zinc finger; Fungal protein of unknown function	g3143.t1	51.38%	yes
GLN3	YER040W	GATA zinc finger	g1456.t1	36.64%	yes
GZF3	YJL110C	GATA zinc finger	g1991.t1	45.23%	yes
*DAL80**	YKR034W	-	-	-	-

**DAL80* is not present in *H. vineae.*

Supplementary Table 4. Gene expression of *AGP1, GAP1, MEP2* and *PUT2* during the first 48 h of fermentation. Gene expression ($2^{-\Delta\Delta Ct}$) of each gene at different time points was determined during the first 48 h, considering 4 h after inoculation as the reference time. The values are expressed as the mean Log10 relative gene expression. The resulting Log10 $2^{-\Delta\Delta Ct}$ values were statistically analyzed using ANOVA and Tukey's post-test. Different letters indicate significant differences in gene expression within each strain, $p < 0.05$.

	Time (hours)	*AGP1*	*GAP1*	*MEP2*	*PUT2*
H. vineae T02/5AF	8	0,037^{a}±0,023	-0,543^{a}±0,146	0,080^{a}±0,332	-0,133^{a}±0,152
	12	-0,557^{a,b}±0,206	-0,734^{a}±0,016	0,080^{a}±0,200	-0,134^{a}±0,016
	16	-0,456^{a,b}±0,185	-0,318^{a}±0,197	0,039^{a}±0,251	0,533^{a,b}±0,403
	20	-0,506^{a,b}±0,358	-0,437^{a}±0,308	0,235^{a}±0,294	0,703^{a,b}±0,338
	24	-0,782^{a,b}±0,085	0,434^{a,b}±0,076	0,573^{a,b}±0,109	1,236^{b,c}±0,018
	30	-1,191^{b}±0,621	1,447^{b,c}±0,661	0,914^{a,b}±0,604	1,061^{b,c}±0,126
	36	-1,019^{a,b}±0,621	1,722^{c}±0,371	1,183^{a,b}±0,159	1,699^{c}±0,116
	48	-0,297^{a,b}±0,180	2,014^{c}±0,106	1,474^{b}±0,266	1,744^{c}±0,177
H. vineae T02/19AF	8	-0,452^{a}±0,346	-0,669^{a}±0,033	-0,535^{a}±0,220	-0,243^{a}±0,130
	12	-0,515^{a}±0,117	-0,666^{a}±0,063	-0,629^{a}±0,118	-0,103^{a}±0,019
	16	-0,521^{a}±0,422	-0,766^{a}±0,012	-0,526^{a}±0,220	0,038^{a,b}±0,436
	20	-0,602^{a}±0,210	-0,573^{a}±0,245	-0,477^{a}±0,351	0,243^{a,b}±0,036
	24	-0,746^{a,b}±0,255	0,236^{b}±0,022	0,271^{a,b}±0,053	0,471^{a,b,c}±0,051
	30	-1,437^{a,b} ±0,238	0,560^{a,b}±0,173	0,870^{b}±0,022	0,716^{b,c}±0,019
	36	-1,762^{b}±0,131	0,642^{a,b}±0,102	0,524^{b}±0,431	1,105^{c}±0,308
	48	-1,269^{a,b}±0,201	0,738^{a}±0,011	0,484^{b}±0,066	1,111^{c}±0,099
S. cerevisiae QA23	8	0,106^{a}±0,040	-0,887^{a}±0,224	-0,330^{a}±0,177	-0,227^{a}±0,097
	12	-0,018^{a,b}±0,003	-0,737^{a}±0,437	-0,442^{a}±0,645	-0,014^{a}±0,321
	16	-0,541^{a,b,c}±0,116	0,086^{a,b}±0,099	-0,149^{a}±0,522	0,108^{a}±0,519
	20	-0,593^{a,b,c}±0,010	0,210^{b,c}±0,077	0,146^{a,b}±0,183	0,031^{a}±0,044
	24	-0,061^{a,b}±0,223	0,590^{b,c}±0,188	0,513^{a,b}±0,241	0,797^{a,b}±0,329
	30	-1,022^{c}±0,089	0,962^{c,d}±0,100	0,565^{a,b} ±0,022	1,603^{b,c}±0,036
	36	-0,705^{b,c}±0,309	1,098^{c,d}±0,162	0,809^{a,b} ±0,252	1,846^{c}±0,171
	48	-0,370^{a,b,c}±0,293	1,567^{d}±0,282	1,385^{b}±0,204	1,922^{c}±0,150

CHAPTER 5

Genetic and phenotypic diversity of *Brettanomyces bruxellensis* strains from wine regions of Catalonia

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Abstract

The yeast *Brettanomyces bruxellensis* is the most reported spoilage microorganism in aged wines mainly due to the production of phenolic off flavors. In the present study 64 strains of *B. bruxellensis* isolated from Catalonian aging wines were genetically evaluated. The isolates were identified at species level by RFLP analysis of the 5.8S-ITS region and 26S rRNA gene sequencing and subsequently subjected to analysis of intra-species variability through the study of intron splice sites (ISS-PCR). The *B. bruxellensis* strains isolated had high intra-specific diversity as they were distributed genetically into 8 clusters mostly depending on their origin. However, this clustering did not necessarily mean that other physiological traits were similar. We also investigated the resistance of the strains to sulfur dioxide (SO_2) under laboratory and wine-like conditions resulting in different responses. Most of the strains grew quickly under laboratory conditions and their growth was affected above 10 mg SO_2/l, whereas diverse responses were observed under wine-like conditions depending on the strain. Interestingly, under wine-like conditions, the tolerance to SO_2 increased with the time of incubation with the consequent importance for the control of this yeast on the wine industry. Nevertheless, the tolerance to SO_2 was no related to the genetic clusters and some of the strains were very resistant and grew up to 60 mg/l of SO_2 under wine-like conditions. Additionally, the spoilage potential of the isolated strains was evaluated under wine-like conditions using p-coumaric and ferulic acids (8:1) as precursors of 4-ethylphenol (4-EP) and 4-ethylguaiacol (4-EG), respectively. The growth of *B. bruxellensis* strains was limited under the wine-like conditions, with most of the isolates decreasing their growth on solid medium across incubation. However, all the evaluated strains were able to produce 4-EP and 4-EG above their detection threshold in variable quantities depending on the strain. This result suggests that even if the cells of *B. bruxellensis* were not culturable, they were able to produce volatile phenols. Thus, both the resistance to SO_2 and the production of volatile phenols varied within the *B. bruxellensis* population and was unrelated to the observed genetic clustering. This complexity must be taken into account in order to optimize the monitoring protocols and the corrective interventions, remarking the importance of an early detection.

Keywords: *Brettanomyces bruxellensis*, wine spoilage, genetic clustering, SO_2 tolerance, phenol production

Introduction

Nowadays, one of the main problems in the wine industry is the biological alteration and spoilage of wine by the activity of certain bacteria and yeast. The most frequent physical and chemical changes observed in wine due to microbiological spoilage are film or sediment formation, cloudiness and the off-odours or off-flavours (Agnolucci et al., 2009).

B. bruxellensis (teleomorph *Dekkera bruxellensis*) is considered the most important spoiler in wine industry (Loureiro and Malfeito-Ferreira, 2003). This yeast has been detected at low frequency in grapes or must (Renouf and Lonvaud-Funel, 2007) but it is typically detected during wine aging in barrels and in bottle due to its tolerance to both high ethanol and low oxygen concentrations (Wedral et al., 2010).

Several studies highlighted both the great genetic and phenotypic diversity of *B. bruxellensis* in different wine regions. In fact, diverse typing methods have been used to explore the genetic diversity of this species in wine environments such as Random Amplified Polymorphism DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), pulsed field electrophoresis (REA-PFGE), SAU-PCR, PCR- DGGE, mtDNA restriction analysis, inter splice PCR (ISS-PCR), partial sequencing of the gene 26S rDNA or microsatellite profiling method (Agnolucci et al., 2009; Albertin et al., 2014; Avramova et al., 2018; Campolongo et al., 2010; Conterno et al., 2006; Curtin et al., 2007; Di Toro et al., 2015; Guzzon et al., 2018; Martorell et al., 2006; Oelofse et al., 2009). Nevertheless, our understanding of the *B. bruxellensis* population structure and the factors that modulate it remains limited. Furthermore, to our knowledge, there are no studies analyzing the biodiversity of this yeast species in any wine region of Spain, one of the most important wine producers together with Italy and France (European Comission, 2018).

The spoilage effect of this contaminant in wine is mainly associated to the production of volatile phenols which are related to unpleasant aromas described as barnyard, horse sweat or medicinal (Chatonnet et al., 1992; Oelofse et al., 2009). Therefore, the presence of *B. bruxellensis* in wine often provokes rejection by consumers and consequent economic losses for wine industry (Wedral et al., 2010). It has been demonstrated that the volatile phenol production by *B. bruxellensis* depends on the concentration of their precursors in the must (mainly *p*-coumaric and ferulic acids) (Rodrigues et al., 2001), and the specific strain implicated (Crauwels et al., 2017; Di Toro et al., 2015). However, from previous studies it is not clear if the production of volatile phenols is associated to the physiological state or growth rate of *B. bruxellensis* and often

controversial results have been presented (Agnolucci et al., 2010; Di Toro et al., 2015; Sturm et al., 2015; Vigentini et al., 2008). For example, various studies related the number of metabolically active *B. bruxellensis* with the amount of phenols produced (Chatonnet et al., 1997; Rodrigues et al., 2001; Vigentini et al., 2008), whereas other studies suggest the existence of viable but non-culturable (VBNC) population able to produce phenols (Agnolucci et al., 2010; Laforgue and Lonvaud-Funel, 2012; Serpaggi et al., 2012).

The most common method to prevent *B. bruxellensis* spoilage is the addition of sulfur dioxide (SO_2) mainly through the addition of potassium bisulfite solution to must and wine (Ribéreau-Gayon et al., 2006). Unfortunately, the high phenotypic diversity of *B. bruxellensis* includes, for example, differences regarding the tolerance to SO_2 (Agnolucci et al., 2014; Avramova et al., 2018; Curtin et al., 2012) and volatile phenols production (Agnolucci et al., 2009; Conterno et al., 2006; Crauwels et al., 2017; Di Toro et al., 2015; Martorell et al., 2006; Romano et al., 2008) making difficult the prediction of its occurrence, behaviour and control. In addition, most of the studies analyzing the ability of *B. bruxellensis* to tolerate SO_2 or to produce volatile phenols have used synthetic laboratory media or concentrations of phenols precursors that are not commonly found in wines, thus the results obtained may be biased. In fact, several authors found that the tendency of volatile phenols production by *B. bruxellensis* strains in synthetic media was quite different from the one in wine matrix or wine-like conditions (Oelofse et al., 2009; Sturm et al., 2015).

The present work aimed to determine for the first time the genetic diversity of *B. bruxellensis* strains isolated from different Catalonian wine regions and their phenotypic ability to tolerate SO_2 and to produce volatile phenols under wine-like conditions.

Materials and methods

Yeast isolation

Samples for the isolation of *B. bruxellensis* strains were taken from wineries of different wine regions of Catalonia including AOC Priorat, Montsant, Tarragona and Penedès. Samples consisted of wines in oak barrels taken at variable time points during the aging, including 4, 5, 8, 12 and 14 months (Supplementary Figure 1). The wine sampled from Tarragona and Montsant had "Brett" character defined by a characteristic barnyard/medicinal aroma. The samples from Priorat or Penedès did not present any aromatic defect.

A volume of 100 µl of each sample was plated directly into modified WLN medium (Difco™ WL Nutrient Medium, BD) with the addition of cycloheximide (100 mg/l). WLN plates were incubated from 7 to 10 days at 28 °C. Slow-growing colonies were microscopically observed and those with typical *Dekkera/Brettanomyces* cellular morphology were streaked on WLN+cycloheximide plates and incubated for the same period. The successfully isolated strains were grown in plates of YPD medium (2% glucose, 2% peptone, 1% yeast extract and 1.7% agar) for subsequently genus and species verification. In addition, *B. bruxellensis* strain CECT (Spanish Type Culture Collection) 1009 isolated from Lambic beer, was grown on YPD and used for comparison purposes with Catalonian wine isolates.

Yeast identification by RFLP analysis of 5.8S-ITS and partial sequencing of ITS1-ITS4 gene region

Isolated yeasts were grown in 4 ml of liquid YPD medium, at 28 °C under agitation of 120 rpm during 48 h. DNA extraction was performed as previously described by Querol et al. (1992) with some modifications. Briefly, cells were spun down in a centrifuge, washed with sterile distilled water and resuspended in buffer solution 1 (0.9 M Sorbitol, 0.1 M EDTA, pH 7.5). Then, zymolyase and lyticase enzymes (10 mg/ml each) were added for cell wall digestion and let them to act at 37 °C for 1 h. After centrifugation, the resulting pellet was resuspended in a buffer solution 2 (50 mM Tris, 20 mM EDTA, pH 7.4). After that, a solution of SDS 10% and potassium acetate 5 M was added and samples were incubated for 5 minutes on ice. Afterwards, samples were centrifuged twice and the supernatant was transferred to a new microfuge tube for DNA precipitation using isopropanol. Finally, the resulting DNA was washed with 70% (v/v) ethanol, air-dried and dissolved in 20 µl of TE solution (10 mM Tris–HCl, 1 mM EDTA, pH 8).

The obtained DNA from the suspected *B. bruxellensis* isolates was used for yeast identification based on the RFLPs of the PCR-amplified ITS-5.8S rDNA region as described by Esteve-Zarzoso et al. (1999). The RFLP patterns obtained were compared with those of the www.yeast-id.org (https://www.yeast-id.org/) based on the method described by Esteve-Zarzoso et al. (1999) and grouped within the profiles of *B. bruxellensis* or other known yeasts species. The isolates presenting *B. bruxellensis* profile were confirmed by sequencing the PCR amplified ITS1-ITS4 region (Macrogen Inc.). The sequences were compared with those of the Type Strains included in

GenBank using BLASTN tool (NCBI). Identification at species level was achieved with homologies with type strains higher than 99%.

Strain typing by Intron Splice Site PCR (ISS-PCR) analysis

Identification of *B. bruxellensis* isolates at strain level was performed by a multiplex Intron Splice Site PCR (ISS-PCR) using 1μl (80-100ng) of the extracted DNA as a template. For that, 2 primers pairs that mapped to ISS, DbEI1 (5′-CTGGCTTGGTGTAAGT-3′)/La2 (5'-CGTGCAGGTGTTAGTA-3') and El1 (5'-CTGGCTTGGTGTATGT-3')/ La2 (5'-CGTGCAGGTGTTAGTA-3'), were used for the reaction as described by Vigentini et al. (2011). The PCR products were separated by electrophoresis on 2% agarose gels containing ethidium bromide (4%) for 8 h at 50 V. The images of the agarose gel were taken and digitalized using a UV transilluminator with a digital camera.

The resulting DNA patterns were processed using GelJ v.2.0 software (Department of Mathematics and Computer Science of University of La Rioja, Spain). A composite dendrogram was constructed with the unweighted pair group method using arithmetic averages (UPGMA) (Guzzon et al., 2018). The similarity matrix was constructed using Dice's similarity coefficient (Vigentini et al., 2011). Strains were clustered together when coefficient of genetic similarity was higher than 90%. To check the reproducibility of this ISS-PCR method, two biological replicates of the collection strain CECT 1009 were tested. As the genetic similarity obtained between both CECT 1009 patterns was 100%, the ISS-PCR method was considered reproducible and reliable.

Resistance to sulfur dioxide under laboratory and wine-like conditions

Two isolates from each genetic cluster were randomly selected to study sulfur dioxide tolerance and volatile phenols production. In the case of cluster 9, considering the high proportion of isolates included in this group, four isolates were chosen for further studies. Finally, cluster 3 included the two biological replicates of type strain CECT 1009 and just one of the CECT 1009 replicates was used as representative.

Thus, a total of 19 *B. bruxellensis* strains were used to study their sulphite tolerance in two different media. The first medium tested consisted in yeast nitrogen base with amino acids (YNB, Difco™) (6.70 g/l) and glucose (5 g/l). The second one, synthetic wine medium (SWM), consisted in YNB with amino acids (Difco™) (1.70 g/l), tartaric acid (4 g/l), glycerol (5 g/l), sodium acetate

(0.134 g/l) and ethanol (10% v/v). Both media were adjusted to pH 3.5 and supplemented with different volume concentration of potassium metabisulphite ($K_2S_2O_5$) solution to obtain the final concentrations of SO_2 to evaluate. Specifically, in this work we have tested the tolerance to molecular SO_2 concentrations of 0, 5, 10, 15, 20, 25, 30, 40, 50 and 60 mg/l. To calculate the total SO_2 it was considered that $K_2S_2O_5$ corresponds to the 50% of total SO_2. The free molecular SO_2 concentration was also assessed on days 0, 7 and 20 from non-inoculated controls of both YNB and SWM with the commercial kit (GAB system) based on the Ripper method (Vahl and Converse, 1980).

For the preparation of the inoculum in YNB we performed as following: For each selected isolate, one colony was grown in 20 ml of YPD medium in sterile tubes at 28 °C and 120 rpm during 48 h. Finally, 2 ml of the grown culture was added to 30 ml of YNB medium and incubated in the previous conditions (28 °C, 120 rpm, 48 h). For the SWM inoculum, we added some steps to adapt the isolates to the concentration of ethanol (10% v/v) that contains this medium. In this case, after incubation in YPD, two consecutive steps in YNB containing 4%(v/v) and 8%(v/v) of ethanol were done before the transferring to SWM. Cells were finally adapted in 100 ml of SWM (28 °C, 120 rpm, 96 h) before the inoculation to SWM containing different concentrations of SO_2.

Finally, each well of a microtiter plate (96 wells) containing 195 µl YNB or SWM with different concentrations of molecular SO_2 was inoculated with 5 µl of the adapted preculture to obtain a final concentration of $2 \cdot 10^6$ cells/ml. Each isolate was tested in triplicate for the different SO_2 concentrations.

The tolerance to SO_2 was followed by growth of the selected *B. bruxellensis* isolates into the two media (YNB and SWM) containing different SO_2 concentrations. Growth was monitored by OD measurements (600nm) with the spectrophotometer SPECTROstar (BMG LABTECH, USA) each 48 h during 7 days for YNB medium and each 4-5 days during 30 days for SWM.

Volatile phenol production under wine-like conditions

The selected *B. bruxellensis* isolates were also evaluated for volatile phenol production under wine-like conditions. Specifically, we analyzed the production of 4-ethylphenol (4- EP) and 4-ethylguaiacol (4-EG) from their hydroxycinnamic acids (HCA) precursors (*p*-coumaric and ferulic acids). In order to evaluate this feature, a concentration of $2 \cdot 10^6$ pre-adapted cells/ml grown in YNB with 8% (v/v) of ethanol were inoculated in duplicate in 100 ml of SWM and incubated

on screw-tap tubes without agitation at room temperature. The SWM contained the HCA precursor's *p*-coumaric and trans-ferulic acids (Sigma Aldrich, St. Louis, Missouri, EEUU) at a ratio of 8:1 with a final HCA concentration of 10 mg/l. This concentration and proportion simulates the amount of HCAs commonly found in red wines (Chatonnet et al., 1992; Hixson et al., 2012). The growth and viability of *B. bruxellensis* isolates was followed by OD (600nm) measurements and CFU (colony forming units) counting on solid YPD plates. For the volatile phenol analysis, 10 ml of each culture was taken after 7 and 30 days of incubation, centrifuged and the supernatant frozen at -20 °C until its analysis by the metabolomics facility of the Centre for Omic Sciences (COS, Reus, Spain). Briefly, the volatile phenols were extracted with dichloromethane as solvent and analyzed by GC-MS with a chromatographic column CP-Sil 24 CB (30 m x 0.25 mm i.d., 0.25 μm film) (Agilent Technologies). Compounds were detected using electronic impact (EI). Standards of 4-ethylphenol and 4-ethylguaiacol were used to prepare the calibration curves using *p*-cresol as Internal Standard.

Results

Yeast identification and *B. bruxellensis* strains typing

During the present study, 172 colonies were selected on plates of WLN+cycloheximide medium inoculated with aging wine samples from different Catalonian wine regions. From these colonies, 64 were confirmed as *B. bruxellensis* strains both by RFLP analysis of the 5.8S-ITS gene region and partial sequencing of the ITS1-ITS4 gene region. The rest of the yeasts colonies had a RFLP pattern that did not correspond to that of *B. bruxellensis.* A high proportion of the identified *B. bruxellensis* strains come from samples of Tarragona, which presented "Brett" character at the time of sampling. However, Montsant samples also presented "Brett" character and the number of isolates were even lower than in the other two regions (Priorat and Penedès).

