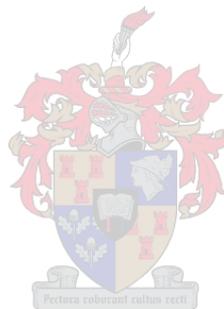


Comparative analysis of fermentative yeasts during spontaneous fermentation of grapes from different management systems

by

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01/03/2014

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Summary

The microorganisms associated with grape berry surface can be influenced by numerous factors such as agronomic parameters. Hence, the focus of this study was comparison between three agronomic farming systems to evaluate their impact on yeast diversity. In addition, the dynamics of the yeast population throughout wine alcoholic fermentation were monitored. Three vineyards (conventional, biodynamic and integrated) were chosen and the experiment was carried out during the 2012 and 2013 vintages. A total of 600 yeast isolates including *Saccharomyces* and non-*Saccharomyces* were obtained from grape must and during different stages of fermentation including beginning, middle and end of alcoholic fermentation, from all three vineyards. Yeast species diversity in grape must and their population dynamics were evaluated by cultivating the yeasts in nutrient media and using "Polymerase Chain Reaction and sequence analysis of the ITS1-5.8S rRNA-ITS2 region. Eight, four and one species were detected from biodynamic, conventional and integrated must in 2012 vintage whereas, 2013 vintage displayed a higher diversity and 12, 11 and 9 different species were identified from biodynamic, conventional and integrated vineyard, respectively. *Aureobasidium pullulans* was the most frequent isolate in all three vineyards whereas *Saccharomyces cerevisiae* was below detection level in grape must and was only isolated in low frequencies in biodynamic must (3% of the total population) in both vintages. In general, the overlap of common yeast isolates (e.g. *M. pulcherrima* and *H. uvarum*) was observed in the musts obtained from different vineyards although unique minor species could be isolated and clearly demonstrated the distinction between the three vineyards. Moreover, biodynamic must displayed a higher degree of diversity in both 2012 and 2013 compared to the conventional and integrated vineyards. The beginning of all spontaneous fermentations was dominated by non-*Saccharomyces* yeast species (e.g. *H. uvarum*, *C. zemplinina*), as the fermentation proceeded, the population of non-*Saccharomyces* species were gradually decreased and strongly fermentative yeast *S. cerevisiae* dominated and completed the fermentations. The dynamics of *S. cerevisiae* strains was also evaluated during different stages of fermentation (beginning, middle and end), using interdelta PCR methods. A high diversity (10-18 strains per fermentation) and the sequential substitution of *S. cerevisiae* strains were observed throughout spontaneous fermentations. In addition, integrated vineyard displayed the highest *S. cerevisiae* strains compared to biodynamic and conventional vineyard.

Opsomming

Die mikro-organismes wat met die oppervlak van druiwe bessies geassosieer word kan deur verskeie agronomiese faktore beïnvloed word. Gevolglik was die fokus van die studie om 'n vergelyking tussen die impak van drie verskillende boerdery sisteme op die invloed op gis diversiteit te bepaal. Die dinamiek van gis populasies tydens alkoholiese fermentasie is bykomstig bestudeer. Drie verskillende wingerde (konvensioneel, biodinamies en geïntegreerd) is gebruik vir die studie tydens die 2012 en 2013 oesjare. In total is 600 gis isolate, insluitend *Saccharomyces* en nie-*Saccharomyces* giste, verky van druiwe mos tydens verskillende fases van die fermentasie proses (begin, middle en einde) vir al drie wingerde. Die diversiteit en populasie dinamika van gis spesies in die druiwe mos is geëvalueer deur die giste in verskillende media op te groei en ook deur die gebruik van die "polymerase ketting reaksie" (PKR) en DNS volgorde bepaling van die ITS1-5.8S rRNA-ITS2 gebied. Tydens die 2012 oesjaar is agt, vier en een afsonderlike spesies geïsoleer, in vergelyking met die 12, 11 en 9 verskillende spesies wat tydens 2013 geïdentifiseer is uit die biodinamiese, konvensionele en geïntegreerde onderskeidelik. *Aureobasidium pullulans* is teen die hoogste frekwensie geïsoleer in al drie wingerde, terwyl *Saccharomyces cerevisiae* onder die deteksie limiet was in druiwe mos en ook slegs in lae getalle in die biodinamiese mos (3% van die totale populasie) in beide oesjare. Oor die algemeen is 'n oorvleueling tussen verwante spesies (bv. *M. pulcherrima* en *H. uvarum*) waargeneem en die mos vanaf verskillende wingerde, terwyl meer geringe spesies deurgans geïsoleer kon word en duidelik 'n verkill tussen die drie wingerde uitgewys het. Druiwe mos uit die biodinamiese wingerd het verder 'n hoër graad van diversiteit en beide 2012 en 2013 vertoon as beide die konvensionele en geïntegreerde wingerde. Die begin van alle spontane fermentasies was gedomineer deur die populasie van nie-*Saccharomyces* gis spesies (bv. *H. uvarum*, *C. zemplinina*), wat geleidelik afgeneem het met die verloop van die fermentasie. Die populasie van die sterk fermentatiewe, *S. cerevisiae*, het toegeneem tydens fermentasie en die fermentasie afgehandel as dominante gis. Die dinamika van *S. cerevisiae* rasse is ook geëvalueer tydens die verskillende fases van fermentasie (begin, middle en einde) deur gebruik te maak van interdelta PKR metodes. 'n Hoë diversiteit (10-18 rasse per fermentasie) en die opeenvolgende verplasing van *S. cerevisiae* rasse was waargeneem deur die verloop van spontane fermentasies. Daarbenewens het die geïntegreerde wingerd die grootste getal *S. cerevisiae* rasse in vergelyking met die biodinamiese en konvensionele wingerde opgelewer.