The typing of the *B. bruxellensis* strains was performed by ISS-PCR and resulted in 9 genetic clusters (C1-C9) at 90% level of similarity (Figure 1). Cluster C3 included the duplicated fingerprints of the collection strain CECT 1009 resulting from different DNA extractions to show both reproducibility (Supplementary Figure 2) and correct clustering of the fingerprints (Figure 1). The rest of the clusters (C1-2, C4-C9) include the 64 *B. bruxellensis* strains isolated during the present study.

Cluster distribution was mostly depending on the wine region from where the strains were isolated. For example, cluster C1 included isolates from Penedès, clusters C2 and C6 included isolates from Priorat and clusters C4 and C5 from Tarragona. Other clusters like C7 and C8 included mostly isolates from Priorat and Tarragona, respectively. However, cluster C9 was the larger one and included isolates from diverse regions (Penedès, Montsant and Tarragona). Nevertheless, wine regions closer geographically did not necessarily cluster together (for example, C6 and C7) although some clusters (like C1 and C5) including isolates from the same geographical area had a high level of similarity (Figure 1, Supplementary Figure 1).

Resistance to sulphur dioxide under laboratory and wine-like conditions

The collection strain CECT 1009, four strains from the larger cluster (C9) and two strains from the rest of the clusters (19 strains in total) were selected for the SO_2 tolerance test. The strains were evaluated for their ability to grow both on YNB and SWM medium in the presence of different concentrations of SO_2 (Total SO_2 0, 5, 10, 15, 20, 25, 30, 40, 50, and 60 mg/l). At day 0, the total and free SO_2 (fSO_2) were coincident for both media at pH 3.5 (Supplementary Figure 3). However, the fSO_2 decreased with time for non-inoculated YNB medium though it remained constant with time for non-inoculated SWM (Supplementary Figure 3).

Under laboratory conditions (growth in YNB) and at initial time of incubation, all the strains were able to grow without SO_2 and at least with 5 mg/l of SO_2 (Figure 2A). Also, most of the *B. bruxellensis* strains grew under 10 mg/l of SO_2 on the third day of incubation and 47.4% of the strains cope with up to 20 mg/l SO_2 at the fifth day of incubation. Furthermore, the tolerance to SO_2 from all strains increased with further incubation time even if their growth was initially affected from 10mg/l onwards (Figure 2A). However, the strains of cluster C6 that during the 7th day of incubation presented high growth at 30 mg/l of total SO_2, were in fact bearing approximately 10 mg/l of fSO_2 because it was demonstrated that the concentration of fSO_2 decreased with time in the non-inoculated control of YNB (Supplementary Figure 3). The isolates belonging to the same cluster usually showed similar SO_2 tolerance, for example clusters C1, C2, C4, C5 and C6. Nevertheless, strains of the same cluster but isolated from different location presented differences in their SO_2 tolerance, for example, isolates from clusters C7 and C8 (Figure 2A). The collection strain CECT 1009, included in the cluster C3, and the strain XIV showed an average tolerance to 5-10 mg/l SO_2 from the beginning to the end of the incubation, being in

general more sensible to SO_2 than most of the strains isolated from wine samples during the present study (Figure 2A).

Under wine-like conditions, the growth of all the *B. bruxellensis* strains was clearly inhibited respect the growth on YNB due to the ethanol concentration (10% v/v) and low carbon source in SWM (Figure 2B). No significant growth was observed up to the 7th day and then, the growth and SO_2 tolerance moderately increased for some strains from the 20th to the 30th day. However, clusters C1, C2, C3 (collection strain CECT 1009) and C4, hardly grew on SWM. Differentially to YNB medium, the concentration of total and fSO_2 remained constant in SWM across time (Supplementary Figure 3). Thus, most of the strains (52.6 % of the tested isolates) were highly tolerant to SO_2 and were growing after 30 days under, at least, 30 mg/l of fSO_2 (Figure 2B). Strains VII and VIII from cluster C5 and some strains from clusters C4, C7 and C9 (strains VI, XVI and XVIII) grew even at 30-60 mg/l SO_2 being the most resistant isolates. Although the trend in SO_2 tolerance was in some cases similar for isolates from the same cluster (for example, cluster V), wine-like conditions generally increased the differences in the observed SO_2 tolerance for the tested strains (Figure 2B).

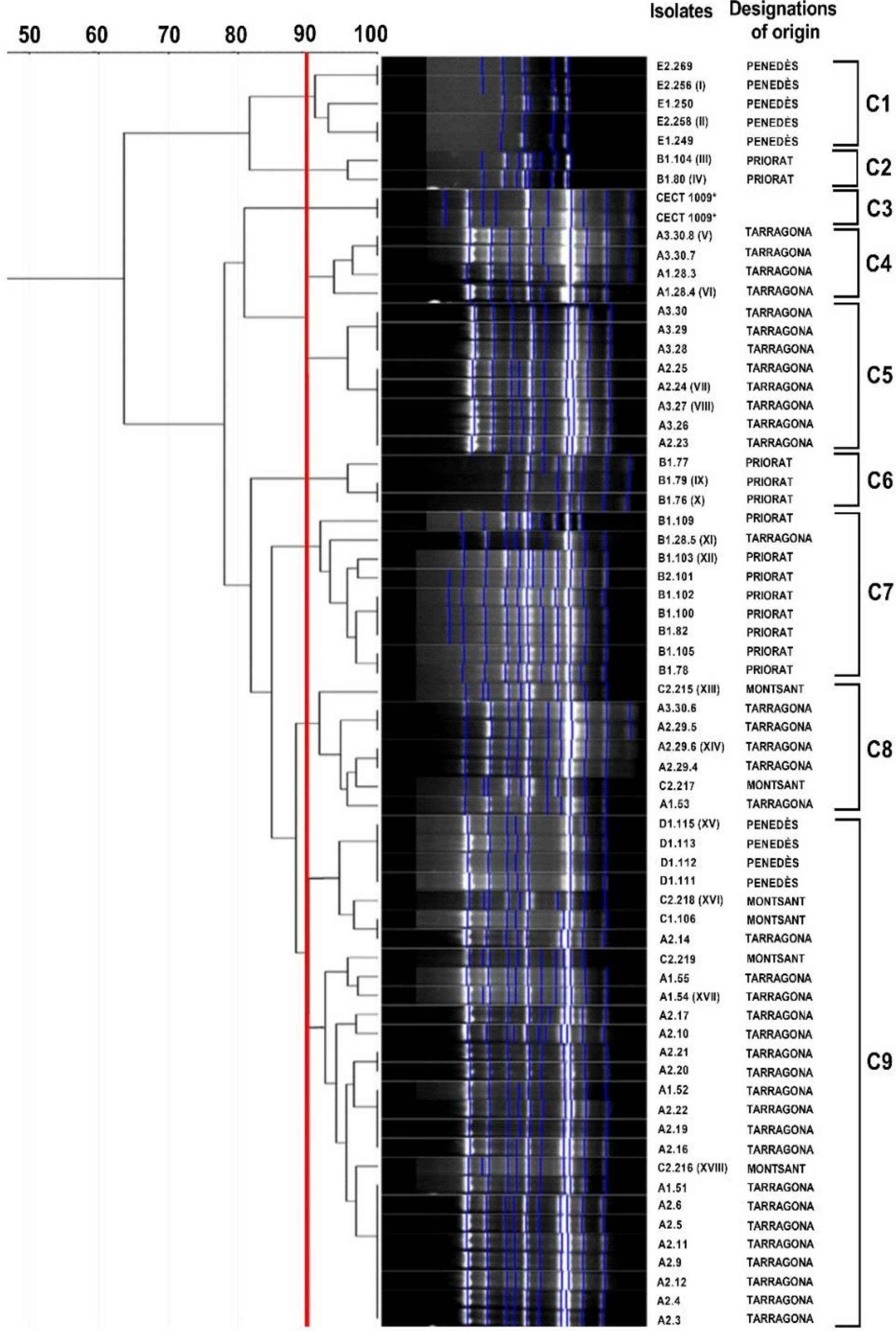
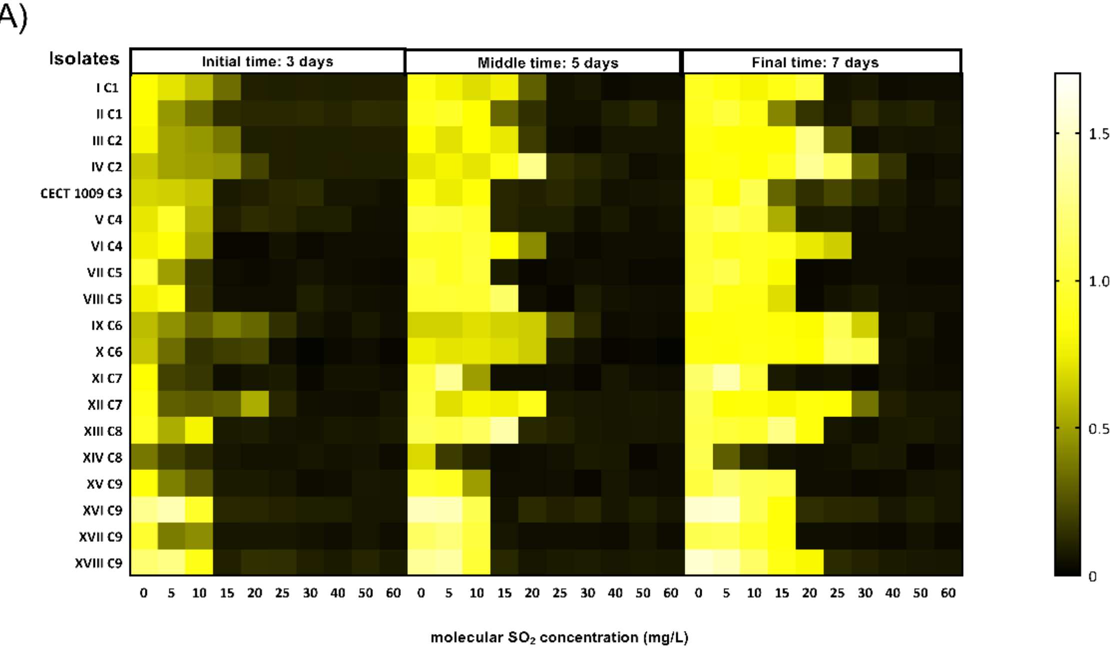


Figure 1. Dendogram of multiplex ISS-PCR profiles from *B. bruxellensis* strains isolated from Catalonian wine regions.



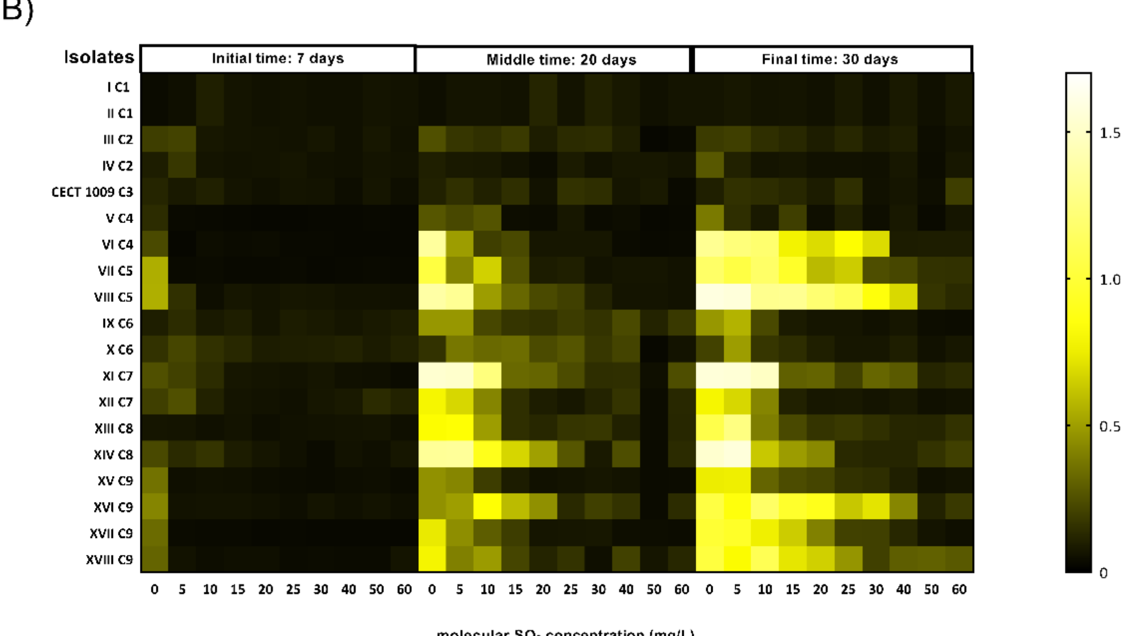


Figure 2. Heat maps representing the growth of the selected strains as OD (600nm) measurements with increasing concentration of SO_2 (0-60 mg/l) under laboratory conditions (A) and under wine-like conditions (B).

Volatile phenol production under wine-like conditions

In order to assess the spoilage potential of the isolated strains, we emulated wine conditions. Each strain was inoculated in SWM containing *p*-coumaric and ferulic acids (ratio 8:1, final concentration of 10 mg/l) as the main HCA precursors of 4-EG and 4-EG, respectively.

Most of the cells were slowly growing along the 30 days of incubation as shown by the OD measurements (Figure 3) with isolates from the same cluster exhibiting a similar final growth value (for example, clusters C4, C5 and C9). Various isolates presented very low growth on wine-like conditions as observed in the previous experiment, for example, clusters C1, C2, C6 and the collection strain CECT 1009 included in cluster C3 (Figure 3). The isolates presented an OD (600nm) at 30st day that ranged from 0.134±0.004 for the isolate I of cluster C1 to 0.443±0.06 for the isolate XVII of cluster C9.

High proportion of the isolates (52.6%) presented lower CFU on YPD plates than the initial inoculum (10^6 cells/ml) even after 30 days of incubation, indicating that the cultivability of those strains decreased with time during incubation (Figure 4). The remaining isolates either showed an increase on CFU at the 7th day but then decreased through the 30th day of incubation (clusters C6 and C7 and the isolates VIII and XIII from clusters C5 and C8, respectively) or they kept increasing up to the 30th day of incubation (isolates XVII and XVIII from cluster C9).

Regarding the volatile phenol production, all isolated strains, even if they presented limited growth, were able to produce 4-EP and 4-EG from their precursors and the concentration of both phenols increased with incubation time. At the 7th day, final of the exponential phase, the maximum registered 4-EP and 4-EG were 2.05±0.06 mg/l and 0.24±0.01 mg/l, respectively, whereas after 30 days of incubation the maximum values were 4.16±0.1 mg/l of 4-EP and 0.52±0.015 mg/l of 4-EG (Figure 5). The production of phenols was not always related to the yeast growth measured as the OD (600nm). For example, strains IV, X and XIII had low final growth (OD ranging from 0.190 to 0.203) but produced similar concentrations of phenols than others strains presenting higher growth (OD ranging from 0.263-0.443) (Figure 3). Additionally, strains with low growth like IV, X and XIII produced higher phenol concentrations than the strain CECT 1009 from cluster C3 (Figure 5) with similar final growth (Figure 3). The *B. bruxelllensis* isolates producing lower phenol compounds were strains form cluster C1 (1.1±0.01 mg/l of 4-EP and 0.17±0.04 mg/l of 4-EG) that also exhibited the lower final growth (OD 0.134±0.004) and the collection strain in cluster C3 (0.91±0.01 mg/l of 4-EP and 0.15±0.05 mg/l of 4-EG). Furthermore,

no relation was found between the number CFU and the amount of volatile phenols. In fact, with the exception of the strains from cluster C9, the number of CFU of the remaining strains decreased from 7th to 30th day but increased both their 4-EP and 4-EG production (Figure 5).

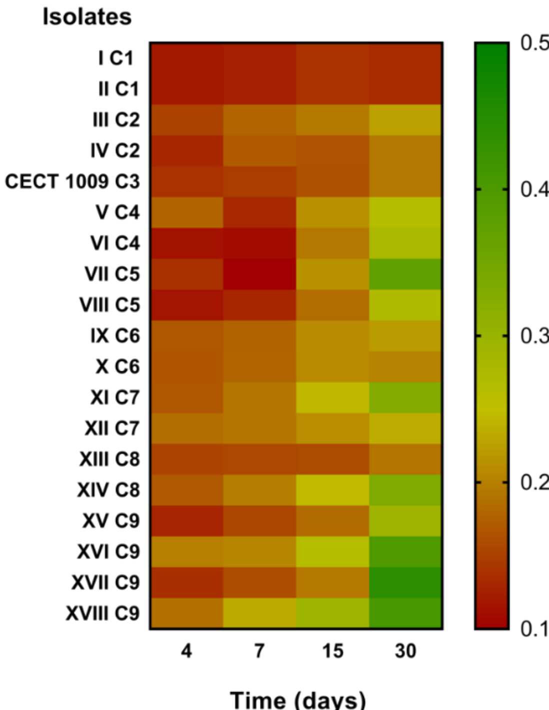


Figure 3. Heat map representing the growth of the selected strains as OD (600nm) measurements under wine-like conditions with *p*-coumaric and ferulic acids as precursors of 4-EP and 4-EG, respectively.

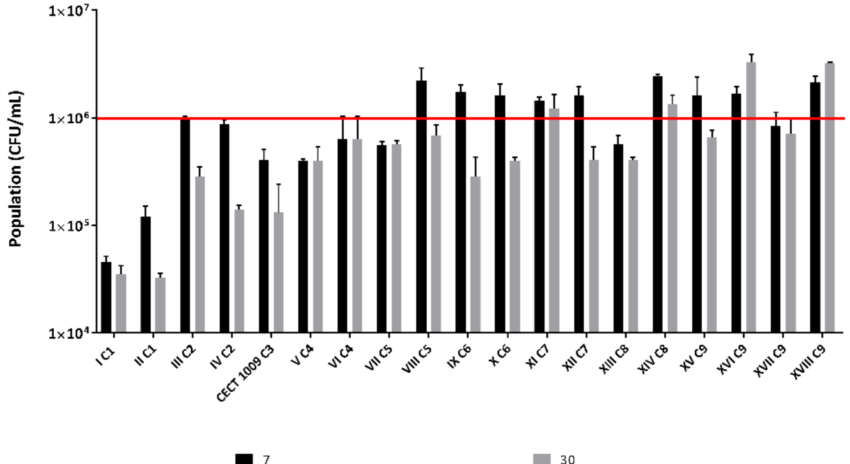


Figure 4. CFU counts of selected strains on YPD plates after 7 and 30 days of incubation under wine-like conditions with SWM and *p*-coumaric and ferulic acids as precursors of 4-EP and 4-EG, respectively.

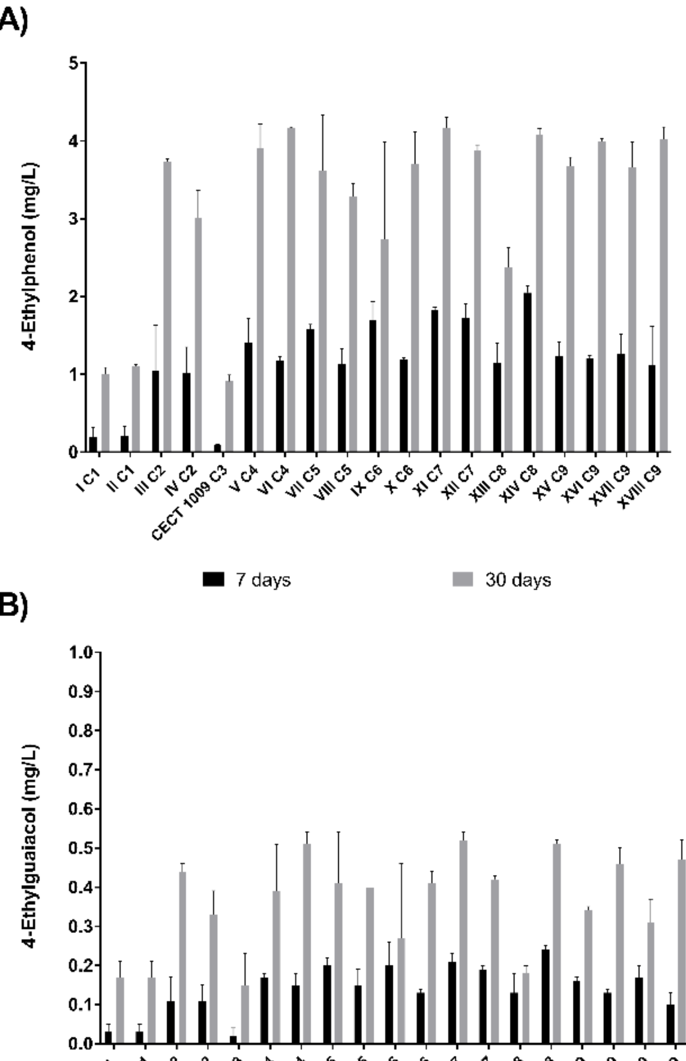


Figure 5. Production of 4-EP (A) and 4-EG (B) by the selected strains after 7 (black bars) and 30 days (grey bars) of incubation under wine-like conditions with SWM and *p*-coumaric and ferulic acids.

Discussion

The negative impact of *B. bruxellensis* on wine industry due to its spoilage activity has led to numerous studies analyzing both phenotypic and genetic diversity of this yeast in different geographical areas (Agnolucci et al., 2009; Curtin et al., 2007; Di Toro et al., 2015). However, little attention has been previously paid to biodiversity of *B. bruxellensis* strains isolated from Spain or Spanish wines, even if it represents an important part of the wine world production (European Comission, 2018). The higher abundance of this yeast have been frequently described during aging of wines in oak barrels and bottled wines (Wedral et al., 2010). In the present study, we

isolated 64 *B. bruxellensis* strains from aging wine samples from different Catalonian wine regions resulting in 8 genetic clusters plus an additional one represented by the collection strain CECT 1009 included for control purposes. The prominent isolation frequency of this species agrees with previous experiences suggesting that, of the five species in this genus, *B. bruxellensis* is the one most frequently isolated from oenological environments (Agnolucci et al., 2009; Guzzon et al., 2018).

The biogeography and genetic diversity of *B. bruxellensis* has been previously studied with controversial results. In cross-national studies, Conterno et al. (2006) found a certain relation between clusters and site of isolation, while Vigentini et al. (2012) and Guzzon et al. (2018) described that strains of distant geographical origin appeared to be closely related at genetic level, indicating a poor correlation of clusters and the geographic location of isolation. Other study analyzing the biodiversity in various winemaking regions of Australia, highlighted the presence of three genotypes found across multiple winemaking regions (Curtin et al., 2007). At a regional level, Campolongo et al. (2010) and Di Toro et al. (2015), highlighted that strains isolated from the same area could be grouped into different clusters. Our results show, however, that in different wine regions from a limited geographical area like Catalonia, each of the 8 clusters at 90% of similarity included mostly isolates from a specific wine region, with the exception of cluster C9 that included isolates from 3 different wine regions. This results highlight a high intraspecific diversity of *B. bruxellensis* strains within Catalonian wine regions. However, the isolates of wine regions geographically closer not always showed higher similarity level than distant regions and did not cluster together. Our findings might be potentially explained by the adaptation of *B. bruxellensis* to the environment of each specific wine region and the lack of spreading of this species in the studied area.

SO_2 is the most common antimicrobial agent used in wine industry to control *B. bruxellensis.* However, the strong legislation and consumer pressure to reduce any kind of wine additives makes it undesirable to obtain wines with high concentrations of SO_2. The maximum allowable limits for the addition of SO_2 by the OIV (Organisation Internationale de la vigne et du vin) is 150 to 300 mg/l of total SO_2. Nevertheless, just the molecular SO_2 appears to exert an antimicrobial action and its concentration in wine depends on many factors such as pH, ethanol, temperature, anthocyanin levels and nutrient content (Sturm et al., 2014). The molecular SO_2 concentration recommended to control *Brettanomyces* spp. ranges from 0.5 to 0.8 mg/l (Oelofse et al., 2008) and

from 20 to 30 mg/l of fSO_2 (Ribéreau-Gayon et al., 2006). Several studies evaluated the resistance to SO_2, with contradictory results depending on the strains and the media used. For example, very tolerant *B. bruxellensis* strains have been previously reported with resistance up to 0.6 mg/l of m SO_2 and 30mg/l of fSO_2 (Avramova et al., 2018; Conterno et al., 2006). In the latter work, isolates from different fermentation niches were evaluated and classified into sensitive or resistant strains depending on their resistance to SO_2. Besides to the tolerance description, they were also able to link this phenotype to specific genotypic groups. Similar results were observed previously by Curtin et al. (2012) when analyzing *B. bruxellensis* strains isolated just from wine environments. However, other authors found no relation between SO_2 resistance and genetic groups though the range to maximum SO_2 tolerance varied from two-to fivefold (Barata et al., 2008; Conterno et al., 2006; Curtin et al., 2012). In the present study we found different response to SO_2 when the strains were growth in YNB or SWM. All the evaluated strains increased the resistance to SO_2 with incubation time. However, the concentration of fSO_2 decreased with time in non-inoculated YNB media, thus the real SO_2 resistance of the strains on YNB had to be corrected. Nevertheless, most of the tested strains were able to display high growth in YNB with at least 10 mg/l of fSO_2 after just 5 days of incubation. On the other hand, the growth of *B. bruxellensis* in SWM was inhibited respect to YNB medium and thus, longer incubation time was necessary to achieve high growth. Nevertheless, the concentration of fSO_2 did not decrease with time in non-inoculated SWM and most strains presented high growth after 30 days of incubation under at least 20 mg/l of fSO_2. Indeed, isolates VI, VII, VIII, XI, XVI and XVIII were able to grow in concentrations of even 30 mg/l. These results suggest an adaptation of the strains to SO_2 with time and it is of remarkable importance for the prevention of their growth by the wine industry. Our results in YNB are in agreement with Agnolucci et al. (2010) as most of *B. bruxellensis* isolates decreased in growth by the same concentration of SO_2. However, under wine-like conditions, the differences in SO_2 tolerance were more evident between the different strains and some of them were able to growth under 60 mg/l of SO_2. Nevertheless, we found no relation between the genetic cluster and the resistance to SO_2 similar to Conterno et al. (2006).

The accumulation of volatile phenols in wine is considered the most detrimental effect directly attributable to *Brettanomyces* (Suárez et al., 2007). Our last experiment evaluated the production of 4-EP and 4-EG of the isolated strains wine-like conditions (SWM). Previous studies used assay conditions, which are often far from vinification conditions including rich media, no ethanol,

elevated pH and/or high concentration of HCA precursors. The results of these studies may be biased and far from the real situation in wine. In fact, various authors acknowledged the need of applying wine-like conditions to better understand the real physiology of *B. bruxellensis* in wine (Oelofse et al., 2009; Sturm et al., 2015). In our experiment, the growth of the *B. bruxellensis* strains was clearly inhibited under wine-like conditions achieving an OD (600nm) that ranged from 0.134±0.004 to 0.443±0.06 after 30 days of incubation. These values are significantly lower than the respective OD values resulting from the SO_2 tolerance experiment on SWM at 0 mg/l SO_2. This fact is probably due to the presence of the HCA in the medium that has been described to have an inhibitory effect on the growth of *B. bruxellensis* (Harris et al., 2010). Besides, no agitation and lower temperature were used in order to simulate wine-like conditions. The lack of agitation reduces the amount of oxygen and inhibits the growth of this yeast increasing the production of phenolic compounds (Curtin et al., 2013). In spite of the scarce observed growth, after 30 days under wine-conditions all the tested strains produced both 4-EP and 4-EG at levels that largely exceed the detection threshold of these compounds in wine (230 and 47 µl, respectively; Chatonnet et al., 1992). The production of phenols from 10 mg/l of HCA as precursors ranged from 2.74 to 4.79 mg/l of 4-EP+4-EG in most of our isolates tested excluding strains I, II and the collection strain of cluster C3. These concentrations are in agreement with the reported previously when using 6 or 11 mg/l of HCA (Curtin et al., 2013; Sturm et al., 2015). It remains unclear whether metabolic capacity to produce volatile phenols or ability to grow in wine is the key determinant of spoilage potential of *B. bruxellensis*. Previous studies indicated that the production of 4-EP and 4-EG was strain dependent (Agnolucci et al., 2009; Barbin et al., 2008; Conterno et al., 2006; Guzzon et al., 2018). However, Curtin et al. (2013) tested three different strains and found no differences on phenol production when wine-like conditions were used. Other studies evidenced a relationship between the physiological state of *B. bruxellensis* and its ability to produce ethylphenols, being culturable populations able to synthesize large quantities (Barata et al., 2008; Sturm et al., 2015). Our results show that the 4-EP and 4-EG production under wine-like conditions, is not always related to the maximum growth of the strains because the proportion of phenols produced by some of the low growing strains was similar to that produced by strains with higher growth. Also, within the low growing strains, differences in phenol production were observed. Additionally, there was no relation between CFU and phenol production as most of the strains showed decreasing CFU counts but increasing phenols production over time. Altogether,

our results suggest that under wine-like conditions most of the strains loose culturability but were still able to produce phenols. Thus, an early detection of *B. bruxellensis* in wines is recommended in order to avoid high populations because even if they enter in a VBNC state for example, by SO_2 addition, they would be able to keep spoiling the wine. In this sense, molecular techniques like qPCR or RNA-FISH with specific primers and probes for *B. bruxellensis* would be useful by their sensitiveness and quick performance (Stender et al., 2001; Tofalo et al., 2012).

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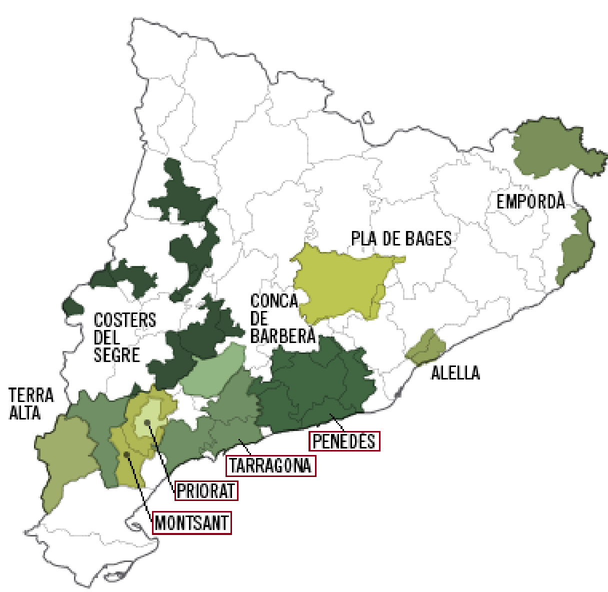
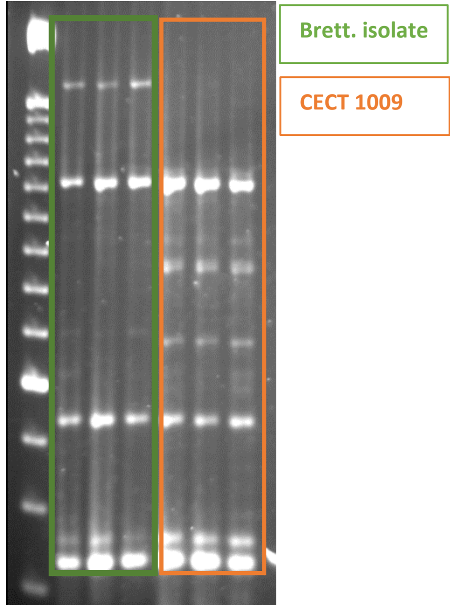
Supplementary material

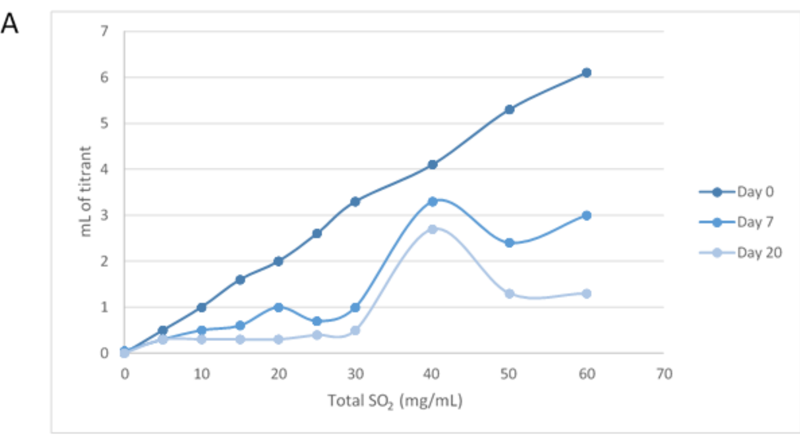


Supplementary Figure 1. Map of the regions in Catalonia with the regions sampled during the study marked within a square.

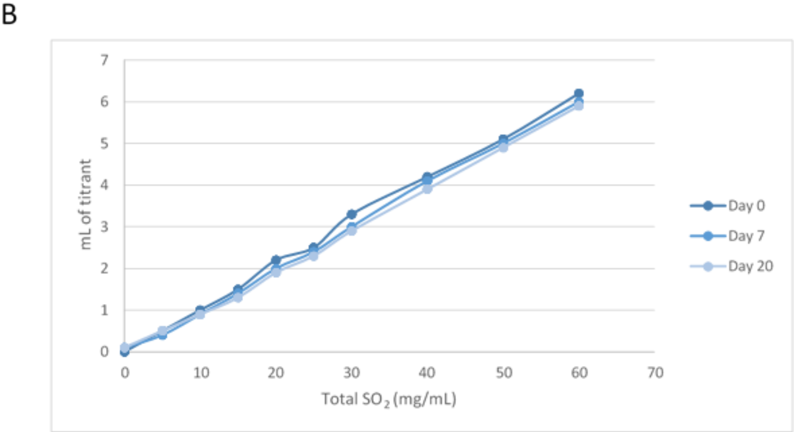


Supplementary Figure 2. Agarose gel of the ISS-PCR pattern of triplicate DNA extraction of one of the isolated strains and the collection strains CECT 1009.

A



B



Supplementary Figure 3. Concentration tendency over time of total and free SO_2 in YPD (A) and SWM (B) medium. The ml of trittant x 10 is equivalent to the free SO_2 (mg/l).

GENERAL DISCUSSION

Alcoholic fermentation is the key process for wine production consisting in the transformation of the sugars present in the grape must into ethanol and carbon dioxide. Although yeasts are the principal actors of the fermentation, they co-exist with a community of microorganisms that can determine the success of the process. In fact, these populations of fungi, yeasts and bacteria are already present on grape surface and different factors will define the final community structure, such as climatic conditions or vineyard management and location (Stefanini and Cavalieri, 2018). Moreover, winemaker's attention is being focused on the employment of the non-*Saccharomyces* yeasts isolated from the berries to produce differential and distinctive wines (Mas et al., 2016). Indeed, mixed and sequential fermentations seem to be the best option to get the positive contribution of non-*Saccharomyces* yeasts and also to guarantee the completion of the process thanks to the *Saccharomyces* oenological aptitudes (Jolly et al., 2014; Padilla et al., 2016). Other non-*Saccharomyces* yeasts with detrimental effects on wine, like *Brettanomyces bruxellensis,* also attract study interest in order to better understand their metabolism and to provide improved tools for their prevention and eradication (Curtin et al., 2015).

Several factors have to be considered during alcoholic fermentation, especially when mixed or sequential fermentations are performed. Interactions between the different microorganisms present during the fermentation, mainly among *Saccharomyces* and non-*Saccharomyces* yeasts, the effect of some environmental conditions on yeast physiology and metabolism and the competition for some pivotal nutrients like nitrogen, direct the fermentation performance and the succession of microbial populations (Bell and Henschke, 2005; Ciani and Comitini, 2015). Furthermore, the application of molecular techniques, particularly NGS technologies, are contributing on deciphering the effects of previous situations on the microbiome of grapes and also during alcoholic fermentation and post-fermentation proceedings (Morgan et al., 2017).

Considering all these aspects, the aim of this thesis was to evaluate the effect of different biotic and abiotic factors on yeast and bacterial communities during alcoholic fermentation and its effect on the fermentation process and the final wine.

Influence of grape health status on wine microbiota

Grape surface displays a complex consortium of fungi, yeasts and bacteria, denominated grape microbiome, that evolves during the ripening period defining the microbial communities present at harvest time and in the resulting grape must. Nevertheless, climatic conditions or vineyard

management can disrupt these microbial populations and promote the development of some spoiler microorganisms, such as *B. cinerea* (Barata et al., 2012b). A primary research goal in grape microbiome research is to elucidate microbiome functions that alter grapevine and fermentation performance. Several researchers tried to differentiate between beneficial, neutral, and detrimental effects of grape microorganisms mainly based on culture dependent methods (Barata et al., 2012a; Nisiotou et al., 2011). As none of these studies used techniques such as qPCR and NGS, in Chapter 1 we aimed to establish the relationship between the health state of the berry with specific changes on the grape microbiota employing culture dependent and independent techniques, including for the first time qPCR and NGS methodologies.

In order to achieve this goal, in Chapter 1 we described spontaneous fermentations of Macabeo grapes exhibiting different health status: healthy, rotten and *Botrytis*-affected. Fungal and bacterial diversity at different time points of the fermentations was assessed by plate culturing in different media and by molecular methods as PCR-DGGE, qPCR and NGS. Similarly to previous works, musts and the initial stage of fermentations from both rotten and *Botrytis*-affected grapes exhibited higher yeast and AAB counts as stated by plating and qPCR. Despite the important contribution of plate culturing, qPCR and PCR-DGGE to assess microbial diversity and quantification, NGS technology was the best tool to decipher the microbiome at the different fermentation stages allowing greater diversity identification than the other techniques (Barata et al., 2012b; Nisiotou et al., 2011). Indeed, this would indicate why current studies on grape microbiota are opting for NGS technologies to achieve a better ecology coverage (Bokulich et al., 2012).

NGS allowed the detection of *Saccharomyces* in all grape musts and *Oenococcus* in the healthy grape must, not detected by the other techniques in this work and poorly recovered in previous reports at this stage (Franquès et al., 2017). *Oenococcus* presence in must was positively related to the healthy status of the grapes and, similarly, *Gluconobacter* genus and *H. uvarum* were more abundant in healthy samples. Meanwhile, higher proportions of *H. osmophila* and *Gluconoacetobacter* were recovered in samples from damaged grapes. While *H. uvarum* has been broadly detected in berries and musts ranging different health states, *H. osmophila* has not been identified in healthy grapes and musts by previous studies. However, Barata et al. (2008b) seldom isolated *H. osmophila* in sour rotten samples. Therefore, linking our findings with those of Barata et al. (2008b), the greater presence of *H. osmophila* could be an indicator of health disruption of

grapes. In the case of bacteria, several genera of AAB have been previously isolated from healthy grapes and musts. In our study we detected by NGS techniques higher proportion of *Gluconacetobacter* in damaged grape samples than in healthy ones which agrees with results from Barata et al. (2012a) in sour rotten samples using culture-dependent techniques. Our study also describes high proportions of *Gluconobacter* in healthy grapes similarly to a previous study using massive sequencing on healthy grapes fermentation (Portillo and Mas, 2016).

Additionally, whereas fungal and bacterial diversity decreased from the middle to the end of healthy grape fermentations, in damaged fermentations diversity remained high and constant. Therefore, this situation would point the greater microbial instability arisen from the disruption of grape health. According to our results, an increased initial biodiversity can difficult the proper fermentation development and represents a warning signal for the fermentation performance regardless of health grape status (Chapter 3). Thus, greater attention should be paid if this diversity level increases or remains constant along the fermentation indicating that there is no a leading yeast species driving the fermentation. Finally, we demonstrated that differences in fungal and bacterial communities not only depends on the fermentation stage but also on the grape health condition.

Thus, in Chapter 1 it was confirmed that NGS technique is an appropriate tool to conduct a microbial phylotyping survey to define a core microbiome, i.e., the microbial taxa consistently present in a healthy grapevine. The next approach would be to employ metagenomics, metatranscriptomics, or metaproteomics to infer functional properties of the whole microbial community or some key microbial taxa within it. Finally, the taxonomic composition of microbiomes could be experimentally manipulated to test hypotheses about microbiome function. It remains challenging to elucidate specific functional roles of the microbiome in shaping grapevine performance traits (e.g., growth, health and overall fitness). Crucial to this challenge is the complexity of microbiome properties, which can be driven by interactions among taxa within the microbiome community and which can vary with both the host genotype and the environment.

Effect of nutrient availability on wine yeasts and fermentation

One of the abiotic factors with a huge impact on wine fermentation and yeast development is the presence of nutrients in the grape must, especially nitrogen and sugar, which are normally

unbalanced. In addition, the climate change is unfolding a new scenario with grapes resulting in greater maturity and, consequently, an increased sugar content. We have to combine this situation with the nitrogen concentration of the must since yeasts require larger amounts of nitrogen in high sugar juices (Martínez-Moreno et al., 2012). Therefore, taking into consideration these nutrient conditions together with the new trends on using non-*Saccharomyces* yeasts for winemaking, we decided to evaluate the effect of sugar excess and limiting nitrogen content on the evolution of mixed and sequential fermentations using three non-*Saccharomyces* yeast species and one *S. cerevisiae* strain (Chapter 2).