This thesis is dedicated to my parents and my family for their love, supports and inspiration.

Biographical sketch

Bahareh Bagheri was born on 21 March 1982. Bahareh enrolled at the Azad University of Varamin in 2002 and obtained her BSc-degree (Food Science) in 2006. Bahareh, joined the food industry in 2006 and enrolled for a MSc-degree in Wine Biotechnology at Stellenbosch University in 2012.

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Preface

This thesis is presented as a compilation of 4 (four) chapters. Each chapter is introduced separately and is written according to the style of the journal International Journal of Food Microbiology to which Chapter 3 (three) is submitted for publication.

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Chapter 1

Introduction and project aims

1.1 Introduction

Grapes are a source of the microorganisms that constitute the wine microbial consortium and mediate the biochemical process that convert grape juice to wine. Wine is therefore a product of complex interactions between common grape microorganisms including, yeasts, bacteria and filamentous fungi (Combina et al., 2005; Renouf et al., 2005; Barata et al., 2012; Milanović et al., 2013). Yeasts, including *Saccharomyces* and non-*Saccharomyces* species are the main agents that perform alcoholic fermentation in winemaking. Several studies have shown that the strongly fermentative yeast, *Saccharomyces cerevisiae* is the dominant species during alcoholic fermentation due to its high fermentative capacity and high resistance to ethanol. However, weakly fermentative non-*Saccharomyces* yeasts have also been shown to contribute in the pre-fermentation and the initial stage of alcoholic fermentation (Tello et al., 2011, Bezerra-Bussoli et al., 2013; Barata et al., 2011). Moreover, several studies, consecutively demonstrated the impacts of yeasts on fermentation speed, wine flavour and wine quality (Longo and Vezinhet, (1993); Querol and Barrio, (1990); Fleet and Heard et al., (1993); Di Maro et al., 2007).

The density and diversity of yeasts on the grape berry surface is affected by numerous factors such as, grape variety (Cordero-Bueso et al., 2011), grape health (Loureiro and Malfeito-Ferreira, 2003; Barata et al., 2008), grape ripeness (Martins et al., 2012), climatic condition and geographic location (Bezerra-Bussoli et al., 2013), application of different chemicals (Milanović et al., 2013), application of different oenological practices (Andorrà et al., 2008) and also application of different farming systems (Cordero-Bueso et al., 2011; Martins et al., 2012; Setati et al., 2012). In general, grapes used for wine fermentation, can be obtained through different farming systems comprising, organic, conventional, biodynamic and integrated approaches. Conventional farming systems were the prevalent farming systems in the twentieth century. Conventional viticulture uses synthetic pesticides, fungicides and fertilizers. Integrated pest management system (IPM) was established in 1970. The use of organic fertilizers is encouraged in this system. However, the use of synthetic fertilizers and pesticides with careful monitoring is allowed. In South Africa, grapes are mainly produced through integrated production of wine (IPW), established by the South African wine industry in 1998 (http://www.wosa.co.za/sa/sustainable_ipw.php). This method promotes the use of biological strategies such as bait and ducks for pest control rather than chemical options. To the contrary, the use of synthetic fertilizers and pesticides are strictly banned in organic viticulture which is one of the best examples of environmentally friendly agriculture. Biodynamic viticulture is an early scheme of organic viticulture which typically uses the natural fertilizers and pesticides under the Demeter regulation is encouraged in this farming practice (www.demeter-usa.org/downloads/Demeter-Farm-Standard.pdf).

Wine has traditionally been produced through spontaneous fermentation, which is characterized by successional development of indigenous yeast species without addition of any starter

culture. This form of fermentation is thought to result in complex, unpredictable wine due to the interactions between the indigenous non-*Saccharomyces* species and different *Saccharomyces* strains during the fermentation (Tello et al., 2011). However, most winemakers rely on the production of wine through inoculated fermentation by using commercial selected *S. cerevisiae* strains as monocultures or in mixed fermentations with non-*Saccharomyces* species. Although the wine produced through inoculated fermentation is more reliable, it is worth mentioning that this method results in less participation of indigenous non-*Saccharomyces* and *Saccharomyces* strains due to the fast dominance of commercial *S. cerevisiae* strains (Beltran et al., 2002). The initial stage of spontaneous fermentation is mainly dominated by the non-*Saccharomyces* species and as the fermentation proceeds, the population of non-*Saccharomyces* species gradually declines due to their sensitivity to anaerobic condition and high ethanol concentration (over 5-7% ethanol). On the other hand, the ethanol tolerant yeast, *S. cerevisiae* dominate and complete the fermentation (Combina et al., 2005; Di Maro et al., 2007; Settanni et al., 2012). Although several studies have been performed on spontaneous fermentation, there is still the lack of comprehensive information about the impacts of farming systems on yeast diversity on grapes and the dynamics of such yeasts during the spontaneous fermentation due to the poor sampling strategies. In most of the previous studies, sampling was limited to the initial and middle stages of fermentation while it has been demonstrated that some non-*Saccharomyces* species (e.g. *H. uvarum*, *T. delbrueckii*) can persist until the final stage of fermentation (Jemec et al., 2001, Tello et al., 2011). Therefore, tracking the dynamics of ethanol tolerant non-*Saccharomyces* species was impossible. The population dynamics was only monitored in the beginning, middle, and after consumption of 70 g/L sugar which might have resulted in loss in yeasts diversity. Recent works (Tello et al., 2011, Cordero-Bueso et al., 2011, Milanović et al., 2013) focused on the differences obtained from yeast communities associated with grape must and fermentations in organic, conventional farming systems demonstrated that organic farming systems display higher biodiversity. In addition, Pancher et al. (2012) demonstrated that fungal endophytic communities in grapevines from organic farming system were different from those associated with grapevines in farming system that use integrated pest management (IPM) systems. In addition, Setati et al. (2012) revealed that although conventional, biodynamic and IPM farming systems contained certain common yeasts, of the were sufficient minor unique species in each farming system that allowed for a clear distinction between the three systems. These studies reported the organic type farming systems to have higher yeast diversity than conventional farming systems. However, such consensus has not been demonstrated when fermentative yeasts in grape must and wine were evaluated. Therefore, the further investigation on the impacts of farming systems on yeast diversity in grape must and monitoring the yeasts dynamics throughout alcoholic fermentation is still necessary.