In this work, we showed that nitrogen limitation has a stronger effect on alcoholic fermentation than higher sugar concentration. Indeed, mixed nitrogen-limited fermentations got stuck and exhibited higher proportion of the non-*Saccharomyces* species at mid and final fermentation stages. As stated by Andorrà et al. (2012) and Medina et al. (2012), the consumption and requirements of nitrogen by the non-*Saccharomyces* yeasts would interfere in the development of *S. cerevisiae* raising the risk of stuck or sluggish fermentations. Furthermore, considering the results of the mixed fermentations under different nutrient conditions and inoculation times of *S. cerevisiae*, we suggested the best time for *S. cerevisiae* inoculation in each situation. Thus, when nitrogen content is suitable (300 mg/l), in musts with a sugar concentration ranging from 200 to 240 g/l, *S. cerevisiae* should be inoculated between the first 24 and 48 h of fermentation to ensure the growth and contribution of the non-*Saccharomyces* yeasts and the availability of nitrogen for *S. cerevisiae* development. Notwithstanding, under nitrogen limiting conditions, the best approach would be to co-inoculate *S. cerevisiae* with non-*Saccharomyces* yeasts as this yeast group is nitrogen-demanding and would impede *S. cerevisiae* growth if it is incorporated later on. Another option apart from co-inoculation could be the supplementation of nitrogen in the moment of *S. cerevisiae* addition in sequential fermentations as proposed by Medina et al. (2012).

As nitrogen demonstrated to have a decisive role on wine fermentation and yeast development, we focused the interest on this nutrient and its effect on non-*Saccharomyces* yeasts. Currently, some studies have arisen about nitrogen preferences and consumption by different non-*Saccharomyces* species (Gobert et al., 2017). However, how nitrogen is metabolized by these yeast species is still unknown. They will probably share with *S. cerevisiae* homologous routes for nitrogen uptake and consumption. In fact, *S. cerevisiae* displays a mechanism called Nitrogen

Catabolite Repression (NCR) through which it selects the best nitrogen sources for growth modifying the expression of the genes responsible of the transport and metabolism of these nitrogen sources and also through the exclusion of the unnecessary proteins for this process (Magasanik and Kaiser, 2002). For that reason, in Chapter 4, we decided to evaluate the presence of this mechanism in *H. vineae*, a non-*Saccharomyces* yeast of oenological interest as stated in Chapter 3. Therefore, we analyzed the expression of four NCR-regulated orthologous genes (*AGP1, GAP1, MEP2* and *PUT2)* in two strains of *H. vineae* in comparison to *S. cerevisiae*, and we also determined the nitrogen consumption using synthetic musts with an established nitrogen content (140 mg YAN/l).

The expression of NCR-regulated proteins along the fermentation has been used as an indirect tool to study this mechanism in *S. cerevisiae* (Beltran et al., 2007). In Chapter 4, we observed a similar gene expression between *S. cerevisiae* and *H. vineae* strains as *GAP1, PUT2* and *MEP2* began to express while *AGP1* became down-regulated as nitrogen was depleted. This fact together with the nitrogen consumption and the homology found on the NCR related proteins between *H. vineae* and *S. cerevisiae* clearly suggested the presence of NCR regulation in *H. vineae*. Regarding to nitrogen utilization, *H. vineae* and *S. cerevisiae* shared similar consumption patterns with differences just in the ammonia, valine, tyrosine and tryptophan consumption. Between *H. vineae* strains, slight divergences in nitrogen utilization were observed, probably responding to different strain nitrogen preferences as noted in *S. cerevisiae* strains (Crépin et al., 2014). Considering all these evidences, we were able to suggest the presence of NCR regulatory system in *H. vineae*. Nevertheless, Gobert et al. (2017) have demonstrated that nitrogen preferences change depending on temperature conditions in both *Saccharomyces* and non-*Saccharomyces* yeasts. This situation is probably the result of temperature effect on the proteins structure responsible for their transport into the cell (Beltran et al., 2007). Thereby, to see the effect of temperature on nitrogen consumption and metabolism in *H. vineae,* fermentations should be performed at different temperature ranges.

In both Chapter 2 and Chapter 3 the importance of nitrogen has been emphasized. In the case of mixed and sequential fermentations, the incoming studies showing non-*Saccharomyces* preferences for specific amino acids would contribute with the supplementation of specific amino acids depending on the strains or species employed. Moreover, other key nutrients like vitamins should be also considered in these approaches. In the case NCR analysis in *H. vineae*, the

generation of mutants could be proposed as a direct tool for the evaluation of this mechanism. In addition, a transcriptomic approach might allow the identification of other mechanisms than NCR exerted by this yeast under nitrogen stress conditions.

The positive and negative contribution of non-*Saccharomyces* yeasts

Recently, the good aptitudes of some non-*Saccharomyces* yeasts on wine production have been highlighted. These yeasts positively contribute to winemaking through their enzymatic activities that influence on wine aroma or even favor some winemaking processes (Jolly et al., 2014; Padilla et al., 2016). Among the most promising non-*Saccharomyces* species, *H. vineae* stands out by its contribution on wine flavor. Concretely, *H. vineae*'s contribution to wine aroma is mainly related to the increased production of the 2-phenetyl acetate ester and the synthesis of benzenoid compounds associated with fruity and floral aromas (Martin et al., 2016; Viana et al., 2009).

Accordingly, we aimed to describe the effect of *H. vineae* inoculation on the fermentation performance and the resulting wines under semi industrial conditions (Chapter 3). In order to achieve this goal, we inoculated *H. vineae* in Macabeo and Merlot musts and followed the fermentation dynamics by plate-culturing and different molecular techniques. Moreover, the resulting wines were chemically and sensory analyzed and compared with those obtained by *S. cerevisiae* inoculation. The inoculation of *H. vineae* in Macabeo must had a stronger effect on the final wine resulting in more fruity and flowery wines thanks to the high production of 2-phenetyl acetate. Indeed, the production of 2-phenetyl acetate by *H. vineae* was 50 times more than the produced in Macabeo wines fermented with *S. cerevisiae*. The contribution of *H. vineae* responded to its ability to dominate the autochthonous microbiota at least until mid-fermentation. In contrast to Macabeo, *H. vineae* was unable to impose over the microbiota present in Merlot must and, as a consequence, no contribution to the resulting wines was noticed. While Macabeo must was subjected to a filtration process that reduced the initial microbial load, Merlot must was maintained in contact with grape skins providing additional yeast and bacteria which would difficult the imposition of *H. vineae*. Furthermore, the 90% of the sensorial panelists preferred Macabeo wines fermented with *H. vineae* over those fermented with *S. cerevisiae*. As expected by the chemical analysis, the expertise panel highlighted the flowery profile of Macabeo wines inoculated with *H. vineae*. Therefore, the present results agrees with other studies performed with

H. vineae (Martin et al., 2018) and demonstrates why the interest on non-*Saccharomyces* yeasts is increasing between oenologists.

Not all non-*Saccharomyces* yeasts contribute positively to wine. In fact, one of the most concerning oenological spoilers, *Brettanomyces bruxellensis*, belong to this non-*Saccharomyces* group. This yeast is known for its ability to develop in aging wines and produce ethylphenols that confer unpleasant odours, ranging from barnyard to horse sweat, to the final wine (Loureiro and Malfeito-Ferreira, 2003). Considering its relevance in winemaking, many research focused on studying *B. bruxellensis* biodiversity have been done in various oenological regions (Agnolucci et al., 2009; Avramova et al., 2018a; Campolongo et al., 2010; Curtin et al., 2012). However, *B. bruxellensis* diversity has not been assessed in an important wine region like Spain. Therefore, in Chapter 5 we analysed the biodiversity of several *B. bruxellensis* isolates from different oenological regions of Catalonia and evaluated their ability to produce ethylphenols and their tolerance to SO_2.

The different isolates were classified in 8 genetic clusters. Most of these clusters included isolates from a specific wine region coinciding with Guzzon et al. (2018) and Vigentini et al. (2012) observations. Nevertheless, similarly to studies from Campolongo et al. (2010) and Di Toro et al. (2015), some isolates exhibited higher genetic similarity with clusters from distant geographical areas. Furthermore, as SO_2 is the most common antimicrobial agent used in wine industry to avoid *B. bruxellensis* development, we evaluated the sulphite tolerance of the isolates in order to decipher if exists a genetic correlation with sulphite resistance. In fact, some research has already linked sulphite tolerance with specific genotype groups (Avramova et al., 2018b; Curtin et al., 2012). Despite this fact, in this experiment, just like Conterno et al. (2006) no relationship between genetic cluster and SO_2 tolerance could be established. When strains were grown in YNB, most of them were affected by SO_2 concentrations higher than 10 mg/l, whereas in wine-like conditions, tolerance to SO_2 increased with incubation time and some of the isolates exhibited an extreme sulphite resistance and grew on SO_2 contents ranging from 30 to 60 mg/l.

In addition, the ability of the isolates for producing ethylphenols was tested in wine-like medium containing the precursors of 4-ethylphenol and 4-ethylguaicol. Despite the prominent inhibition of *B. bruxellensis* strain's growth in these conditions, most isolates produced ethylphenols at levels that exceed the detection threshold. While many studies have related the production of these compounds with *B. bruxellensis* growth (Barata et al., 2008a; Sturm et al.,

2015), our results suggested that *B. bruxellensis* is able to produce ethylphenols despite its growth inability, emphasizing the importance of considering viable but non-culturable (VBNC) populations during wine aging. Therefore, it would be necessary the employment of alternative techniques to plate culturing in order to detect the presence of VBNC populations of *B. bruxellensis* and, consequently, to apply the appropriate measures for its eradication. From the possible methods, qPCR using specific primers for *B. bruxellensis* represents a suitable and sensitive technique for its detection.

In summary, through the present thesis it was demonstrated how health status of grapes alters the fungal and bacterial populations present along the fermentation. The presence of specific bacterial and fungal genera and species was also evidenced to be related with the initial health status of the grape berries. Furthermore, during this thesis was proven that both initial microbial diversity and load can affect the correct progress of the fermentation and the imposition of the inoculum. In addition, the great contribution of NGS techniques was highlighted. This methodology not only corroborated the results obtained by other techniques but also contributed with new data enlarging the knowledge of the field.

Moreover, we were able to demonstrate the pivotal influence of nutrients, especially nitrogen, on wine performance when mixed and sequential fermentations with non-*Saccharomyces* yeasts were employed. According to our results, nitrogen supplementation or the early inoculation of *S. cerevisiae* would be some of the practices needed to ensure the correct performance of mixed and sequential fermentations when either the initial nitrogen concentration is low or the sugar content is considerably high. Furthermore, our results also suggested that *H. vineae* displays a NCR mechanism to select the best nitrogen sources for its development in a similar way to *S. cerevisiae*. Indeed, *H. vineae* also showed to contribute positively to Macabeo wines conferring a more flowery and fruity aroma profile.

On the other hand, strains from the non-*Saccharomyces* spoiler *B. bruxellensis* demonstrated to genetically classify according to the wine region of isolation. Moreover, all these *B. bruxellensis* isolates were able to produce ethylphenols despite their slow growth or absence of culture plate recovery. Finally, although no correlation between genetic clustering and sulphite tolerance could be established, some *B. bruxellensis* strains grew under high concentrations of SO_2, which supports the oenologists concern about this yeast during winemaking. Thus, the inability to isolate *B. bruxellensis* in plates though their presence in the media and their ability to produce ethylphenols

makes necessary the utilization of other techniques, such as qPCR, to ensure their detection and control.

Thus, the results from this thesis confirm the hypothesis that certain non-*Saccharomyces* yeasts can exert a positive effect on alcoholic fermentation and final wine quality depending on the available nutrients during fermentation and the interaction of these non-*Saccharomyces* yeasts with other microorganisms intrinsically present or inoculated in the must.

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GENERAL CONCLUSIONS

1. Health status of grapes defines the fungal and bacterial communities present on berry surface and during fermentation process.
    - Specific bacterial and fungal genera and species are identified depending on grape's health state. While *Hanseniaspora osmophila* and and *Gluconoacetobacter* predominate in damaged samples, *Hanseniaspora uvarum, Oenococcus oeni* and *Gluconobacter* are abundantly present in healthy samples.
    - Fungal and bacterial diversity decrease from the middle to the end of healthy grape fermentations. Meanwhile, in rotten and *Botrytis*-affected fermentations, diversity increase during this period.
    - NGS is the current technique providing a deeper view of both fungal and bacterial ecology of grapes and fermentation.

2. Nitrogen limitation has a stronger influence than high sugar concentration on the evolution of mixed and sequential fermentations.
    - A limiting nitrogen concentration results in increased fermentation times and stuck fermentations if *S. cerevisiae* is inoculated from 24 hours onwards.
    - An excessive sugar content lead to slower fermentation performances.
    - Under optimal nutrient conditions, 48 hours after the beginning of the fermentation is the best time for *S. cerevisiae's* inoculation to take advantage of non-*Saccharomyces* yeasts metabolism. Nevertheless, when nitrogen is limiting, *S. cerevisiae* should be either co-inoculated with the non-*Saccharomyces* yeasts or inoculated together with nitrogen addition to ensure the availability of nitrogen and, thereby, its development.

3. *Hanseniaspora vineae* is a promising non-*Saccharomyces* yeast for wine fermentation which exhibits a similar nitrogen regulation to *S. cerevisiae*.
    - *H. vineae* is able to overcome the autochthonous microbiota of musts when the initial load and diversity of this microbial populations is low.
    - The presence of *H. vineae* until mid-fermentation confers more fruity and flowery aromas to the resulting wines through the increased production of some aromatic compounds, especially 2-phenetyl acetate.

- *H.vineae* and *S. cerevisiae* have similar expression patterns of the NCR regulated genes *GAP1, AGP1, MEP2* and *PUT2* and similar nitrogen consumption profiles suggesting the presence of the NCR regulation mechanism in this non-*Saccharomyces* yeast.
- The homology found on NCR related proteins between *H. vineae* and *S. cerevisiae* strengthen the evidence of this mechanism in *H. vineae.*

4. Strains of *B. bruxellensis* isolated from oenological regions of Catalonia are genetically distributed depending on the region of isolation and are able to produce considerable amounts ethylphenols.
    - The different *B. bruxellensis* isolates exhibit different tolerance to SO_2. However, this phenotype is not linked to their genetic distribution.
    - *B. bruxellensis* is able to produce ethylphenols at higher levels than the detection threshold, even when their growth is inhibited.

ANNEX 1

Materials and Methods

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Culture Media **244**

YPD (Yeast Peptone Dextrose) 244

WLN (Wallerstein Laboratory Nutrient) 244

YNB (Yeast Nitrogen Base) 244

YNB (Yeast Nitrogen Base) with amino acids 245

Lysine (Lysine Agar) 245

MRS (De Mann, Rogosa and Sharp Agar) 245

GYC (Glucose-Yeast Extract-$CaCO_3$) 245

SM (Synthetic Must) 246

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Culture Media

YPD (Yeast Peptone Dextrose)

Glucose	20 g/l
Yeast Extract	10 g/l
Bacteriological Peptone	20 g/l
Agar (Solid Medium)	17 g/l

WLN (Wallerstein Laboratory Nutrient)

WL Nutrient Agar (Oxoid, England)	75 g/l

WLN (Wallerstein Laboratory Nutrient) with Cycloheximide for *B. bruxellensis* isolation

WL Nutrient Agar (Oxoid, England)	75 g/l
Cycloheximide Stock Solution (5 mg/ml)	20 ml/l

Cycloheximide Stock Solution of 5 mg/ml for *B. bruxellensis* isolation

Cycloheximide	2.5 g
96% Ethanol	0.5 l

YNB (Yeast Nitrogen Base)

Glucose	20 g/l
Yeast Nitrogen Base without amino acids and ammonium sulfate	1.7 g/l
$(NH_4)SO_4$	150 mg/l

YNB (Yeast Nitrogen Base) with amino acids

Glucose	5 g/l
Yeast Nitrogen Base with amino acids and ammonium sulfate	6.7 g/l

Lysine Agar Medium

Lysine Medium	66 g/l
Lactate Potassium	4 ml/l
Lactic Acid	1 ml/l

MRS (De Mann, Rogosa and Sharp Agar)

MRS Medium	55 g/l
Fructose	5 g/l
DL-Malic acid	6 g/l
Cysteine	0.5 g/l
Agar	20 g/l
Nystatin*	100 mg/l
Sodium azide 0.1%**	40 µl/plate

* Fungicide to inhibit fungal growth.

**Inhibit growth of most non-LAB bacteria. It is added directly to the plate before plating the sample.

GYC (Glucose-Yeast Extract-$CaCO_3$)

Glucose	50 g/l
Yeast Extract	10 g/l
$CaCO_3$	10 g/l
Agar	15 g/l
Natamycin*	1 mg/l

* Fungicide to inhibit fungal growth.

SM (Synthetic Must)

Glucose	100 g/l
Fructose	100 g/l
Citric acid	5 g/l
Malic acid	0.50 g/l
Tartaric acid	3 g/l
KH_2PO_4	0.75 g/l
K_2SO_4	0.50 g/l
$MgSO_2$ 7 H_2O	0.25 g/l
$CaCl_2$ 2 H_2O	0.15 g/l
NaCl	0.20 g/l
NH_4Cl (120 mg N/l)	0,460 g/l
Amino acids (180 mg N/l) Stock Solution	10 ml/l
Oligo-elements Stock Solution	1 ml/l
Vitamins Stock Solution	10 ml/l
Anaerobic Factors Stock Solution	1 ml/l

Oligo-elements Stock Solution

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

Amino Acid Stock Solution

Tyrosine	1.95 g/l
Tryptophan	17.42 g/l
Isoleucine	3.25 g/l
Aspartic acid	4.42 g/l
Glutamic acid	11.96 g/l
Arginine	36.79 g/l
Leucine	4.81 g/l
Threonine	7.54 g/l
Glycine	1.82 g/l
Glutamine	49.92 g/l
Alanine	14.56 g/l
Valine	4.42 g/l
Methionine	3.12 g/l
Phenylalanine	3.77 g/l
Serine	7.8 g/l
Histidine	3.38 g/l
Lysine	1.69 g/l
Cysteine	2.08 g/l
Proline	59.93 g/l

Vitamins Stock Solution

Myo-inositol	2 g/l
Pantothenate calcium	0.15 g/l
Thiamine hydrochloride	0.025 g/l
Nicotinic acid	0.2 g/l
Pyridoxine	0.025 g/l
Biotine	3 ml (from stock 100mg/l)

Anaerobic Factors Stock Solution (0.1 l)

Ergosterol	1.5 g
Oleic acid	0.5 ml
Tween 80	50 ml
Ethanol (absolute)	Until 100 ml

SW (Synthetic Wine)

Yeast Nitrogen Base with amino acids and ammonium sulfate	1.7 g/l
Tartaric acid	4 g/l
Glycerol	5 g/l
Sodium acetate	0.134 g/l
Ethanol (10% v/v)	100 ml/l

Protocols

DNA extraction

Adapted from Querol et al. (1992).

1. Wash cells pellet with 1 ml of sterile distilled water. Centrifuge for 3 min at 10000 rpm.
2. Resuspend the resulting pellet with 500 µl of buffer solution 1 (0.9 M Sorbitol, 0.1 M EDTA, pH 7.5).
3. Incubate the samples with 30 µl of both zymolyase and lyticase enzymes (10 mg/ml each) for 1 h at 37 °C.
4. Centrifuge for 2 min at 10000 rpm. Discard the supernatant.
5. Resuspend the pellet in 500 µl of buffer solution 2 (50 mM Tris, 20 mM EDTA, pH 7.4).
6. Add 13 µl of SDS 10% and mix well by shaking. Incubate for 5 min at 65 °C.
7. Add 200 µl of potassium acetate 5M and mix well by inversion. Incubate in ice for 5 min.
8. Centrifuge for 10 min at 4°C and 12000 rpm.
9. Transfer the supernatante to a new eppendorf with 700 µl of isopropanol. Incubate for 5 min at room temperature.
10. Centrifuge for 10 min at 12000 rpm. Discard the flow-through.
11. Add 500 µl ethanol (70% v/v).
12. Centrifuge for 5 min at 12000 rpm. Discard the flow-through.
13. Dry the pell across emptiness.
14. Resuspend the pellet with 20 µl of TE solution (10 mM Tris–HCl, 1 mM EDTA, pH 8).

DNA extraction with DNeasy Plant Mini Kit (Qiagen)

Based on DNeasy Plant Mini Kit Protocol (Qiagen) and Hierro et al. (2006).