1.2 Project aims

Nowadays a number of viticultural and winemaking practices are being investigated to improve wine quality. However, the impact of such practices on yeast diversity and dynamics is often neglected. Consequently, there is still a lack of sufficient quantitative and qualitative data to establish general conclusions about the impact of farming systems on yeast diversity and the impact of the yeast communities on fermentation processes and wine quality. The current study aimed to:

1. Isolate and identify yeasts in grape musts obtained from conventional, integrated and biodynamic farming systems.
2. Monitor the dynamics of non-*Saccharomyces* yeast species during alcoholic fermentation.
3. Evaluate *Saccharomyces cerevisiae* strain diversity and dynamics throughout alcoholic fermentation.

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Chapter 2

Literature review

**Yeast diversity and dynamics during
spontaneous fermentation**

2 Yeast diversity and dynamics during spontaneous fermentation

2.1 Grape microbial community

The grape berry surface contains a complex microbial community that plays a critical role in wine quality. After the first microbial investigation by Pasteur (1872), demonstrating the presence of microbes on grape surface, several studies consecutively confirmed that the grape berry surface harbours a wide variety of yeasts, filamentous fungi and bacteria (Martini et al., 1996; Mortimer and Polsinelli, 1999; Combina et al., 2005 (a); Renouf et al., 2005; Barata et al., 2012 (a); Martins et al., 2012; Furukawa et al., 2013; Milanović et al., 2013). Yeasts are mainly responsible for the conversion of grape must sugar to ethanol, while lactic acid bacteria (mainly *Oenococcus oeni*) are considered contribute to wine quality through conversion of malic acid to lactic acid (Bartowsky, 2009; Martins et al., 2012; González-Arenzana et al., 2012). On the contrary, the acetic acid bacteria such as species of *Acetobacter* and *Gluconobacter* are often implicated in spoilage of wine (Sengun and Karabiyikli, 2011; Martins et al., 2012), while filamentous fungi have not been shown to make significant contribution in winemaking (Barata et al., 2012(a)).

Yeasts are the most important microorganisms in wine production due to their influence on fermentation speed, wine flavour and wine quality (Chavan et al., 2009; Combina et al., 2005). Therefore, further investigation on the quantitative and qualitative diversity of yeast communities present on grape surface as well as grape must is important. The density and diversity of the yeast population on grape berries is affected by numerous factors such as, grape variety (Cordero-Bueso et al., 2011), grape health (Loureiro and Malfeito-Ferreira, 2003; Barata et al., 2008), grape ripeness (Martins et al., 2012), climatic condition and geographic location (Bezerra-Bussoli et al., 2013; Bokulich et al., 2013), application of different chemicals (Milanović et al., 2013), application of different oenological practices (Andorrà et al., 2010) as well as application of different farming systems (Cordero-Bueso et al., 2011; Martins et al., 2012; Setati et al., 2012).

2.1.1 Impact of grape health on yeast diversity

The skin of grape berry might get damaged due to several reasons. These include heavy rainfall, attack by insects, attack by birds (Somers and Morris, 2002) or damage caused by phytopathogenic moulds such as grey rot (Barata et al., 2012 (a)). Several studies have demonstrated that physical damages to the grape berries can influence yeast population, density and diversity as well as population composition. For instance, while sound grape berries contain low yeast levels ranging between, $10^2 - 10^3$ cfu/mL, damaged grapes exhibit a drastic increase of total yeast diversity as well as total yeast counts between $10^6 - 10^8$ cfu/mL (Loureiro and Malfeito-Ferreira, 2003; Barata et al., 2008). Sound grape berries mainly harbour the

basidiomycetous yeasts (e.g. *Rhodotorula* spp. and *Cryptococcus* spp.), and to a lesser extent ascomycetous yeasts such as the apiculate yeast (*Hanseniaspora uvarum*), the oxidative yeasts such as *Candida* spp. and the film forming *Pichia* spp. (Barata et al., 2012 (b)). On the other hand, damaged grape berry skins contain high amounts of sugar which provides a selective environment for the growth of different ascomycetous species with higher fermentative activity, such as *Pichia membranifaciens* and *Issatchenkia terricola*, as well as, osmophilic and osmotolerant genera such as *Torulaspota* and *Zygosaccharomyces*, mainly, *Z. bailii* (Loureiro and Malfeito-Ferreira, 2003; Barata et al., 2008; Barata et al., 2012(b)). The main wine fermentation yeast, *Saccharomyces cerevisiae* has also been isolated from damaged berries albeit occasionally (Renouf et al., 2005; Milanović et al., 2013).