1. Wash cells pellet with 1 ml of sterile distilled water.
2. Lysate the cells in tubes containing 700 µl **AP1** buffer and 1 g glass beads (0.5 mm diameter) in a mini bead-beater (Biospec Products Inc., Okla) using three times of 1 min at maximum rate with intervals of 1 min on ice.
3. Centrifuge for 1 min at 4 °C and 10000 rpm.
4. Take the supernatant to a new eppendorf tube for RNA treatment.
5. RNA treatment: add 4 µl of **RNase A** in cell suspension and incubate for 10 min at 65 °C.
6. Incubate in ice for 5 min with 130 µl **P3** buffer. Centrifuge for 5 min at 4 °C and 14000 rpm.
7. Transfer the supernatant into **QIAshredder Mini Spin Column**. Centrifuge for 2 min at 14000 rpm.
8. Transfer the flow-trhough into a new eppendorf tube and add 675 µl of **AW1** buffer. Mix well by pipetting.
9. Transfer 650 µl of the mix into a **DNeasy Mini Spin Column**. Centrifuge for 1 min at 10000 rpm.
10. Discard the flow-through and add the remainder mix volume. Centrifuge for 1 min at 10000 rpm.
11. Place the DNeasy Mini Spin Column into a new Collection Tube and add 500 µl of **AW2** buffer.
12. Centrifuge for 1 min at 10000 rpm and discard the through-flow.
13. Add 500 µl of **AW2** buffer and centrifuge for 2 min at 14000 rpm. Discard the flow-through and place the column into a new eppendorf tube.
14. Add 100 µl of **AE** buffer elution. Incubate for 5 min at room temperature and centrifuge for 1 min at 10000 rpm.

Reagents and components provided by the commercial kit are indicated in bold.

RNA extraction with RNeasy Mini kit (Qiagen)

Based on RNeasy Plant Mini Kit Protocol (Qiagen) and RNase-Free DNAse Set (Qiagen).

1. Resuspend cells pellet with 600 µl of **RLT** buffer containing mercaptoethanol (10 µl mercaptoethanol/ml RLT buffer).
2. Lysate the cells resuspended in **RLT** buffer in tubes containing 1 g glass beads (0.5 mm diameter) in a mini bead-beater (Biospec Products Inc., Okla) using five times of 1 min at maximum rate with intervals of 1 min on ice.
3. Centrifuge for 3 min at 4 °C and 14000 rpm.
4. Transfer the suspension into an eppendorf tube with 600 µl of ethanol (70% v/v). Mix by pipetting.
5. Transfer 700 µl of the mix to an **RNeasy Mini spin column** placed in a 2 ml collection tube. Centrifuge for 15 sec at 10000 rpm. Discard the flow-through. Repeat twice.
6. Add 350 µl of **RW1** buffer to the **RNeasy Mini spin column**. Centrifuge for 15 sec at 10000 rpm.
7. DNAsa digestion step: Add 80 µl of DNAsa digestion mix (10 µl **DNAsa** + 70 µl **RDD** reagent) to the **RNeasy Mini spin column**. Incubate for 15-20 min at room temperature. Add 350 µl **RW1** buffer and centrifuge for 15 sec at 10000 rpm. Discard the flow-through.
8. Add 500 µl **RPE** buffer to the **RNeasy Mini spin column**. Centrifuge 2 min at 10000 rpm.
9. Place the RNeasy Mini spin column into a new eppendorf tube. Add 30 µl of **RNase-free water** to the spin column membrane and centrifuge 1 min at 10000 rpm.
10. Measure the final concentration of RNA in NanoDrop (ThermoFisher Scientific, US).

Reagents and components provided by both commercial kits are indicated in bold.

Reverse Transcription

RNA was synthetised into cDNA by reverse transcription as following (Applied Biosystems, USA):

1. Adjuste RNA concentration to 320 ng/µl using Nuclease free water (Invitrogen, USA).
2. Add 1 µl of oligo-dT (Invitrogen) and incubate 5 min at 65 °C. Place 1 min at 4 °C.
3. Add 7 µl of the following mix as described by SuperScript III Reverse Transcriptase (Invitrogen):
    - 4 µl of buffer
    - 1 µl dNTPs
    - 2 µl of DTT
4. Incubate the mix at 42 °C for 5 min. Then, add 1 µl of RT enzyme.
5. Incubate final suspension at 42 °C for 50 min followed by 15 min at 70 °C.
6. The resulting cDNA can be used for gene expression anylisis by qPCR.

SO_2 Determination

Based on Ripper's method. SO_2 concentration was determined according to manufacturer's instructions (GAB Systems, Spain):

Free SO_2

1. Level up the burette to 25 ml with **Standard solution**.
2. Pour into an Erlenmeyer flask 25 ml of the sample. Add 1-2 ml of **Indicator** solution and 5 ml of **Acid** solution.
3. Place the Erlenmeyer flask under the burette and add the **Standard solution** until the color of the Erlenmeyer's flask solution changes and remains constant for 10-15 sec.
4. Multiply ten times the volume spent to find out the concentration of free SO_2 (mg/l).

Total SO_2

1. Level up the burette to 25 ml with **Standard Solution**.
2. Pour into an Erlenmeyer flask 25 ml of the sample and 10 ml of **Alkali** solution. After covering and shaking, incubate for 15 min.
3. Add 1-2 ml of **Indicator** solution and 5 ml of **Acid** solution.
4. Place the Erlenmeyer flask under the burette and add the **Standard Solution** until the color of the Erlenmeyer's flask solution changes and remains constant for 10-15 sec.
5. Multiply ten times the volume spent to find out the concentration of total SO_2 (mg/l).

Reagents and components provided by the commercial kit are indicated in bold.

HPLC Analysis

Amino Acid Determination

Amino acids were analysed by HPLC method described by Gómez-Alonso et al. (2007).

Sample preparation:

- 400 µl of sample
- 700 µl of borate buffer (1M, pH 9)
- 300 µl of methanol
- 15 µl of diethyl ethoxymethylenemalonate (DEEM) (Fluka, Germany)
- 10 µl L-2-aminoadipic acid (internal standard) (1 g/l)

Incubation of the samples during 2 h at 80 °C for derivatization. After that, 50 µl of each sample is directly injected into the HPLC.

Chromatograph specifications: Agilent 1100 Series HPLC (Agilent Technologies, Germany). Quaternary pump, an autosampler and a multiple wavelength detector at 269, 280 and 300 nm. Nitrogen compound separation of sample was peformed using a 4.6 x 250 mm x 5 µm Hypersyl ODS column (Agilent Technologies, Germany) with a guard column (ACE5 C18-HL) through a binary gradient:

- Phase A: 25 mM acetate buffer pH 5 with 0.02% sodium azide
- Phase B: mixture of acetonitrile and MeOH (80:20)
- Flow: 0.9 ml/min

The target compounds were identified according to the retention time f corresponding standards and were quantified using the internal standard method.

Molecular methods

5.8S-ITS-RFLP analysis

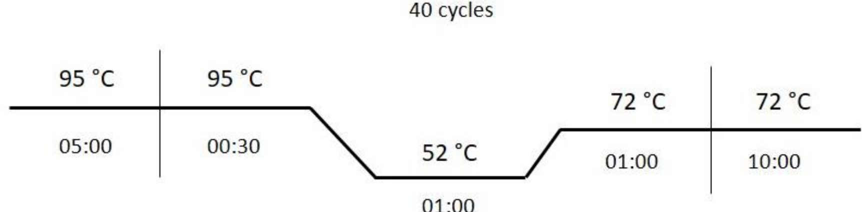
For yeast identification. Based on Esteve-Zarzoso et al. (1999).

Primer	Sequence
ITS1	5'- TCCGTAGGTGAACCTGCGG-3'
ITS4	5'- TCCTCCGCTTATTGATATGC-3'

The PCR mix was prepared as follows:

- 5 µl Buffer 10X w/o Mg^{2+}
- 3 µl $MgCl_2$
- 1 µl primer ITS1 (10 µM)
- 1 µl primer ITS4 (10 µM)
- 1 µl dNTPs (10 mM each dNTP)
- 2.5 U Taq DNA polymerase
- 1 µl DNA
- Sterile Milli-Q water q.s. 50 µl

PCR program:



To visualize PCR products: 1.5 % agarose gel electrophoresis containing ethidium bromide.

Restriction of the PCR products with *Hinf*I, *Hae*III, *Cfo*I, *Dde*I and *Mbo*I performing the following mixture:

- 2 µl of buffer (specific for each restriction enzyme)
- 1 µl restriction enzyme
- 7 µl sterile Milli-Q water
- 10 µl PCR product

Incubate overnight at 37 °C and visualization of the restriction profiles in a 3 % agarose gel electrophoresis containing ethidium bromide.

26S rDNA D1/D2 domain analysis

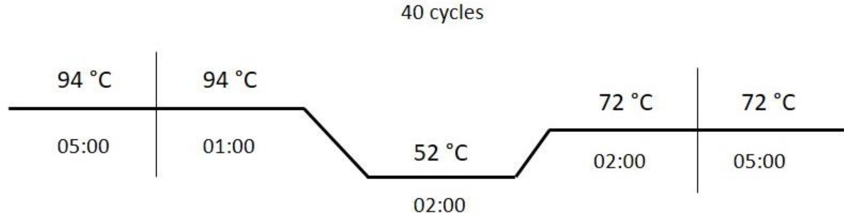
For yeast species identification through sequencing 26S rDNA D1/D2 domain. Based on Kurtzman and Robnett (1998).

Primer	Sequence
NL1	5'- GCATATCAATAAGCGGAGGAAAAG-3'
NL4	5'- GGTCCGTGTTTCAAGACGG-3'

The PCR mix was prepared as follows:

- 5 µl Buffer 10X w/o Mg^{2+}
- 1.5 µl $MgCl_2$
- 1 µl primer NL1 (10 µM)
- 1 µl primer NL4 (10 µM)
- 1 µl dNTPs (10 mM each dNTP)
- 1.25 U Taq DNA polymerase
- 1 µl DNA
- Sterile Milli-Q water q.s. 50 µl

PCR program:



To visualize PCR products: 1.5 % agarose gel electrophoresis containing ethidium bromide.

PCR products are sent to Macrogen (Korea) for purification and sequencing. For yeast species identidification, the resulting sequences will be analysed through BLAST using sequence alignment against the NCBI database (https://blast.ncbi.nlm.nih.gov/).

Intron Splice Site PCR (ISS-PCR) analysis

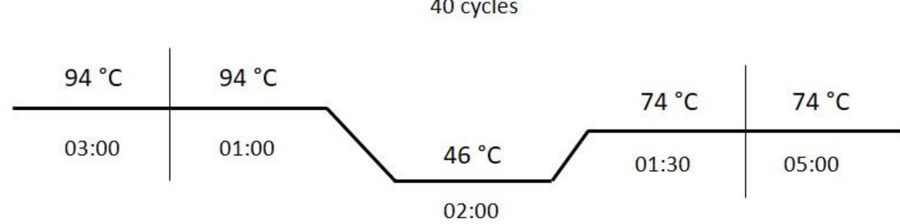
For *Brettanomyces bruxellensis* typification. Based on Vigentini et al. (2011).

Primer	Sequence
DbEI1	5′-CTGGCTTGGTGTAAGT-3′
La2	5’-CGTGCAGGTGTTAGTA-3’
El1	5’-CTGGCTTGGTGTATGT-3’

The PCR mix was prepared as follows:

- 2.5 µl Buffer 10X w/o Mg^{2+}
- 0.5 µl $MgCl_2$
- 1.9 µl primer DbEI1 (10 µM)
- 1.9 µl primer La2 (10 µM)
- 1.9 µl primer El1 (10 µM)
- 0.5 µl dNTPs (10 mM each dNTP)
- 1 U Taq DNA polymerase
- 1 µl DNA
- Sterile Milli-Q water q.s. 25 µl

PCR program:



To visualize PCR products: 2 % agarose gel electrophoresis containing ethidium bromide for 8 h at 50 V.

DGGE (Denaturing Gradient Gel Electrophoresis)

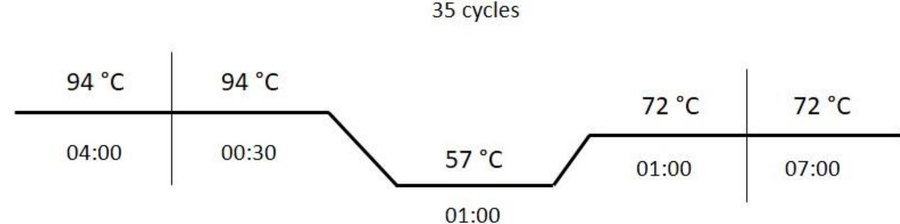
For fungal and bacterial diversity analysis. Based on Meroth et al. (2003) and Muyzer et al. (1993).

Primer	Sequence
U1^{GC} fungi	5'-GCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCG CCCCGTGAAATTGTTGAAAGGGAA -3'
U2 fungi	5'-GACTCCTTGGTCCGTGTT-3'
341f^{GC} bacteria	5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCAC GGGGGGCCTACGGGAGGCAGCAG -3'
518r bacteria	5'-ATTACCGCGGCTGCTGG -3'

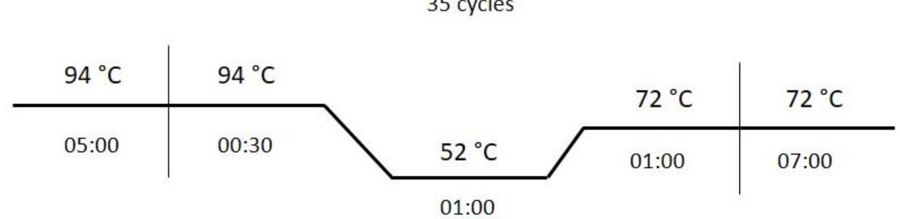
The PCR mix was prepared as follows:

- 5 µl Buffer 10X w/o Mg^{2+}
- 1 µl $MgCl_2$
- 1 µl primer U1^{GC}/341f^{GC} (10 µM)
- 1 µl primer U2/518r (10 µM)
- 1 µl dNTPs (10 mM each dNTP)
- 1.25 U Taq DNA polymerase
- 1 µl DNA
- Sterile Milli-Q water q.s. 50 µl

PCR program for fungi (U1^{GC}/U2)



PCR program for bacteria (341f^{GC}/518r)



The polyacrylamide gel with gradient from 35% to 55% was carried out using solution A (0% denaturing solution) and solution B (100% denaturing solution) by Econo Gradient Pump (Bio-Rad, USA).

Solution A 0% Denaturing		**Solution B 100% Denaturing**	
40% Acrylamide/Bis	20 ml	40% Acrylamide/Bis	20 ml
50X TAE buffer*	2 ml	50X TAE buffer	2 ml
dH_2O	78 ml	Formamide (deionized)	40 ml
		Urea	42 g
Total volume	100 ml	dH_2O	to 100 ml

*50X 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).

20 µl of TEMED (N, N, N, N'-tetra-methyl-ethylenediamine) and 200 µl of ammonium persulphate (0.1 g/l) are added to 20 ml of each solution. Then, both A and B solutions are used for building the gradient gel with a gradient of denaturalization of 35% to 55% using Econo Gradient Pump (Bio-Rad, USA). The pump programme is the following:

- Min. 0 50% solution B
- Min. 0.5 50% solution B
- Min. 3 32% solution B
- Min. 3.5 32% solution B

Pump speed: 10 ml/min
Polimerization time: ≈ 1 hour

15 µl of the PCR products are mixed with 5 µl of loading buffer (2% bromophenol blue, 2% xylene cyanol, 100% glycerol). The resulting 20 µl mixture is loaded to gel. Then, electrophoresis is run for 3 h at 200 V. Finally, the gel is stained with ethidium bromide for its visualization.

The bands are excised from the gels, and the DNA is eluted overnight in 40 µl of 10 mM Tris pH 8 and 1 mM EDTA (TE) at 4 °C. After that, the DNA is re-amplified with the same pair of primers without the GC-clamp and sequenced by Macrogen (Korea) for species identification.

Microbial population analysis by qPCR

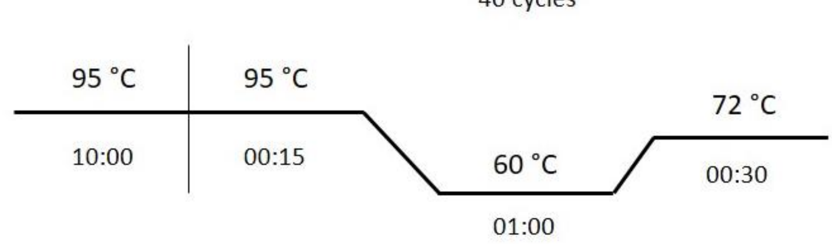
For general quantification of yeast and bacterial groups and species of oenological interest.

Target	Primers	Ribosomal gene region	References
Total yeast	200F/324R	26S rRNA	Hierro et al. (2006)
Saccharomyces spp.	CESPF/SCERR	ITS2 and 5.8S rRNA spanning region	Hierro et al. (2007)
Hanseniaspora spp.	CESPF/HUVR	ITS2 and 5.8S rRNA spanning region	Hierro et al. (2007)
S. bacillaris	AF/200R	D1/D2 (26S rRNA)	Andorrà et al. (2010)
T. delbrueckii	TODSL2/TODSR2	ITS (between 18S rRNA and 26S rRNA)	Zott et al. (2010)
Metschnikowia spp.	MP5FW/MP3BW	26S rRNA	Díaz et al. (2013)
B. cinerea	BC3F/BC3R	ITS (between 18S rRNA and 28S rRNA)	Suarez et al. (2005)
LAB	WLAB1/WLAB2	16S rRNA	Neeley et al. (2005)
AAB	AQ1F/AQ2R	16S rRNA	González et al. (2006)

The PCR mix was prepared as follows:

- 10 µl SYBR *Premix ExTaq* II (2X) (Takara)
- 0.4 µl primer Forward (10 µM)
- 0.4 µl primer Reverse (10 µM)
- 0.4 µl ROX Reference Dye (50X)
- 2 µl DNA
- 6.8 µl sterile purified water

PCR program run in 7300 Fast Real-Time PCR System (Applied Biosystems):



Gene expression analysis by qPCR

For gene expression analysis of NCR genes in *S. cerevisiae* and its orthologs in *H. vineae*. Primers used for *S. cerevisiae* are described by Beltran et al. (2004) and Gutiérrez et al. (2013).

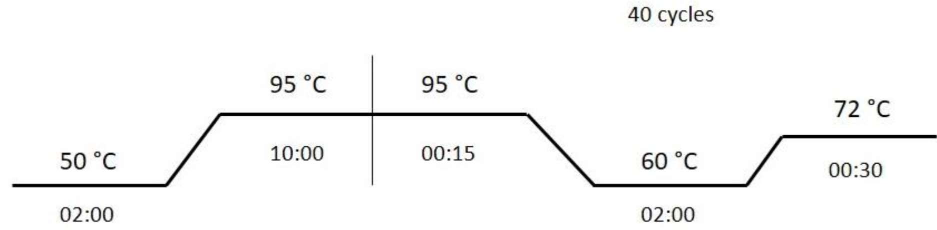
Primers designed for *H. vineae* are listed below:

Gene	Name	Oligonucleotide sequence (5'-3' end)
ACT1	ACT-F	GGCTTCTTTGACCACTTTCCAA
	ACT-R	GATGGACCACTTTCGTCGTATTC
IPP1	IPP-F	CATTCACAACTACGGTGCTTTCC
	IPP-R	GGTTTCTGGGTGAACTTCGTTT
AGP1	AGP1-F	ATTGCTGGGTGACGGTTCTT
	AGP1-R	TGACATTGGTAGCGGCAATAAC
GAP1	GAP1-F	CAAAGGTTTGCCATCTGTCATC
	GAP1-R	TGCAGAGTTACCGACAGACAACA
MEP2	MEP2-F	TCGATGACGGGTTGGATGTT
	MEP2-R	CATAACGGCACCACTTAAACCA
PUT2	PUT2-F	GATACGACATGTTGGCAGCAA
	PUT2-R	TTTCGCCTTGGAAAACGTTT

The PCR mix was prepared as follows:

- 10 µl SYBR *Premix ExTaq* II (2X) (Takara)
- 0.4 µl primer Forward (10 µM)
- 0.4 µl primer Reverse (10 µM)
- 0.4 µl ROX Reference Dye (50X)
- 2 µl cDNA
- 6.8 µl sterile purified water

PCR program run in 7300 Fast Real-Time PCR System (Applied Biosystems):



- The expression of *AGP1, GAP1, MEP2* and *PUT2* is normalized with *ACT1* and *IPP1*.
- Relative gene expression is determined using the $2^{-\Delta\Delta Ct}$ method, where the Ct value corresponds to the number of cycles needed to achieve the background fluorescence.

Library Construction for NGS

For fungal and bacterial microbiome description.

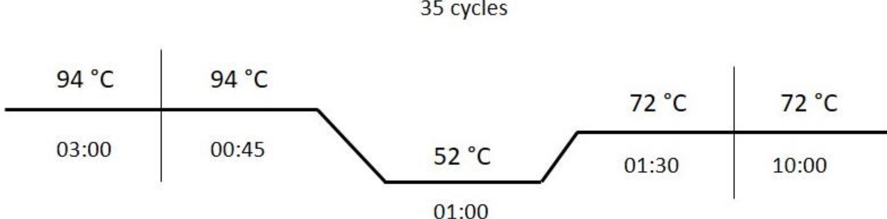
Primer	Sequence	References
FR1 fungi	5’-ANCCATTCAATCGGTANT -3′	Prévost-Bouré et al. (2011)
FF390 fungi	5’-CGATAACGAACGAGACCT-3’	Prévost-Bouré et al. (2011)
515F bacteria	5’-GTGCCAGCMGCCGCGGTAA-3’	Caporaso et al. (2011)
806R bacteria	5’-GGACTACHVGGGTWTCTAAT -3’	Caporaso et al. (2011)

The library construction is performed amplifying each sample with both universal fungal and bacterial primer pairs. Each primer contains an Iontorrent tag and a 10-bp barcode unique to each amplified sample.

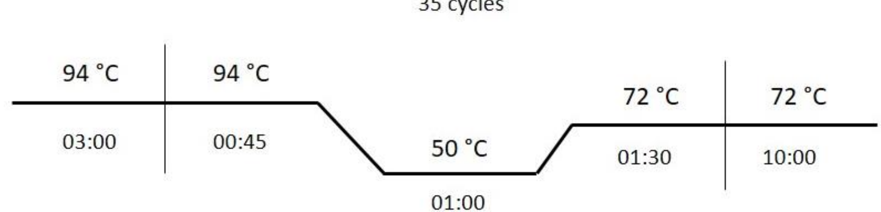
The PCR mix was prepared as follows:

- 5 µl Buffer 10X w/o Mg^{2+}
- 1.25 µl $MgCl_2$
- 1 µl primer Forward (10 µM)
- 1 µl primer Reverse (10 µM)
- 1 µl dNTPs (10 mM each dNTP)
- 1.25 U Taq DNA polymerase
- 1 µl DNA (5-100 ng DNA)
- Nuclease-Free water q.s. 50 µl

PCR program for fungi (FR1/FF390)



PCR program for bacteria (515F/806R)



- The PCR products are pooled by sample and cleaned using a GeneRead Size Selection kit (Qiagen, Hilden, Germany) following manufacturer's instructions.
- The cleaned PCR products are submitted to the Centre for Omic Sciences (Reus, Spain) where their quality is checked by a Bionalyzer and their quantity adjusted for sequencing.
- The adjusted samples are then sequenced by the equipment PMG from Ion Torrent with chips 318.

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ANNEX 2

Other publications derived from this thesis

UNIVERSITAT ROVIRA I VIRGILI
INFLUENCE OF NON-SACCHAROMYCES YEAST ON WINEMAKING AND QUALITY
Jéssica Lleixà Daga

Development of a genetic transformation toolkit for *Brettanomyces bruxellensis*

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Research Article

RESEARCH ARTICLE

Development of a genetic transformation toolkit for *Brettanomyces bruxellensis*

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ABSTRACT

Brettanomyces bruxellensis is usually considered a spoilage microorganism, responsible for significant economic losses during the production of fermented beverages such as wine, beer and cider, though for some styles of beer its influence is essential. In recent years, the competitiveness of this yeast in bioethanol production processes has brought to attention its broader biotechnological potential. Furthermore, the species has evolved key fermentation traits in parallel with *Saccharomyces cerevisiae*. Attempts to better understand *B. bruxellensis* physiology through genomics-driven research have been hampered by a lack of functional genomics tools. Genetic transformation for *B. bruxellensis* has only been developed recently and with limited efficiency. Here we describe gene transformation cassettes tailored for *B. bruxellensis*, which provide multiple drug-resistant markers and the ability to tag *B. bruxellensis* with different fluorescent proteins. All marker cassettes resulted in increased transformation efficiency compared to the maximum reported in literature, with one cassette, *TDH1p* natMX, showing five times greater efficiency. Transformation cassettes encoding fluorescent proteins enabled discrimination between subpopulations of transformed *B. bruxellensis* cells by flow cytometry and fluorescent microscopy. Thus, the genetic transformation toolkit described here unlocks several molecular applications such as strain tagging, insertional mutagenesis and potentially targeted gene deletion.

Keywords: *Brettanomyces*; wine; transformation

INTRODUCTION

Brettanomyces bruxellensis is mainly considered a spoilage yeast which is associated with several fermented beverages including, wine, beer and cider (Curtin, Varela and Borneman 2015; Varela and Borneman 2017) and can also contaminate bioethanol production processes (Liberal *et al.* 2007; Basillo *et al.* 2008). *Brettanomyces bruxellensis* produces several metabolites that negatively affect the sensory properties of fermented beverages with wine being particularly affected. Most prominent amongst these are the volatile phenols 4-ethylphenol (4-EP), 4-ethylguaiacol (4-EG) and 4-ethylcatechol (4-EC), which impart 'elastoplast'/'bandaid', 'medicinal', 'barnyard' and 'earthy' aromas (Chatonnet *et al.* 1992; Chatonnet and Dubourdieu 1995). Others include N-heterocycles responsible for 'mousy' taint (Grbin and Henschke 2000) and methylbutanoic (isovaleric) acids which elicit a 'rancid' aroma (Fugelsang and Zoecklein 2003; Curtin *et al.* 2013).

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Despite its association with the spoilage of fermented beverages, *B. bruxellensis* makes a positive contribution in other fermentation settings. These include the production of Belgian lambic and gueze ales (Vanbeneden *et al.* 2008), the production of cachaça, a distilled spirit made from sugar cane (Parente *et al.* 2015) and the production of bioethanol (Blomqvist *et al.* 2011; Galafassi *et al.* 2011). In fact, *B. bruxellensis* has several traits relevant for industrial applications. It is highly tolerant to both ethanol and low pH (Conterno *et al.* 2006; Curtin, Varela and Borneman 2015), can assimilate a wide range of carbon sources, including chitin and cellobiose (Blondin *et al.* 1982; Curtin *et al.* 2012; Reis *et al.* 2014), and can assimilate nitrate as a nitrogen source, which provides *B. bruxellensis* with a competitive advantage over *Saccharomyces cerevisiae* (de Barros Pita *et al.* 2013; Neto *et al.* 2014).

The combination of significant economic losses in fermented beverage industries and the potential for biotechnological applications has renewed research focus upon *B. bruxellensis*. Genome sequences are now available for several *B. bruxellensis* strains from a variety of fermentation sources including beer, wine and biofuel (Curtin *et al.* 2012; Piškur *et al.* 2012; Borneman *et al.* 2014; Crauwels *et al.* 2015; Olsen *et al.* 2015). These datasets are being used to make discoveries which are enabling rapid advances in our understanding of *B. bruxellensis* biology. For example, amplification of the alcohol dehydrogenase gene family which suggests a parallel evolution between *B. bruxellensis* and *S. cerevisiae* (Curtin *et al.* 2012; Piškur *et al.* 2012), and expansion in amino acid permeases that may facilitate survival in relatively low nutrient environments which provides insight into the evolution of traits that differentiate *B. bruxellensis* from other species (Curtin *et al.* 2012). In addition, these genomic studies have shown that the *B. bruxellensis* genome is highly dynamic. Triploid strains (possibly formed via interspecific hybridization with an unknown closely related species) have been observed (Curtin *et al.* 2012; Borneman *et al.* 2014; Avramova *et al.* 2018) and the species is characterised by extensive karyotype heterogeneity (Hellborg and Piskur 2009).

Genetic transformation is a foundational technology that enables the comprehensive study of a species by applying a multitude of molecular biology tools, such as gene overexpression, gene deletion, incorporation of marker genes for competition experiments and tagging for visualisation or purification. Transformation has been available in *S. cerevisiae* for well over 30 years, with much of the knowledge that has been generated for this species being due to the early adoption of this technique. However, genetic transformation for *B. bruxellensis* has only been developed very recently, with both random genomic insertion and episomal plasmids available for the introduction of heterologous DNA (Miklenic *et al.* 2015; Ishchuk *et al.* 2016; Schifferdecker *et al.* 2016). Here, we describe the development of gene transformation cassettes tailored for *B. bruxellensis* which increase non-homologous transformation efficiency. These cassettes provide multiple drug-resistant markers and the ability to tag *B. bruxellensis* with different fluorescent proteins.

MATERIAL AND METHODS

Strain and media

Brettanomyces bruxellensis AWRI2804 (UC Davis collection UCD2041) was obtained from the Australian Wine Research Institute (AWRI) Wine Microorganism Culture Collection. This strain was the recipient strain for all genetically modified constructs. Cryogenically preserved (–80°C) strains were cultured and maintained on YMPG plates (3 g/L malt extract, 3 g/L yeast extract, 5 g/L peptone, 10 g/L glucose, 40 g/L $CaCO_3$, 20 g/L agar) and stored at 4°C. Strains were grown in YPD medium (yeast extract 10 g/L; peptone 20 g/L; glucose 20 g/L) or in minimal medium containing 5 g/L glucose and 6.7 g/L YNB with amino acids pH 3.5 (MM5).

Construction of DNA cassettes

Plasmids containing genes conferring resistance to different antibiotic were obtained as follows. *Brettanomyces bruxellensis TDH1* promoter was synthesised by Invitrogen GeneArt (ThermoFisher Scientific, MA, USA) and cloned in plasmids pMK-T and pMA generating plasmids pMK-T-TDH1pr and pMA-TDH1pr (Table 1). PCR fragments containing the antibiotic resistance cassettes kanMX, natMX and hygMX, and the Ag*TEF2* terminator were amplified from pUG6, pAG25 and pAG32, respectively, using the primers listed in Table 2. Plasmids pUG6, pAG25 and pAG32 were obtained from the Euroscarf collection (University of Frankfurt, Germany). PCR products were purified, digested with SalI and SpeI, and cloned into pMK-T-TDH1pr, generating the plasmids pMK-T-TDH1pr-kanMX, pMK-T-TDH1pr-natMX and pMK-T-TDH1pr-hygMX (Table 1). Similarly, a PCR fragment containing natMX and the Ag*TEF2* terminator was cloned into pMA-TDH1pr generating plasmid pMA-TDH1pr-natMX (Table 1).

DNA cassettes containing fluorescent proteins were obtained as follows. GFP (green fluorescent protein) was amplified from pADH1-tc3-GFP (Euroscarf collection, University of Frankfurt, Germany), digested with NotI and KpnI and cloned in pCV2_BB, whereas TagBFP (blue fluorescent protein) was synthesised by Invitrogen GeneArt (ThermoFisher Scientific) and cloned in the same plasmid generating plasmids pCV2_BB-GFP and pCV2_BB-BFP, respectively (Table 1). Thus, both proteins were flanked by the strong *S. cerevisiae* promoter Sc*FBA1p* and the Sc*PGK1* terminator. PCR fragments containing the gene encoding a fluorescent protein and the Sc*PGK1* terminator were amplified from pCV2_BB-GFP and pCV2_BB-BFP using primers listed in Table 2. These primers introduced restriction sites for cloning and a flexible linker of four amino acids (gly-gly-ser-gly) to generate a fusion protein between natR and the fluorescent proteins. PCR products were digested with SphI/XbaI and cloned into pMA-TDH1pr-NatMX digested with SphI/SpeI, replacing the Ag*TEF2* terminator with the Sc*PGK1* terminator and generating plasmids pMA-TDH1pr-NatMX::GFP and pMA-TDH1pr-NatMX::BFP (Table 1). All cloning steps were performed in *E. coli* DH5α.

Transformation

Brettanomyces bruxellensis AWRI2804 was transformed by electroporation following the protocol described by Miklenic *et al.* (2015). Briefly, strains were grown overnight in liquid YPD medium, then 1 mL of culture was used to inoculate 200 mL of fresh YPD medium and incubated on an orbital shaker (140 rpm, 28°C) until a concentration of 5×10^7 cells/mL was achieved (mid-exponential phase). Cells were then centrifuged (3000 rpm, 4 min, room temperature), supernatant was carefully decanted and cells were washed three times with 50 mL sterile deionised water. Cells were resuspended in 20 mL of 35 mM of dithiothreitol and 100 mM lithium acetate and incubated for 45 min at 28°C with gentle shaking (140 rpm). Cells were then isolated by centrifugation (3000 rpm, 4 min, 4°C), with the resulting pellet washed twice with 20 mL of ice-cold sterile deionised water and

Table 1. Plasmids used in this study.

Plasmid	Description/genotype	Source/reference
pMK-T	*E. coli* replicating plasmid, kanamycin^R	Invitrogen GeneArt
pMA	*E. coli* replicating plasmid, ampicillin^R	Invitrogen GeneArt
pUG6	*E. coli* replicating plasmid kanMX cassette (geneticin^R)	Euroscarf collection
pAG25	*E. coli* replicating plasmid natMX cassette (clonNAT^R)	Euroscarf collection
pAG32	*E. coli* replicating plasmid hygMX cassette (hygromycin^R)	Euroscarf collection
pMK-T-TDH1pr	*E. coli* replicating plasmid containing the *B. bruxellensis TDH1* promoter, kanamycin^R	This study
pMA-TDH1pr	*E. coli* replicating plasmid containing the *B. bruxellensis TDH1* promoter, ampicillin^R	This study
pMK-T-TDH1pr-kanMX	kanR flanked by the *B. bruxellensis TDH1* promoter and the *TEF2* terminator, kanamycin^R	This study
pMK-T-TDH1pr-natMX	natR flanked by the *B. bruxellensis TDH1* promoter and the *TEF2* terminator, kanamycin^R	This study
pMK-T-TDH1pr-hygMX	hygR flanked by the *B. bruxellensis TDH1* promoter and the *TEF2* terminator, kanamycin^R	This study
pMA-TDH1pr-natMX	natR flanked by the *B. bruxellensis TDH1* promoter and the *TEF2* terminator, ampicillin^R	This study
pCV2_BB	*E. coli* replicating plasmid *FBA1p*-MCS*-*PGKt*, kanMX, construct flanked by EcoRI/SpeI 5′end and XbaI 3′ end	This study
pADH1-tc3-GFP	*E. coli* replicating plasmid, *ADH1* promoter, three tetracycline aptamers, GFP	Euroscarf collection
pCV2_BB-GFP	GFP flanked by the *S. cerevisiae FBA1* promoter and the *PGK1* terminator	This study
pCV2_BB-BFP	BFP flanked by the *S. cerevisiae FBA1* promoter and the *PGK1* terminator	This study
pMA-TDH1pr-natMX::GFP	natR::GFP fusion flanked by the *B. bruxellensis TDH1* promoter and the *PGK1* terminator, ampicillin^R	This study
pMA-TDH1pr-natMX::BFP	natR::BFP fusion flanked by the *B. bruxellensis TDH1* promoter and the *PGK1* terminator, ampicillin^R	This study

MCS, multiple cloning site.

Table 2. Primers used in this study.

Primer	Sequence
kanMX-SalI-F	5′- catg gtcgac atgggtaaggaaaagactcacgttt -3′
natMX-SalI-F	5′- catg gtcgac atgggtaccactcttgacgacacgg -3′
hygMX-SalI-F	5′- catg gtcgac atgggtaaaaagcctgaactcaccg -3′
tTEF2-SpeI-R	5′- catg actagt tcgacactggatggcggcgttagtat -3′
GFP-NotI -F	5′- catg gcggccgc atggctagcaaaggagaagaac -3′
GFP-KpnI -R	5′- catg ggtacc ttatttgtagagctcatccatgccatg -3′
SphI-link-GFP-F	5′- catg gcatgccctgcccc ggtggtagcggc atggctagcaaaggagaagaac -3′
SphI-link-BFP-F	5′- catg gcatgccctgcccc ggtggtagcggc atgtctgaattgatcaaagaaaacatgc -3′
tPGK1-XbaI-R	5′- catg tctaga cctagatagcttaaaactgcataaaggc -3′
M13-F	5′- gtaaaacgacggccagtg -3′
M13-R	5′- ggaaacagctatgaccatg -3′

Underlined sequences indicate flexible linker for protein fusions.

then twice with 20 mL of ice-cold 1M sorbitol solution. Finally, cells were resuspended in 1M sorbitol (ice-cold), to a final volume of 500 μL, with 50 μL aliquots used for each transformation reaction. A volume of 1 μL containing DNA for transformation (28–56 ng/μL) was then introduced into the samples, mixed thoroughly and incubated on ice for 5 min. Samples were then placed in separate 0.2 cm-gap electroporation cuvettes (Bio-Rad) previously kept on ice, and pulsed (1.8 kV, 5 ms) with Bio-Rad Gene Pulser Electroporator (600 Ω, 25 μF). Immediately after the pulse, 1 mL of 1M sorbitol:YPD (1:1) ice-cold solution was added, and the samples were incubated at room temperature for 20 min without shaking. After the incubation step, the cells were placed in 10 mL sterile tubes and 1 mL of YPD was added. The samples were incubated overnight at 28°C with shaking (140 rpm) and then spread on plates containing the appropriate antibiotic. Plates were then incubated at 30°C, and transformants were visible after 5–7 days.

DNA cassettes for transformation were obtained by PCR using the primers M13-F and M13-R (Table 1). PCR products were purified using the Wizard® SV Gel and PCR clean-up system (Promega, Madison, USA) and used for transformation as indicated above.

Flow cytometry

Over 150 individual transformants for each fluorescent protein were assessed for mean fluorescence by flow cytometry using a Guava® easyCyte 12HT instrument (Merck Millipore). Transformants were grown in 96-well plates containing 200 μL of MM5 medium. Microplates were sealed with a Breathe-Easy® sealing membranes (Diversified Biotech) and incubated at 28°C for 2 days. Cell cultures were diluted in PBS buffer (NaCl 8 g/L, KCl 0.2 g/L, Na_2HPO_4 1.44 g/L, KH_2PO_4 0.24 g/L pH 7.4) to ensure a cell concentration lower than 5×10^5 cells/mL before being analysed in the flow cytometer. Forward and side scatter detectors were used to determine particle size and estimate cell numbers, blue fluorescence was detected using violet (405 nm solid state laser) excitation and a 448/50 nm

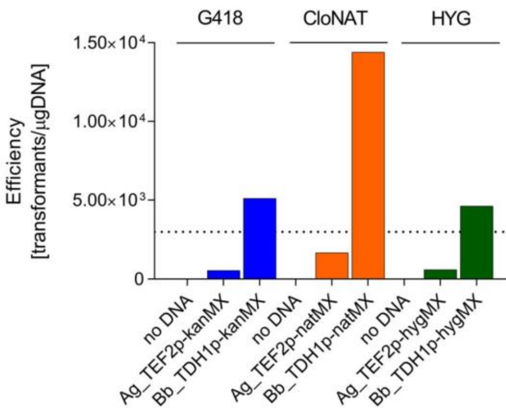


Figure 1. *Brettanomyces bruxellensis* transformation efficiency with selectable marker cassettes containing the *A. gossypii TEF2* promoter (AgTEF2p) or the *B. bruxellensis TDH1* promoter (BbTDH1p). The dotted line indicates the maximum efficiency reported in the literature for *B. bruxellensis* (2.8×10^3 transformants/μg DNA).

detection filter, while green fluorescence was detected using blue/green (488 nm solid state laser) excitation and a 525/50 nm detection filter. For all analyses, a minimum of 5000 events were acquired while cell throughput was kept under 500 cells/μL to ensure accuracy. Results were analysed with inCyte software version 3.2.

One positive transformant for each fluorescent protein, AWRI2804_natR::GFP and AWRI2804_natR::BFP, and the parental strain AWRI2804 were grown in 10 mL test tubes containing 2 mL of MM5 medium for 2 days (28°C, rotary incubator). Individual cultures and mixed cultures of AWRI2804_natR::GFP and AWRI2804_natR::BFP at different ratios (3:1, 1:1 and 1:3) were then used to estimate the ability of the flow cytometer to discriminate between green, blue and absence of fluorescence, using the parameters indicated above.