2.1.2 Impact of grape berry ripening on yeast diversity

The distribution of non-*Saccharomyces* yeasts on the grape skin is also affected by the degree of berry maturation and grape ripeness (Loureiro and Malfeito-Ferreira, 2003; Renouf et al., 2005; Barata et al., 2008). The significant increase in total yeast composition and yeast diversity at different stages of berry developments has been reported previously (Renouf et al., 2005; Barata et al., 2008). Basidiomycetous yeasts of the genera *Rhodotorula*, *Rhodosporidium*, *Sporobolomyces* and *Cryptococcus* as well as ascomycetous yeasts such as *Candida* spp. are typically predominant from berry set to harvest (Table 1). The yeast-like fungus, *Aureobasidium pullulans* has also been shown to be the dominant isolate at berry set and not at harvest (Renouf et al., 2005; Barata et al., 2008). However, its presence at full ripeness has been shown using the culture independent PCR-DGGE method (Prakitchaiwattana et al., 2004). These yeasts can survive in nutrient poor environments and have been shown to produce exopolysaccharides and form biofilms that protect them against environmental stress (Renouf et al., 2005; Barata et al., 2012 (a)). Similarly, biofilm formation has been indicated as the reason for the prevalence of *Candida*, *Rhodotorula* and *Cryptococcus* spp., during the ripening stage (Renouf et al., 2005). The increase in total yeast population and species diversity has been associated with chemical evolution in the berries and increase in cell wall elasticity (Renouf et al. 2005). Moreover an increase in the population of weakly fermentative yeasts (e.g. *Candida* spp.) to the level of 5×10^5 cfu/berry and a decrease in the population of *A. pullulans* was also reported previously (Renouf et al., 2005). The population of weakly fermentative ascomycetous yeast such as *Candida zemplinina*, *Pichia membranifaciens* and *H. uvarum* increases after véraison (Table 2.1). This has been attributed to the reduction in fungicide application closer to harvest, increase in nutrients on the berry surface due to the elasticity of the riper berry skin and leakage of the berry juices to the surface (Renouf et al., 2005; Renouf et al., 2007; Barata et al., 2008).

Table 2.1 Yeast population dynamics during ripening stage. Data compiled from Renouf et al., 2005 with the modification from Barata et al., 2008.

Berry development stage	Yeast species
Berry set	<i>Aureobasidium pullulans</i>
	<i>Cryptococcus</i>
	<i>Rhodotorula</i>
	<i>Candida, Hanseniaspora</i>
	<i>Rhodospiridium</i>
	<i>Sporobolomyces</i>
	<i>Yarrowia</i>
Véraison	<i>Metschnikowia / Pichia</i>
	<i>Hanseniaspora / Candida</i>
	<i>Aureobasidium pullulans</i>
	<i>Cryptococcus</i>
	<i>Rhodotorula</i>
	<i>Sporobolomyces</i>
	<i>Rhodospiridium</i>
	<i>Bulleromyces</i>
<i>Kluyveromyces</i>	
Harvest	<i>Cryptococcus / Saccharomyces</i> sp
	<i>Candida / Kluyveromyces</i>
	<i>Pichia / Issatchenkia</i>
	<i>Rhodotorula / Debaryomyces</i>
	<i>Hanseniaspora / Sporobolomyces</i>

2.2.3 Wine grape production methods

Grapes used for wine production can be obtained through different farming systems including: Organic, Conventional, Biodynamic and Integrated viticulture (Cordero-Bueso et al., 2011; Tello et al., 2011; Martins et al., 2012). Conventional farming systems (Conv) have been the most employed agricultural system in the twentieth century. Conventional viticulture typically uses inorganic or synthetic fertilizers, pesticides and herbicides (Hole et al., 2005). Several criticisms have been made against conventional farming systems due to global concerns and negative impacts of chemical synthetic additives on the environment (Villanueva-Rey et al., 2013). Consequently, farmers have adopted environmentally friendly strategies such as integrated pest management systems in the wine growing regions such as United States, South Africa, Spain, France and Germany. Integrated pest management system (IPM) arose in 1970 in the agriculture sector. Although this scheme does not have a regulated certification system, the preference in this scheme is application of organic fertilizers and using the biological strategies such as bait and ducks for pest control rather than chemical options. However, the use of chemical fertilizers, pesticides and fungicides with careful monitoring is allowed. In South Africa grapes are mainly produced through integrated production of wine (IPW), established by the South African wine industry in 1998 (http://www.wosa.co.za/sa/sustainable_ipw.php).

Organic viticulture is one of the other examples of environmentally friendly farming practices that prohibit the use of chemical and synthetic fertilizers or pesticides. In this system, the application of tillage or grass cutting to control the weeds as well as the application of green manure, natural fertilizers and pesticides, are common practices (Coll et al., 2011; Villanueva-Rey et al., 2013). Biodynamic agriculture was suggested by Rudolf Steiner in 1920 as an individual self-sufficiency unit (Paull, 2011). Biodynamic viticulture is an early scheme of organic viticulture with the emphasis on providing the resource through soil, plant and animals. In this farming specific compost preparations are applied during specific times and the practices are regulated under the Demeter guidelines (www.demeter.net). The Demeter biodynamic farm and processing standard, include necessary elements of the farm and organism, soil fertility management, crop protection, green house management and the use of preparation (www.demeter-usa.org). For instance, disease and insect control are addressed through botanical species diversity, predator habitat.

Table 2.2: A summary of wine grape production methods and regulations for vineyard and cellar management. Data compiled from (www.demeter.net).

Definition	Vineyard	Processing facilities	yeast	Sulfites
Organic	No synthetic pesticides, herbicides, fungicides, fertilizers	Facility is certified to meet organic standards	Native	Up to 100 ppm
Biodynamic	No synthetic pesticides, herbicides, fungicides, fertilizers	Facility is certified to meet biodynamic standards	Native	Up to 100 ppm
Conventional	No certification, Typically uses synthetic pesticides, herbicides, fungicides, fertilizers	Conventional winery	Native/commercial	Up to 300ppm
Integrated	IPW certification. uses synthetic pesticides, herbicides, fungicides, fertilizers with careful monitoring	Integrated winery	Native/commercial	Up to 300ppm

Organic and biodynamic farming systems have been shown to have beneficial impacts on soil fertility as well as microbial biodiversity. A previous study that was conducted by Maeder et al.

(2002) clearly demonstrated higher microbial diversity in wheat soil under biodynamic farming system in comparison with the organic wheat soil.

The impact of farming systems on the diversity of microbial communities associated with grape berries have been recently demonstrated (Cordero-Bueso et al., 2011; Martins et al., 2012; Milanović et al., 2013; Setati et al., 2012). These studies, clearly demonstrated significant differences in microbial composition (type of species and their biological relevance) and microbial diversity (number of species and strains) of grapes obtained from different farming systems. In the study by Pancher et al. (2012), significant differences in the type of fungal communities in grapevines obtained from organically managed farms in comparison with IPM farms was clearly demonstrated. For instance, *Leptosphaerulina chartarum* was only isolated from the organic farm and *Botryosphaeria* sp. was only isolated from the IPM farm. Schmidt et al. (2011) reported a strong dominance of *A. pullulans* in an organic farming system compared to a conventional farming system. This dominance was attributed to the ability of this fungus to detoxify inorganic sulphur. In the study conducted by Cordero-Bueso et al. (2011), the least treated grape must from organic farming system exhibited higher species richness and lower dominance while conventional farm exhibited the higher dominance and lower species richness. Similarly Setati et al. (2012) reported higher species richness and lower dominance in a biodynamic farming system compared to conventional and integrated farming systems. On the contrary, Milanović et al. (2013) demonstrated higher diversity and species richness in conventional farming system than organic farm. In all cases, phytosanitary treatments have been suggested as a contributing factor. However, the extent of their impact needs to be further investigated. In conclusion, previous studies by Setati et al. (2012) and Pancher et al. (2012) clearly indicated an overlap between the yeast species and the fungal community found in different farming systems. However, individual species were isolated from each farming system. Therefore, the further investigation regarding the impacts of farming systems on yeast communities associated with grape must and during the fermentation is essential due to the inconsistent results from the studies (Milanović et al., 2013; Setati et al., 2012; Cordero-Bueso et al., 2011).

2.2 Yeast diversity in the grape must

Freshly crushed grape must typically contains different yeast species at approximately $10^2 - 10^4$ cfu/mL. However, higher values have been reported due to various degree of grape health at harvest time (Jemec et al., 2001; Barata et al., 2012(b); Šuranská et al., 2012). *Cryptococcus*, *Rhodotorula*, *Filobasidium*, *Candida*, *Pichia*, *Hanseniaspora*, *Metschnikowia*, *Issatchenkia*, *Aureobasidium*, *Kluyveromyces* and *Torulaspora*, are the most abundant genera in grape must (Loureiro and Malfeito-Ferreira, 2003; Jolly et al., 2003; Chavan et al., 2009; Šuranská et al., 2012). The yeast community associated with grape must can be divided in to three main categories, (i) oxidative yeasts such as *A. pullulans*, *Cryptococcus* spp. and *Rhodotorula* spp.

that have no fermentation ability, (ii) weakly fermentative yeasts such as *Candida* spp. and *Pichia* spp., as well as (iii) strongly fermentative yeasts such as *Torulaspora delbrueckii*, *Lachancea thermotolerans* and *Saccharomyces* spp. The strongly fermentative yeast, responsible for wine fermentation, *Saccharomyces cerevisiae*, is usually present below the detection level in grape must (Martini et al., 1996; Mortimer and Polsinelli, 1999; Mercado et al., 2007; Di Maro et al., 2007).

The heterogeneous yeast community in the grape must (Table 2.2), originates from different habitats in the farming system (e.g. soil, bark, leaves, animal vectors and leaves) or from the winery equipment (Bezerra-Bussoli et al., 2013; Bokulich et al., 2013), as well as the air in the cellar (Loureiro and Malfeito-Ferreira, 2003; Barata et al., 2012(a), Ocón et al., 2010). A recent study, demonstrated that members of the genera *Cryptococcus*, *Rhodotorula* and *Sporidiobolus* are permanently present in the winery air and bottling line (Ocón et al., 2013). However, these yeasts have no ability to grow in wine and therefore pose no risk for contamination. However, other yeasts such as *Zygosaccharomyces*, *Pichia* as well as *Brettanomyces* spp. that pose greater risk to wine quality have also been detected in winery air (Ocón et al., 2013). Yeasts present on grapes and winery equipment are known to initiate a spontaneous fermentation of grape must. Since these yeasts are capable of anaerobic as well as aerobic growth, some of them may persist during fermentation and contribute secondary metabolites which affect the bouquet of the final

Table 2.3: Dissemination and technological significance of microbial species isolated from the vineyard and winery environment. Data compiled from Barata et al., 2012(a) with the modification from Ocón et al., 2010; Cordero-Bueso et al., 2011; Sun et al., 2009; González et al., 2007 ; Bezerra-Bussoli et al., 2013; Bokulich et al., 2013.