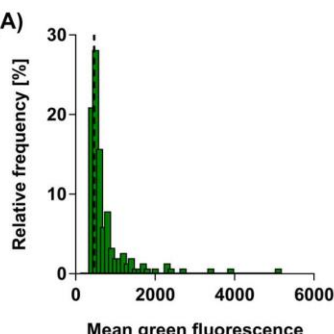
Fluorescence microscopy

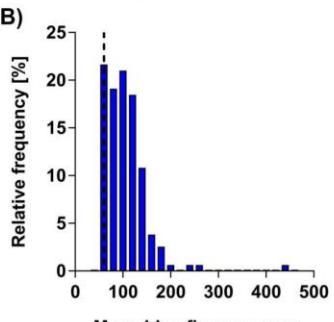
A 1:1 mixed culture of AWRI2804_natR::GFP and AWRI2804_natR::BFP prepared as indicated above was used for fluorescence microscopy. The culture was visualised with a NIKON50i microscope using a Plan Fluor ×100 oil immersion objective. A Nikon Intensilight mercury arc lamp was used as excitation source. Blue fluorescence was imaged using a UV-2A (Nikon) filter block, whereas green fluorescence was imaged using a GFP-B (Nikon) filter block. Photographs were acquired with a Digital Sight DS-2MBW$_C$ camera and processed with the NIS Elements Basic Research software version 4.6.

RESULTS AND DISCUSSION

Improved efficiency for *B. bruxellensis* transformation

Most existing selectable antibiotic marker cassettes (that were developed for *S. cerevisiae*) show high efficiency when used in *S. cerevisiae* (up to 1×10^6 transformants/μg DNA) (Gietz and Schiestl 2007), but only limited efficiency in *B. bruxellensis* (up to 2.8×10^3 transformants/μg DNA) (Miklenic *et al.* 2015). As *B. bruxellensis* is distantly related to *S. cerevisiae*, it was hypothesised that this reduction in efficiency may be due to the heterologous promoter used so far. A homologous promoter was therefore sought from *B. bruxellensis*, with gene expression data (Tiukova *et al.* 2013) indicating that the *TDH1* gene promoter could provide the high and consistent expression necessary to drive the expression of drug-resistant DNA cassettes. A set of DNA vectors





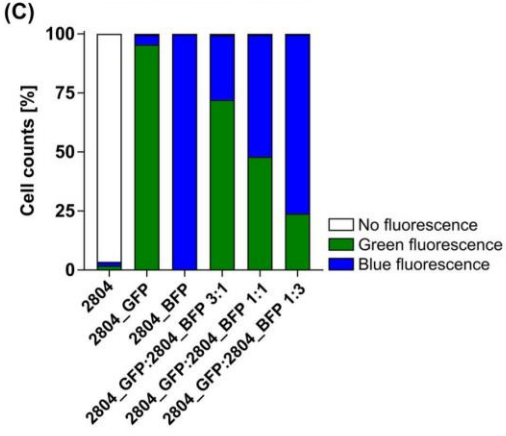


Figure 2. Flow cytometry for AWRI2804 transformed with GFP and BFP. **(A)** Frequency distribution of mean green fluorescence for GFP transformants. **(B)** Frequency distribution of mean blue fluorescence for BFP transformants. More than 150 individual transformants for each fluorescent protein were analysed. The dashed line indicates the value for the parental strain AWRI2804. **(C)** Cell counts according to fluorescence for individual cultures of AWRI2804, AWRI2804_natR::GFP and AWRI2804_natR::BFP and for mixed cultures of AWRI2804, AWRI2804_natR::GFP and AWRI2804_natR::BFP at different proportions.

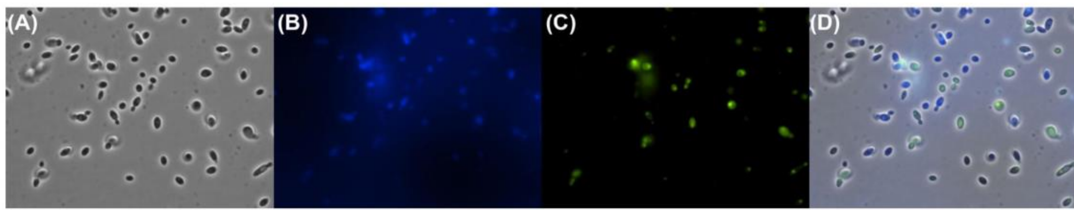


Figure 3. Microscopy for a mixed culture of AWRI2804_natR::GFP and AWRI2804_natR::BFP (1:1 ratio). (A) Normal visualisation. (B) Blue fluorescence. (C) Green fluorescence. (D) Superimposed image. Culture was visualised with a ×100 oil immersion objective.

was then created in which the *Ashbya gossypii TEF2* promoter was replaced with the *B. bruxellensis TDH1* promoter, such that this new promoter would drive the expression of three different drug-resistant genes, kanMX, natMX and hygMX, encoding resistance to G418, CloNAT and hygromycin, respectively (Goldstein and McCusker 1999).

Following transformation into *B. bruxellensis* AWRI2804, all of the marker cassettes that contained the *BbTDH1* promoter showed an increased transformation efficiency compared to those driven by the *AgTEF2* promoter (Fig. 1). The kanMX, natMX and hygMX *TDH1p* cassettes showed nine, eight and seven times higher efficiency, respectively, than their *AgTEF2* counterparts. Indeed, all *TDH1p* marker cassettes exhibited a higher efficiency than the maximum reported previously (2.8×10^3 transformants/μg DNA), with the natMX *TDH1p* cassette being five times higher (1.44×10^4 transformants/μg DNA) (Fig. 1).

Tagging *B. bruxellensis* with fluorescent proteins

Fluorescent markers, such as GFP, provide an attractive methodology for labelling cells *in vivo*. Selectable marker cassettes were constructed whereby the natR (encoding for CloNAT resistance) resistance gene (driven by *BbTDH1*) was fused, in-frame to the open reading frame of either the GFP or BFP fluorescent proteins. These fluorescent marker cassettes were then used to tag *B. bruxellensis* AWRI2804 via random integration. Both GFP and BFP transformants showed asymmetrical distributions for mean fluorescence with approximately 60% of all transformants having up to 2-fold higher fluorescence than AWRI2804 (Fig. 2A and B). Maximum mean fluorescence values for GFP and BFP were 10-fold and 7-fold higher than AWRI2804, respectively. Since *B. bruxellensis* transformation is mediated almost exclusively by non-homologous end joining (Wang, Choi and Lee 2001; Klinner and Schafer 2004), it is likely that the high fluorescence observed in some transformants was the result of multiple copies of the transformation cassette being inserted randomly in the *B. bruxellensis* genome. Approximately 20% of cells displayed no increase in fluorescence compared with the parental strain, which may be due to disruption of the GFP/BFP coding sequence during integration.

One randomly selected transformant for each fluorescent protein (with a mean fluorescence value 2–4 times higher than the parental strain background) was selected for investigating the use of GFP and BFP to discriminate between subpopulations of cells in mixed cultures using flow cytometry. In control cultures of AWRI2804, flow cytometry showed that more than 96% of the cell population showed no fluorescence (below the threshold for fluorescence detection), while a small proportion were classified as fluorescent (green 1.9%, blue 1.6%) (Fig. 2C). In AWRI2804 tagged with GFP, 95.5% of the population showed green florescence, with the remainder showing blue (4.1%) or no florescence (0.4%). Of those cells tagged with BFP, 99.5% were classified as displaying blue florescence with a minimal proportion categorised as green (0.3%) or non-fluorescent (0.2%).

Mixed fluorescent populations were then constructed by combining known numbers of GFP- and BFP-tagged cells of AWRI2804. Following classification by cell sorting, all of the cultures containing AWRI2804_natR::GFP and AWRI2804_natR::BFP at different proportions showed results within ±4% of the expected population ratios (Fig. 2C). This demonstrates the ability to discriminate between green and blue fluorescent cells by flow cytometry in mixed cultures and provides the means to accurately track the prevalence of two different cell populations growing in a competitive environment.

A mixed culture of AWRI2804_natR::GFP and AWRI2804_natR::BFP in a ratio 1:1 was then visualised with fluorescence microscopy (Fig. 3). Green and blue fluorescence signals were observed for different cells indicating minimal cross-fluorescence. A superimposed image enabled a clear identification of AWRI2804_natR::GFP and AWRI2804_natR::BFP cells (Fig. 3D). This indicates that the fluorescent DNA cassettes presented here can be successfully used to visualise *B. bruxellensis* cells by microscopy.

CONCLUSIONS

Genetic transformation cassettes tailored for *B. bruxellensis* significantly increased the transformation efficiency of this species. This higher efficiency combined with multiple drug-resistant markers and fluorescent proteins unlocks several molecular applications such as strain tagging, cellular localisation, gene overexpression and competitive growth assays, which were not available, or were impractical, using previously described techniques. We expect that this genetic transformation toolkit for *B. bruxellensis* will be a valuable resource for the yeast research community.

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Conflicts of interest. None declared.

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Massive Sequencing: A new tool for the control of alcoholic fermentation in wine?

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INFLUENCE OF NON-SACCHAROMYCES YEAST ON WINEMAKING AND QUALITY
Jéssica Lleixà Daga

Review

Massive Sequencing: A New Tool for the Control of Alcoholic Fermentation in Wine?

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Abstract: In wine industry, there is a prevalent use of starter cultures to promote a controlled and efficient alcoholic fermentation preventing the growth of spoilage microbes. However, current trends in enology aim to combine the guaranteed success of monitored process and the complexity of fermentations either by inoculating autochthonous starters or by performing spontaneously to produce distinctive wines. To understand the complex roles of microorganisms on wine fermentation, we must understand their population dynamics and their relationships with wine quality and metabolome. Current metagenomics techniques based on massive sequencing are gaining relevance to study the diversity and evolution of microbial population on every stage of the wine making process. This new tool and technique increases the throughput and sensitivity to study microbial communities. This review focuses on the current knowledge about wine alcoholic fermentation, the contribution of massive sequencing techniques and the possibility of using this tool for microbial control.

Keywords: next-generation-sequencing; alcoholic fermentation; bioinformatics; high-throughput sequencing; wine; microbiota; metagenomics

1. Introduction

Wine is an alcoholic beverage with great cultural and economic importance, which results from the alcoholic fermentation process. During this process, yeasts derive energy by consuming sugars that occur naturally in the grapes and at the same time produce ethanol and carbon dioxide as byproducts [1]. From the yeasts genera the most widely used, due to its fermentation capacities, is the yeast *Saccharomyces* [1], whereas non-*Saccharomyces* yeasts contribute to wine flavor, although they can also spoil wines [2–4].

In addition to the different fermentation techniques used currently by the industry, the characteristics of the wine also depend upon other factors such as climate, soil and grape variety where variation of these factors attribute to the distinctiveness of the wine [5]. Moreover, the interplay between the wine microbiota and the microbiota of the fermentation facilities has been verified but not completely understood [6]. Due to this complexity of interactions between microorganisms in the wine itself, during fermentation, but also between wine microbiota and environment, the wine industry has adopted the use of starter cultures as a mean of control and quality improvement [7]. Nevertheless, more in-depth knowledge in needed in order to understand how microbial interactions may affect the wine quality.

The diversity of the vineyard and grape microbiota has been long ago investigated via traditional microbiological techniques involving agar plate cultivation, microscopy and biochemical characterization, focusing primarily on identifying pathogenic microorganisms or microorganisms that have been associated with wine spoilage. Nevertheless, these techniques fail to identify unculturable microorganisms that comprise a considerable fraction of the wine microbiota [8,9].

Nowadays, molecular techniques such as qPCR (quantitative polymerase chain reaction) and PCR-DGEE (polymerase chain reaction denaturing gradient gel electrophoresis) are widely used for detection and monitoring of microbial communities in wine. The former technique is more appropriate for detection and monitoring of a desired microorganism, whereas the latter for microbial community profiling. Although both techniques are supplemented with culture-dependent methods, however PCR-DGEE fails to detect species in low abudance, and qPCR suffers from scalability problems when many strains should be targeted [10,11].

These drawbacks of the aforementioned culture-dependent and molecular techniques come to solve recent novel techniques that are based on massive sequencing, and which in recent years have been regarded as the tool of choice for studying microbial communities during the various stages of alcoholic fermentation. Although there have been encouraging findings demonstrating the superiority of the massive sequencing over the classical methods concerning speed, sensitivity and accuracy, however most of the research has been confined to describing the constituents microorganisms and their abundance fluctuation over time. Therefore, the aim of this review, apart from exhibiting the contribution of massive sequencing to monitoring alcoholic fermentation, is to demonstrate the possibility of using this method as a tool for microbial control.

2. Sequencing Methods

The metagenomic analysis of wine samples is mainly performed via amplicon-based sequencing which through marker-genes amplification facilitates the taxonomic and phylogenetic profiling of the microbiome [12]. After the Sanger sequencing, that was developed back in 1977 by Sanger et al. [13] and was considered the first generation sequencing, second (SGS) and third (TGS) generation sequencing, collectivelly referred as next-generation sequencing (NGS), have been introduced in research as fast and cost-effective solutions.

Despite the fact that TGS solves many of the disadvantages of the SGS, still is under development and not widely applied in research. From the area of SGS, which is based on "sequencing by synthesis" method, the most popular platforms will be presented.

2.1. Ion Torrent

Introduced back in 2010 [14], Ion Torrent sequences the template DNA strand by detecting hydrogen ions that are released during the polymerization process. As a technology, with an error rate of 1.71% [15], it does not require modified nucleotides and it generates reads of around 200 bp in length allowing for multiple runs and more data generation [16].

2.2. Pyrosequencing

The most recent variant of pyrosequencing, 454 pyrosequencing, was introduced back in 2005 [17], and was the first affordable platform allowing whole genome sequencing. As a technology, it relies on the light signal detection that is emitted after the release of phosphate during the incorporation of a nucleotide by the DNA polymerase. With an error rate below 1% [18] 454 pyrosequencing is capable of generating reads of over 400 bp in length [19].

2.3. Illumina

With the first Illumina sequencer being available back in 2006, Illumina technology is based on the usage of fluorescently labeled dNTP terminators and the detection of light signal upon incorporation. Recent Illumina machines, HiSeq and MiSeq, have decreased the error rate below 1% and are capable of generating reads of around 300 bp in length [20].

From the aforementioned platforms, Illumina is the most widely used, with 52% of the published research citing it, followed by pyrosequencing that holds 48% of the total citations [21]. However, pyrosequencing technology has been discontinued, and currently Illumina is being considered as the largest contributor to SGS.

3. Amplified Genomic Regions

Apart from choosing the most appropriate sequencing platform, researchers have to decide the genomic region that is going to be used for the taxonomic classification of the metagenomic wine sample. As far as bacteria are concerned, the 16S ribosomal RNA (rRNA) gene is the common target that is used in research for taxonomic assignment.

The 16S rRNA gene contains nine hypervariable regions (V1–V9), which all have been used as potential classification targets generating different results. For instance, Bokulich et al. [22] used the V4 and V5 domain so as to ascertain which one is the most taxonomically informative for profiling bacterial communities. Based on the results, the V4 domain was regarded as more suitable for profiling lactic acid bacteria (LAB), as it gave more taxonomic depth comparing to the V5 domain.

Campisano et al. [23] used a 700 bp region that includes the domains from V5 up to V9 in order to assess the impact of pest management on bacterial endophytic communities of Merlot and Chardonnay grapevines, with the results indicating abundance differences of operational taxonomic units (OTUs) between organic and intergrated pest management (IPM) grapevines. The same genomic region was also targeted by Perazzolli et al. [24] in a study of leaf microbiota, that resulted in identification of beneficial microbial communities that could be used as a tool for crop protection. In the same manner, in the past years other researchers have been focusing on other domains for classification purposes. For instance, Sundquist et al. [25] favored the domains V1,V2 and V4, Liu et al. [26] the domains V2, V3 and V4, and Chakravorty et al. [27] the domains V2 and V3.

Regarding fungal classification, researchers have also displayed variability concerning genomic region preference. For instance, David et al. [28] sequenced the 18S rRNA gene to show that 454 pyrosequencing is much more reliable than classical techniques for studying yeast communities in alcoholic fermentation. Holland et al. [29] pyrosequenced the D1–D2 regions of the 26S rRNA, demonstrating that changes in arbuscular mycorrhizal fungal communities do not depend on irrigation frequency. Bokulich and Mills [30] targeted the IT1, ITS2 and the whole ITS in order to compare their classification efficiency by utilizing a mock community. Although they favored the IT1 region, nevertheless they urged for caution as none of these regions reconstructed reliably the whole mock community. Encouraging results targeting the ITS region have been yielded also from the researches of Pinto et al. [31] and Stefanini et al. [32] indicating this region as a suitable target for yeast classification.

4. Bioinformatic Tools

Regardless the NGS platform a researcher decides to utilize, the sequencing of wine metagenomic samples generates a significant amount of data that necessitate the use of bioinformatic pipelines. Despite the plethora of bioinformatic tools available, the most widely used will be presented.

4.1. QIIME

QIIME, which stands for Quantitative Insights Into Microbial Ecology, is a bioinformatic package, offering a variety of microbial community analyses and visualizations, that wraps other software packages with python code [33]. Some of the most frequent wrapped applications include mothur [34], blast [35], PyNAST (Python Nearest Alignment Space Termination) [36], RDP (Ribosomal Database Project) Classifier [37], FastTree [38] and USEARCH (unique word count search) [39].

4.2. MOTHUR

Mothur is a bioinformatic package that re-implements in C and C^{++} code other software packages removing that way any external dependecies during installation. Some of the re-implemented algorithms include DOTUR (Distance-Based OTU and Richness), SONS (Shared OTUs and Similarity), TreeClimber, LIBSHUFF, and UniFrac, and additioanlly the mothur team has incorporated its own analytical features to the platform [34].

4.3. MG-RAST

MG-RAST, which stands for Metagenomics Rapid Annotation using Subsystem Technology, is a server based platform with initial aim the annotation of complete or draft microbial genomes [40]. Currently, MG-RAST offers an automated solution for phylogenetic classification and functional classification of metagenomic samples.

A comparison of these three bioinformatic pipelines has been conducted by Plummer et al. [41] using 16S rRNA gut microbial data. The study concluded that all of the three pipelines were able to generate similar and reliable results with common limitation the ability to classify at the species level due to the type of data. The main differences between the pipelines concerned the usability and duration of analysis. MG-RAST is a more user friendly pipeline compared to the command-line based QIIME and MOTHUR, whereas QIIME required approximately 1 h to complete the analysis with MOTHUR and MG-RAST 10 h and 2 days respectively.

5. Databases

One of the most crucial steps of metagenomic analysis is the taxonomic classification of the microbial community. Apart from other factors, such as the sequence length, the parameters used for quality filtering and the implemented algorithm, this step can be greatly influenced by the chosen database. Currently, there are a number of highly curated databases available, such as Greengenes for 16S rRNA [42], SILVA for small (16S/18S, SSU) and large (23S/28S, LSU) subunit rRNA [43], UNITE for ITS region [44] and RDP for 16S and 28S rRNA classification [45]. However, classification based on these databases should be regarded as a rough estimation of the microbial composition as genera abundances or even taxonomic assignments can be greatly influenced by the chosen percentage of homology.

6. Analysis of Alcoholic Fermentation

There are numerous studies dedicated to the microbial analysis of wine alcoholic fermentation, but until now great focus has been given on describing microbial abundance succession during the various stages of alcoholic fermentation. These studies have attested the superiority of NGS over classical methods [28] and offered novel insights into the microbial communities.

Although bacteria are not directly connected to wine quality, acetic acid bacteria (AAB) and lactic acid bacteria (LAB) play a significant role to the final wine product. Portillo and Mas [46], in a Grenache variety wine fermentation study, showed that AAB and LAB are more abundant than previously thought, with a dominance of *Gluconobacter* during the mid fermentation. The latter finding contradicts the previous notion that *Gluconobacter*, being alcohol sensitive, usually declines during the alcoholic fermentation [47–49]. Similar results have also been yielded in other studies of low-sulfited or unsulfited wine fermentations [50].

Additionally, NGS analysis has created the notion that apart from AAB, other bacteria, not previously described, may be present during the process. Support to this hypothesis came from Godálová et al. [51] in a study of Blaufränkisch and Grüner Veltliner vines, where in addition to genera already found in other studies, such as *Sphingomonas*, *Variovorax*, *Pantoea*, *Enterobacter* and *Tatumella*, new genera were detected, namely *Amycolatopsis*, *Hydrogenophilus*, *Snodgrassella*, *Telluria*, *Gilliamella*, *Lelliottia*, and *Lonsdale quercina*. However, the possible impact of these newly described genera is still to be demonstrated.

Other studies come to supplement existing ones. For instance, Bokulich et al. [22] showed that *Acetobacter*, *Gluconobacter*, and *Gluconoacetobacter* are dominant in winemaking processes, whereas Campanaro et al. [52] in a grape marc study added that *Gluconobacter* and *Gluconoacetobacter* do not survive a prolonged grape marc storage period.

Moreover, the empirically based concept of the coined term "terroir", that is distinction of wine quality due to regional features, has been put under the prism of NGS analysis and verified recently.