Group	Metabolism	Genus	Relevant species	Main Sources
Basidiomycetous Yeasts	Oxidative	<i>Cryptococcus</i>	<i>C. adeliensis</i> / <i>C. albidus</i> / <i>C. saitoi</i> <i>C. carnescens</i> <i>C. magnus</i>	Grape musts, Grape surface, Air
		<i>Rhodotorula</i>	<i>R. rubra</i> , <i>R. nothofagi</i> <i>R. glutinis</i> <i>R. aurantiaca</i>	Grape must, Grape surface, Insect
		<i>Pseudozyma</i>	<i>P. aphidis</i>	Grape must
		<i>Sporobolomyces</i>	<i>S. biogenesis</i> <i>S. roseus</i>	Grape must, grape surface
Ascomycetous Yeasts	Oxidative	<i>Aureobasidium</i>	<i>A. pullulans</i>	Grape must, Grape surface, Insect, Air
	Oxidative or Weakly fermentative	<i>Hanseniaspora</i> / <i>Kloeckera</i> (<i>apiculate yeast</i>)	<i>H. uvarum</i> / <i>K. apiculata</i> <i>H. guilliermondii</i> <i>H. vineae</i>	Grape must, Grape surface

		<i>Candida</i> (film forming yeast)	<i>C. zemplinina</i> <i>Zygoascus hellenicus</i> / <i>C. steatolytica</i> <i>C. sorboxylosa</i> , <i>C. stellimalicola</i> <i>C. parapsilosis</i> , <i>C. versatilis</i>	Grape must, Insect; Air
		<i>Metschnikowia</i>	<i>M. pulcherrima</i> , <i>M. fructicola</i> <i>M. reukaufii</i>	Grape surface, grape must
		<i>Pichia</i> . (film forming yeast)	<i>P. anomala</i> , <i>P. fermentans</i> <i>P. membranifaciens</i> <i>P. guilliermondii</i> , <i>P. kluyveri</i>	Grape must, Grape surface, insects, Winery equipment
		<i>Debaryomyces</i>	<i>D. hansenii</i>	Grape must, Grape surface, Insect
		<i>Lachancea</i>	<i>L. thermotolerans</i> <i>L. fermentati</i>	Grape must
		<i>Issatchenkia</i>	<i>I. terricola</i> , <i>I. occidentalis</i> <i>I. orientalis</i>	Grape must, Winery equipment
	Fermentative	<i>Torulaspora</i>	<i>T. delbrueckii</i>	Grape must
		<i>Zygosaccharomyces</i>	<i>Z. bailii</i> , <i>Z. bisporus</i> <i>Z. rouxii</i> / <i>Z. verona</i>	Grape must , Insect
		<i>Dekkera/ Brettanomyces</i>	<i>D. bruxellensis</i>	Grape must
		<i>Schizosaccharomyces</i>	<i>S. pombe</i>	Grape must
		<i>Saccharomyces</i>	<i>S. cerevisiae</i> , <i>S. bayanus</i> <i>S. paradoxus</i> , <i>S. pastorianus</i>	Grape must, winery surface
		<i>Saccharomycodes</i>	<i>S. ludwigii</i>	Grape must

2.3 Spontaneous wine fermentation

Wine is a natural product which results from many biochemical reactions that begin at berry ripening and continue during harvesting, throughout the alcoholic fermentation, clarification and after bottling (Romano et al., 2003). The fermentation of grape juice to wine is a complex microbiological process which is characterized by the sequential development of various yeasts and lactic acid bacteria. Traditionally, wine is produced through a spontaneous (natural) fermentation process which is mediated by the indigenous microbiota often referred to as the wine microbial consortium (WMC) present on the grapes, and winery equipment (Barata et al., 2012(a); Di Maro et al., 2007; Cordero-Bueso et al., 2011). This consortium generates multitudes of by-products that impart flavour and aroma to the wine. Although the WMC comprises yeasts, lactic acid bacteria and acetic acid bacteria, the yeasts are the main agents of alcoholic fermentation which is the conversion of grape sugars to ethanol and CO₂. The yeast population which can be divided into two main categories viz. *Saccharomyces* and non-*Saccharomyces* yeasts, consists of oxidative, weakly fermentative and strong fermentative

species as described in section 2.2 (Barata et al., 2012 (a)). Studies performed on the yeast population in grape musts and during the different courses of fermentation have consistently demonstrated a rapid and successional development of different non-*Saccharomyces* and *Saccharomyces* species and strains during spontaneous fermentation (Cocolin et al., 2002; Combina et al., 2005; Di Maro et al., 2007; Bezerra-Bussoli et al 2013).

2.3.1 Non-*Saccharomyces* yeast population dynamics during spontaneous fermentation

The non-*Saccharomyces* yeast species available in grape musts, initiate alcoholic fermentation at the level of $10^3 - 10^5$ cfu/mL and often the population density increases up to $10^6 - 10^8$ cfu/mL during the tumultuous phase of fermentation (Jemec et al., 2001; Combina et al., 2005; Di Maro et al., 2007). However, several non-*Saccharomyces* species (e.g. *Cryptococcus* and *Rhodotorula*) have been shown to be sensitive to anaerobic conditions and high ethanol levels (Pina et al., 2004; Romano et al., 2003). Therefore, rapid decline in the non-*Saccharomyces* species diversity is often apparent in the first 2 – 3 days of fermentation, followed by a decrease in population density as the concentration of ethanol increases above 6 – 7% (v/v) (Jemec et al., 2001; Combina et al., 2005; Di Maro et al., 2007; Wang and Liu, 2013). This decline could be due to the other factors such as the nutrient limitation, temperature and the presence of inhibitory factors (Perrone et al., 2013). Only a few species of non-*Saccharomyces* yeasts have been shown to persist under wine making conditions (Table 2.3). Typically, the early stages of the alcoholic fermentation are dominated by yeasts with a low fermentative power. These yeasts, (e.g. strains of *Hanseniaspora uvarum*, *Candida zemplinina*, *Issatchenkia terricola*, and *Issatchenkia orientalis*) are often prevailing until the middle of fermentation (Table 2.3). Of these, *H. uvarum* is most frequently the principal non-*Saccharomyces* yeast present in most of the fermentations. As the weakly fermentative yeasts die-off, they are quantitatively replaced by strong fermentative yeasts, mainly *Saccharomyces cerevisiae*. However, there are a few non-*Saccharomyces* species including, *Torulaspota delbrueckii* and *Lachancea thermotolerans* that have been shown to tolerate up to 10 -12% ethanol and therefore prevail to the final stages of alcoholic fermentation (Di Maro et al., 2007; Settanni et al., 2012). Although non-*Saccharomyces* yeasts have been regarded as wine spoilage organisms in the past decades, the positive contribution of these species in wine aroma and flavour has been demonstrated by several authors (Ciani and Maccarelli, 1998; Jolly et al., 2003; Lopandic et al., 2008; Clavijo et al; 2010). Fermentative non-*Saccharomyces* species compete with *Saccharomyces* for nutrients, and interact in different ways thus contributing significantly to the final organoleptic properties of wine.

Table 2.4. Examples of non-*Saccharomyces* yeast species present during different stages of alcoholic fermentation.

Yeast group	Metabolism	Relevant genus & species	Frequency of occurrence (% relative abundance)			References
			BF	MF	EF	
Basidiomycetous	Oxidative	<i>Rhodotorula mucilaginosa</i>	9.2	07.3	0	Sun et al., 2009; Jemec et al., 2001
		<i>Cryptococcus carnescens</i>	0.9-13.9	0	0	Milanović et al., 2013
Ascomycetous	Oxidative	<i>Aureobasidium pullulans</i>	4.1	0	0	Milanović et al., 2013
	Oxidative or weakly fermentative	<i>Hanseniaspora uvarum</i>	10-100	4-80.4	0.3-13	González et al., 2007; Beltran et al., 2002; Sun et al., 2009; Jemec et al., 2001; Milanović et al., 2013; Cordero-Bueso et al., 2011
		<i>Candida zemplinina</i>	8.4-39.9	13.51	34.4-63	González et al., 2007; Sun et al., 2009
		<i>Metschnikowia pulcherrima</i>	4-26	2	0	González et al., 2007; Beltran et al., 2002; Jemec et al., 2001; Cordero-Bueso et al., 2011
		<i>Pichia kluyveri</i>	11	1.18-9	10	González et al., 2007; Cordero-Bueso et al., 2011
		<i>Issatchenkia terricola</i>	11	18		Beltran et al., 2002; Jemec et al., 2001; Bezerra-Bussoli et al., 2013
		<i>Issatchenkia occidentalis</i>	8.4-22.7	8.4 - 9		Sun et al., 2009; Bezerra-Bussoli et al., 2013
		<i>Issatchenkia orientalis</i>	11.5	5	5-25	Sun et al., 2009; Bezerra-Bussoli et al., 2013
	Fermentative	<i>Zygosaccharomyces bailii</i>		4.32-9.6	2	González et al., 2007; Torija et al., 2001
		<i>Schizosaccharomyces</i> spp.	0	9.6		Torija et al., 2001
<i>Lachancea thermotolerans</i>		8.3	12.4	4.6	Cordero-Bueso et al., 2011	

		<i>Torulaspora delbrueckii</i>	6.1	11.2	6.8	Cordero-Bueso et al.,2011
		<i>Saccharomyces cerevisiae</i>	0-21.3	7-93	63-100	Beltran et al., 2002; Sun et al., 2009; Cordero-Bueso et al., 2011; Bezerra-Bussoli et al., 2013

2.3.2 Methods used to evaluate non-*Saccharomyces* yeast dynamics

The dynamics of yeast during wine fermentation have traditionally been monitored by cultivation-dependent methods which often involve presumptive morpho-physiological identification and characterization of yeast isolates followed by identity confirmation using molecular methods. Although these methods have yielded valuable information, they can be laborious, time-consuming and biased towards yeasts adapted to the cultivation conditions used for isolation while excluding minor species (Cocolin et al., 2011; Renouf et al., 2007; Xufre et al., 2006; Zott et al., 2010). The collection of culture dependent and culture independent studies have been used to investigate the population dynamics of non-*Saccharomyces* species during wine fermentation (Renouf et al., 2007; Zott et al., 2008; Zott et al., 2010; Bezerra-Bussoli et al., 2013). However, this technique has shown to be biased for the growth of the minor species, (e.g. *S. cerevisiae* in the grape must) when the population is below detection level (Prakitchaiwattana et al., 2004; Mercado et al., 2007; Di Maro et al., 2007). On the other hand, the molecular techniques such as “sequencing of the D1/D2 of the large sub-unit 26S ribosomal DNA” and “PCR-RFLP based on restriction analysis of ribosomal DNA followed by “PCR amplification of the rDNA regions” are the common methods for monitoring the yeast population dynamics (Prakitchaiwattana et al., 2004). More recently, cultivation-independent techniques such as Denaturing Gradient Gel Electrophoresis or DGGE (Cocolin et al., 2000; Renouf et al., 2007; Di Maro et al., 2007; Cocolin et al., 2011), fluorescence *in situ* hybridization or FISH (Xufre et al., 2006) and quantitative real time PCR (Zott et al., 2008; Zott et al., 2010) have been introduced for monitoring the yeast population. These methods allow the detection and identification of microorganisms directly from the environment without cultivation and isolation since DNA or RNA is extracted directly from the matrices and subsequently analysed by methods able to highlight microbial diversity (Cocolin et al., 2011). In PCR-DGGE, total DNA is extracted from the ecosystem (e.g. grape must and wine) and selected molecular markers such as the ITS1-5.8S rRNA-ITS2 region or the D1-D2 region of the 26S rRNA subunit are amplified by PCR using specific universal primers. The amplicons are then separated by DGGE, sequenced and identified by sequence comparison with existing sequences in rRNA sequence databases (Prakitchaiwattana et al., 2004). In contrast, FISH analysis relies on species-specific fluorescently labelled probes designed to detect rRNA molecules (Xufre et al., 2006), while RT-QPCR employs species-specific primers (Zott et al., 2010). Although these methods have been deemed more sensitive, and rapid, they also have some bias. For instance, it has been demonstrated that for PCR-DGGE a species present at 10^3 cfu/mL in a mixture will be detected, whereas other yeasts are present at 10^6 cfu/mL or more will not be detected (Prakitchaiwattana et al., 2004; Cocolin et al., 2011). In addition, as reported by Prakitchaiwattana et al. (2004), some yeast such as members of the genera *Rhodotorula*, *Cryptococcus* and *Rhodospiridium* may not be detected through this technique. FISH and qRT-PCR also suffer some disadvantages as they can only follow the dynamics of targeted yeast species due to the