Results from Zarraonaindia et al. [53] suggested that the soil serves as a bacterial reservoir for the vines and subsequently Bokulich et al. [54], in a 200 commercial wine fermentations study, demonstrated the correlation of wine microbiota, wine performance and wine metabolome. These authors even predicted the metabolome of the wine from the microbial composition by using machine learnig techniques [54]. Similar results have also been generated from other studies [55,56].

Besides bacteria, NGS analysis has also given significant insights into the yeast population during fermentation. The most frequent fungi described by NGS analysis are *Saccharomyces*, *Hanseniaspora*, *Issatchenkia*, *Rhodotorula*, *Penicillium*, *Cladosporium*, *Botrytis*, *Sporobolomyces*, *Aspergillus*, *Cryptococcus* and *Pichia* [56–58], with most studies reporting high abundance of *Hanseniaspora* and *Saccharomyces* during the mid and end of the fermentation respectively. Stefanini et al. [32] in a Vino Santo study, found that fungal species composition undergoes a dynamic change with a declining tendency overtime, and that small changes in fermentation procedures may result in significant differences in microbial communities. As advocates to these findings come older studies that have demonstrated that aerobic yeasts are the first to decrease in abundance, and that the mid fermentation yeast genera, such as *Hanseniaspora*, *Candida*, *Metschnikowia* and *Torulaspora*, cannot be not recovered on plates at high ethanol concentration in presence of *Saccharomyces* [59,60]. Interestingly, *S. cerevisiae*, found in very low abundance at the beginning of the fermentation, manages to rise in dominance at the end of the it. In accordance to this, Lleixà et al. [61] drew a comparison between the dynamics of *Saccharomyces cerevisiae* and *Hanseniaspora vineae* after inoculation in Macabeo and Merlot grape varieties. The results indicated that fermentation of *S. cerevisiae* inoculated must was faster than the one with *H. vineae* inoculation, and that inoculation with *S. cerevisiae* is necessary as *H. vineae* alone leads to incomplete alcoholic fermentation. However *H. vineae* was able to dominate the microbiota in Macabeo must but not the Merlot perhaps due to high exhibited yeast diversity of Merlot must.

Another important question that NGS analysis has been called to answer, is whether grapes are the source of spoilage microorganisms [62], or the wine-making equipment [63]. Even though there is no clear answer to this debate, studies from Suárez et al. [64] and Pinto et al. [56] seem to support the latter hypothesis.

7. Control of Alcoholic Fermentation

Controlling the alcoholic fermentation of wine-making is a very complex process. Unlike fed-batch alcoholic fermentation in bioreactors, where algorithms have been developed for the estimation of parameters that may lead to higher biomass concentrations and yield of a specific compound [65], wine alcoholic fermentation incorporates higher order of complexity, as it concerns (i) the determination of all the microbial composition throughout the fermentation process; (ii) the comprehension of the interplay between different microbial communities; (iii) the definition of a series of metabolites that contribute to the wine quality, and (iv) the integration of all these information into a predictive machine-learning model.

In the past, a series of studies have set the ground for controlling alcoholic fermentation by monitoring or modifying certain fermentation parameters, but most of the results were empirical and their interpretation was not an easy task. Various studies have shown that yeasts increase their production of volatile compounds at low fermentation temperatures [66–68]. Therefore, wine-makers that aim at enhancing wine aroma could take advantage of this factor. Another popular method, is choosing a specific yeast strain for improving specific aspects of the wine, with studies having used this technique so as to improve wine characteristics of Sauvignon [69] and Chardonnay [70]. Furthermore, addition of certain nutrients that will prevent the fermentation from stucking, is a common practice. For instance, Cramer et al. [71] developed a fermentation kinetic model which showed that fermentation rate can be increased upon addition of ammonium salts, whereas Birch et al. [72] supported that yeast growth rate and sugar degradation could be influenced by magnesium concentrations. On the other hand, adaptive evolution approaches are aiming towards the creation of non-recombinant yeast strains that could modify wine characteristics, as for instance in

the study of McBryde et al. [73]. Additionally non-*S. cerevisiae* yeasts are known of adding distinct flavors to the wine but due to the fact that they can easily become replaced by *S. cerevisiae*, authors such as Soden et al. [74] have suggested the use of mixed cultures controlled by sequential inoculation.

Although all the above practices are means of manipulating specific aspects of wine fermentation towards a specific outcome, they treat alcoholic fermentation as a black-box without controlling the microbial composition of the wine and consequently the wine quality consistency they are aiming to provide may not be certain. NGS analysis is aiming to tackle these obstacles, but as a relatively new approach so far has yielded descriptive results on the bacteria and yeast genera abundances that have been encountered during the various fermentation stages. Until now, studies from the food industry have already evinced this type of analysis as a promising strategy for the detection of previously undescribed spoiler bacteria [75,76], underlying its suitability for controlling alcoholic fermentation. Nevertheless, NGS analysis has as an intrinsic difficulty the overwhelming amount of metagenomic analysis tools, machine-learning algorithms, databases and parameters that the researcher has to choose from. Because small changes of parameters may result in singificantly altered taxonomic assignment results [26], a possible solution may come from the use of mock communities datasets with known species compositions [77]. This strategy has already been implemented in studies such as the one by Bokulich et al. [78] in order to compare the performance of different classifiers. Even though mock communities datasets cannot lead to the development of a standardized NGS analysis with fixed parameters, as metagenomic samples are bound to laboratory protocols, NGS platforms, environmental and grape variety differences, they may nonetheless serve as a way to validate the robustness of a bioinformatic pipeline or as a starting point for the subsequent metagenomic analysis.

Setting a solid ground for metagenomic analysis is of paramount importance, so additional analyses such as metatranscriptomics and metabolomics can function as determinant factors for the development of system-biology networks aiming for the understanding of microbial communities interaction, and machine-learning prediction models focusing on the quality of the final wine product. With encouraging results coming from studies such as the one by Bokulich et al. [54] where it has been demonstrated that microbial composition of grape must can predict wine metabolome, the future of controlling alcoholic fermentation via NGS analysis seems nothing but promising.

8. Conclusions

The aim of this review is to cite contemporary contributions of massive sequencing techniques to wine alcoholic fermentation, and the possibility of being used as a tool for microbial control. Wine alcoholic fermentation is a complex process that encompasses an intricate and dynamic interaction between microbial populations that leads towards the composition of a wine metabolome that defines the final wine quality and characteristics.

As a way of controlling alcoholic fermentation, the industry has adopted various techniques, such as starter cultures and process monitoring and modification, but these approaches rely on empirical results as little is known about the relationships within the wine microbiome and its correlation to the final wine product.

High-throughput sequencing, based on NGS platforms, has been presented as a metagenomic analysis tool that offers higher speed, accuracy and taxonomic resolution compared to classical culture-dependent and molecular techniques. Till now, the implementation of this technology has yielded significant yet descriptive research results on microbial dynamics in connection to the fermentation stages. Although, NGS metagenomic analysis comprises a vast amount of bioinformatic tools, databases and machine-learning algorithms, however publicly available mock communities datasets may serve as ways of algorithm benchmarking, robustness check of bioinformatic pipelines, and parameters initialization.

These mock communities and highly curated taxonomic databases could set a solid foundation for the metagenomic analysis, upon which metatranscriptomics and metabolomics will be based and provide all the necessary knowledge for the development of system-biology networks and

prediction models for deciphering microbial population dynamics and prediction of final wine product, correspondingly. Regarding the latter, research has provided encouraging results highlighting the potential and benefits of massive sequencing as a tool for controlling alcoholic fermentation.

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Aplicación de técnicas de secuenciación de nueva generación para la detección de *Brettanomyces* y otros contaminantes microbiológicos en el vino de crianza y durante el embotellado

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Las contaminaciones microbiológicas del vino son muy perjudiciales por las considerables pérdidas económicas que representan para el sector vitivinícola y porque, si no son detectadas a tiempo, repercuten gravemente sobre la reputación y economía de la bodega productora. Esta situación es aún más grave en el caso de los vinos de crianza por el valor añadido de los mismos.

Brettanomyces bruxellensis (fig. 1) ha sido caracterizada históricamente como el principal agente responsable de la formación de fenoles volátiles como el 4-etilfenol, 4-etilguaiacol y tetrahidropiridinas, que producen aromas desagradables que alteran el vino sobre todo en las últimas etapas. Aunque no es necesariamente muy abundante, *Brettanomyces* se ha detectado en la uva y en el equipo de la bodega,[1] y prolifera durante la crianza del vino y embotellado donde las poblaciones aumentan de manera lenta pero sin competencia, originando los principales riesgos.

Figura 1. Imagen al microscopio de un cultivo de *Brettanomyces bruxelensis*

Es destacable señalar que es muy probable que ninguna región vitivinícola esté libre de este microorganismo y que no hace falta un gran número de células de *Brettanomyces* para desarrollar la alteración, ya que por encima de 1000 células/mL la calidad organoléptica del vino está seriamente comprometida. Sin embargo, la relación presencia de *Brettanomyces*-deterioro del vino no es siempre evidente. En algunos estudios de nuestro grupo de investigación se han detectado especies alterantes como *Brettanomyces* tanto en vinos deteriorados como en vinos sin ninguna alteración aparente.[2]

Entre los motivos de su proliferación se encuentran:

- un estado sanitario deficitario de la uva de partida con lo que la concentración de levaduras iniciales sería inaceptablemente elevada;
- etapas prefermentativas, aunque se realicen a baja temperatura, ya que el crecimiento de *B. bruxellensis* solo se ve inhibido por debajo de 8ºC;
- exceso de nutrición durante la fermentación que produzca azucares residuales o aminoácidos y sales amoniacales que la levadura podrá usar para su crecimiento después de la fermentación;
- una relación inadecuada entre pH y contenido de sulfuroso, puesto que los pH elevados disminuyen la cantidad de SO_2 molecular, bajando así su efecto antimicrobiano;

- una limpieza inadecuada de las barricas;
- presencia de oxígeno que estimule la producción de acidez volátil.

Detección en bodega

La detección en bodega de *Brettanomyces* se suele realizar mediante análisis sensoriales o de cromatografía gaseosa de los vinos en crianza para detectar la presencia de 4 etilfenol/ guayacol. Sin embargo, este método suele ser confirmativo, pues cuando se detecta el compuesto implica que la población de esta levadura es bastante elevada con lo que reparar la alteración resulta muy complicado.

Otros métodos de detección se basan en el empleo de medios de cultivo, como el DBDM,[3] con agentes selectivos y diferenciales para evitar el crecimiento de otros microorganismos como levaduras, bacterias lácticas, etc., que poseen tasas de crecimiento mayor. Por otro lado, es necesario tratar volúmenes elevados de vino porque las poblaciones de *Brettanomyces* que suponen riesgo en vino se han determinado en 10 UFC/mL y el crecimiento lento de esta levadura hace que los tiempos de incubación sean de 8-10 días, posponiendo así la detección del problema.

Además, otro factor que complica su detección en el vino es la presencia de células en estado viable pero no cultivable (VPNC),4 efecto que viene dado por la adaptación de estos microorganismos a condiciones extremas del vino (concentraciones de etanol, polifenoles, SO_2, ausencia de oxígeno, etc.). El fenotipo de VPNC se caracteriza por la incapacidad de las células de crecer en medio de cultivo aunque mantengan su actividad metabólica o celular de forma que pueden volver a ser cultivables cuando las condiciones ambientales sean favorables. Este fenómeno podría inducir a falsos negativos o infravaloración en placa de vinos con una considerable concentración de la levadura en este estado fisiológico, lo que supondría un riesgo para el vino analizado.[2,4]

Ventajas de las nuevas técnicas moleculares

Recientemente, la utilización de técnicas moleculares basadas en el material genético supone una ventaja respecto a las técnicas clásicas basadas en el cultivo. Las técnicas moleculares utilizadas incluyen análisis como la restricción del DNA mitocondrial,[5] PCR-RFLP,[6,7] PCR-RAPD,[5] PCR con cebadores específicos[8] y PCR anidada.[9]

Sin embargo, muchas de estas técnicas necesitan un paso de enriquecimiento previo para extraer el DNA de forma que son técnicas semicuantitativas. Por ello, se está tendiendo al uso de qPCR para la detección específica y cuantificación de *B. bruxellensis* directamente del vino. Aunque estas técnicas reducen el tiempo de análisis con respecto a las técnicas de cultivo y detectan incluso las células VPNC, al usar el DNA se detectan tanto células vivas como muertas, pudiendo dar lugar a una sobreestimación del número de levaduras en la muestra.[2]

Gracias a la introducción del bromuro monoazódico de etidio (EMA) o bromuro moazódico de propidio (PMB) combinados con la qPCR se han podido detectar únicamente las células vivas.[2] Sin embargo, es necesario optimizar la concentración de EMA para ensayos diferentes y la concentración de etanol afecta los resultados.[2]

El RNA es considerado como un buen indicador de viabilidad ya que se degrada más rápidamente que el DNA[10] aunque también es dependiente del gen usado para la detección. El problema principal de la qPCR a partir tanto de DNA como de RNA es que la qPCR solo detecta y cuantifica los microorganismos diana para los cuales hayamos diseñado cebadores de amplificación específicos y se desconoce si dichos microorganismos son los únicos implicados en el deterioro del vino.

En la actualidad, el estudio de la diversidad microbiana puede realizarse mediante una nueva técnica molecular denominada *secuenciación masiva o HTS* (de sus siglas en inglés *high throughput sequencing).*Consiste en la secuenciación de miles de secuencias por cada muestra tras la extracción directa de ácidos nucleicos de la matriz que se esté estudiando, en nuestro caso el vino. Existen diferentes tecnologías de secuenciación masiva[11] cada una con sus ventajas y desventajas.[12,13]

El estudio de la composición microbiana se realiza de forma habitual mediante la amplificación de genes de interés taxonómico (normalmente el gen *rRNA 16S* para bacterias y el gen de *rRNA 18S* o el *ITS* para hongos) y puede ofrecer la proporción de los distintos grupos taxonómicos dentro de un alimento mediante la secuenciación de estos genes y su comparación con las bases de datos de referencia que proporcionarán la identificación de las distintas secuencias con lo que, además, tiene carácter cuantitativo.

En los últimos años, la secuenciación masiva se ha aplicado en prácticamente todos los campos de investigación de microbiología incluidos estudios sobre alimentos aunque el coste de estos análisis y el requerimiento de habilidades específicas bioinformáticas aún limitan su aplicación industrial.

Muchos de estos estudios tenían un marcado carácter de ecología microbiana (revisado en Ercolini).[14] Por ejemplo, en el ámbito del vino se ha utilizado esta técnica para describir el microbioma del viñedo[15-19] y las bodegas e instalaciones.[15] Zarraonaindia *et al.*[18] demuestran que la microbiota de la vid viene influenciada por las bacterias del suelo en el que se encuentran, las cuales son una importante fuente para las bacterias encontradas en la planta y uva. En esta misma línea, Portillo *et al.*[19] demuestran que no solo la variedad de uva y el viñedo influyen en la microbiota residente en los racimos, sino que, además, factores como la orientación geográfica del viñedo marcan las diferencias en la composición bacteriana de las uvas dentro de la denominación de origen del Priorat (fig. 2).

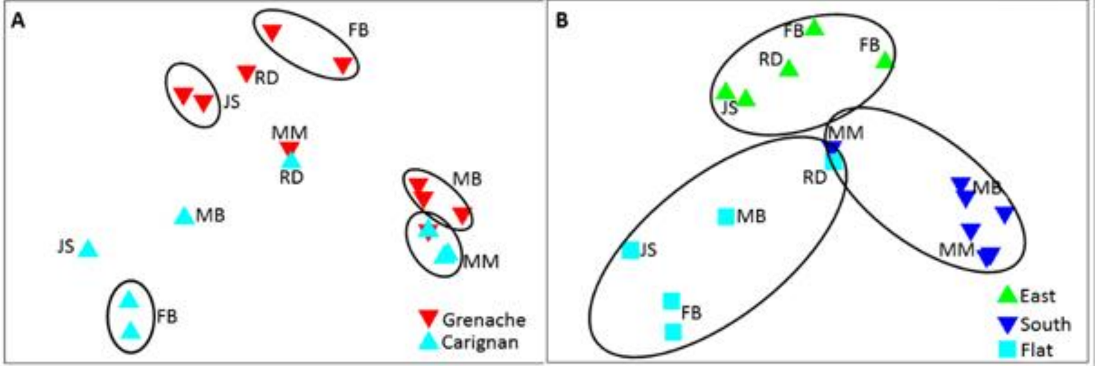

Figura 2. Gráfica de escalado multidimensional (EMD) de las distancias Unifrac (matrices de distancia entre muestras generadas tanto con la composición taxonómica de cada muestra como con la abundancia de cada OTU) entre las muestras de mostos de garnacha y cariñena pertenecientes a cinco viñedos del Priorat, FB: Ferrer Bobet; MM: Mas Martinet; JS: Jaume Sabaté; RD: Roca las Dotze; MB: Mas Botó. **(A)** Agrupamiento de las comunidades bacterianas según la variedad con las réplicas de muestras incluidas en círculos negros (R_{ANOSIM}= 0,191; *P*=0,005). **(B)** Agrupamiento de las muestras de mostos de garnacha y cariñena según la orientación geográfica de los viñedos (R_{ANOSIM}= 0,84; *P*=0,001).

Brettanomyces no ha sido detectada entre las cientos de especies descritas como componentes del microbioma de la vid, mientras que *Saccharomyces cerevisiae* sí fue detectada, aunque poco

Por lo tanto, la identificación de los nichos naturales donde *B. bruxellensis* se encuentra es uno de los retos que limitan nuestro entendimiento sobre su biología y cómo se ha dispersado globalmente. Podríamos esperar que con el descenso en los costes de secuenciación próximamente se pueda revelar la presencia de esta especie fuera del ambiente de las bodegas.

Las técnicas de HTS también se han aplicado ampliamente al estudio de la fermentación de alimentos o su deterioro microbiano[14] y, recientemente, se ha puesto de manifiesto la capacidad y potencial de estas técnicas de HTS para poder detectar contaminaciones en alimentos y su posible trazabilidad en el entorno en el cual se procesan dichos alimentos.[20] Hay que recordar que en muchos alimentos existen microorganismos pertenecientes al mismo género y en estos casos los estudios de HTS basados en secuencias muy cortas a nivel de género no serían de utilidad para diferenciar entre dichas especies. En estos casos, para obtener información a nivel de especie habría que tener como diana fragmentos más largos incluyendo más regiones variables de los genes taxonómicos o bien, complementar la técnica de HTS con alguna técnica de tipificación de especies como la RFLP (por ejemplo, Bokulich *et al.*[21]).

Hacia un mapa mundial de perfiles microbiológicos en regiones vitivinícolas

Un ejemplo de aplicación de HTS en el mundo comercial lo constituye Biome Makers, una empresa fundada por españoles con sede en San Francisco que ha apostado por introducir las técnicas genómicas en el mundo del vino. Actualmente ofrecen el estudio de suelo de viñedos que permite crear un mapa mundial de los perfiles microbiológicos en las diferentes regionales de vino y ver su evolución a lo largo de los años, con el objetivo de descifrar la influencia del suelo tanto en el proceso de vinificación como en el tipo de variedad de uva.

Esta empresa ha sido seleccionada por el primer programa de aceleración de empresas biotecnológicas en el área de la genética existente a escala mundial, promovido por la multinacional Ilumina (empresa líder en tecnología de secuenciación masiva).

La ventaja indudable que las técnicas HTS aportarían al estudio del deterioro en el vino es que, al obtener miles de secuencias para una única muestra, se puede tener una descripción detallada de todos los microorganismos presentes en el deterioro y el cambio poblacional previo a la proliferación de un determinado microorganismo responsable de la alteración, con lo que podría tener carácter predictivo.

Además, las técnicas HTS pueden procesar cientos de muestras simultáneamente y se pueden realizar tanto a partir de DNA como de RNA, con lo que se podría tener información tanto de los microorganismos presentes, como los metabólicamente activos en el momento del deterioro, respectivamente. Por otro lado, mediante la metatranscriptómica (secuenciación masiva de todos los genes que se están transcribiendo en un determinado momento) se podría tener información de las interacciones metabólicas entre los distintos microorganismos implicados en el deterioro del vino. Todas estas técnicas basadas en secuenciación masiva, sin duda ofrecen una oportunidad para un estudio más detallado de los microbios responsables de la fermentación del vino y en su caso, de su posible deterioro.

Aunque las técnicas HTS no sean aplicables a corto plazo por las bodegas como rutina debido a los elevados costes de inversión en la tecnología necesaria y el grado de conocimiento específico que se necesita para realizar dicho análisis e interpretación de datos, sí que se pueden desarrollar servicios a bodegas, en la línea de la compañía Biome Makers ya citada, para que realicen análisis periódicos de poblaciones en vinos de crianza y así poder prevenir posibles contaminaciones microbianas.

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