species-specific probes and primers. Therefore, unknown yeast species may be missed in the analysis. It is worth mentioning that in spite of the bias in both culture-dependent and culture-independent techniques; similar yeast dynamics trends during spontaneous fermentation have been observed (Zott et al., 2010; Xufre et al., 2006; Xufre et al., 2011; Cocolin et al., 2000; Renouf et al., 2007). Both approaches have consistently demonstrated the dominance of non-*Saccharomyces* species in the initial stages of alcoholic fermentation, up to 5-7% ethanol concentration and the major dominance of *S. cerevisiae* in the final stage of fermentation. Therefore, the combination of culture-dependent and culture-independent approaches can be used to achieve better understanding of microbial diversity in grape ecosystems.

2.3.3 Dynamics of *Saccharomyces cerevisiae* in spontaneous fermentation

Although the non-*Saccharomyces* yeasts may play a significant role in the early stages of wine fermentation, the ultimate conversion of grape must to wine is mainly performed by the more alcohol tolerant *Saccharomyces* species, especially *Saccharomyces cerevisiae* (Perrone et al., 2013; Lopandic et al., 2008; Mercado et al., 2007). This species is rarely isolated from nature such as grape surfaces and is only present at low concentrations in fresh must (Mortimer and Polsinelli, 1999; Mercado et al., 2007; Di Maro et al., 2007). However, its population levels increase considerably as the fermentation progresses. *S. cerevisiae* generally dominates the middle and end phases of fermentation reaching up to $10^6 - 10^9$ cfu/ml (Combina et al., 2005). Researchers have been able to demonstrate that alcoholic fermentation is modulated by a consortium of different strains of *S. cerevisiae*. The evaluation of *S. cerevisiae* strain dynamics has clearly demonstrated a sequential replacement of some strains by others. However, no common trend was observed in the number of strains present throughout alcoholic fermentation and the dominance of strains (Querol et al., 1994; Wang and Liu et al., 2013; Cordero-Bueso et al., 2011; Lopandic et al., 2008; Mercado et al., 2007; Schuller et al., 2012). For instance, in the study conducted by Perrone et al. (2013), only one strain was found to be dominant from the beginning through the final stage of fermentation, while in other studies as many as 22 - 43 strains were found throughout different stages of alcoholic fermentation and only a few strains dominated the final stage of fermentation (Mercado et al., 2007; Schuller et al., 2012). In contrast, studies conducted by Lopandic et al. (2008) and Hall et al. (2011) revealed an increase in the diversity of *Saccharomyces* strains during the different courses of fermentation from 4 strains in the beginning to 5 - 10 strains in the middle and final stage of alcoholic fermentation. In the studies by Mercado et al. (2007) and Schuller et al. (2012), the decrease in the diversity of *Saccharomyces* strains and the dominance of a single strain through alcoholic fermentation was observed. On the other hand, in another scenario several strains were observed throughout alcoholic fermentation without detection of any dominant strains in any stage of fermentation (Torija et al., 2001). Several hypotheses exist regarding the variable dominance behaviour of *S. cerevisiae* strains during alcoholic fermentation. Frazier and

Dubourdieu, (1992) suggested that the dominance behaviour of some strains during the courses of fermentation might be due to the fact that these strains become stabilized and dominant in the winery over the time and therefore establish themselves rapidly and in high levels in grape must after crushing. Competition between the strains in order to find the space to survive, the killer activity in different yeasts genera and the presence of non-dominant *Saccharomyces* strains below 10^5 cfu/mL have also been suggested by previous authors (Zagorc et al., 2001; Howell et al., 2005; Arroyo-lopez et al., 2010; Perrone et al., 2013) as reasons for the dominance of certain strains.

2.3.4 Genotypic characterization of *Saccharomyces cerevisiae* strains during spontaneous fermentation

Developments in molecular methods have provided better understanding of wine microbiology and allowed the identification and characterisation of *S. cerevisiae* at the strain level. Similar to the dynamics of non-*Saccharomyces* yeasts, several methods have been employed to evaluate the dynamics of *S. cerevisiae* strains during wine fermentation. However, in this case, the methods are used in conjunction with culture-dependent methods, where the yeasts are first isolated at various stages of fermentation followed by strain identification using molecular techniques. The most common molecular approaches for genotypic characterisation of *S. cerevisiae* are divided into PCR based approaches and non-PCR based approaches.

PCR-base methods include random amplification of polymorphic DNA (RAPD), based on using different primers with random sequence, (PCR) analysis of repetitive genomic DNA (microsatellites and minisatellites), amplification of interdelta sequences of the TY1 retrotransposon and amplified fragment length polymorphism (AFLP). Non-PCR based methods including hybridisation techniques, pulsed field gel electrophoresis (PFGE) of chromosomes and the restriction analysis of mitochondrial DNA. The hybridisation techniques are based on the variation in the restriction sites of non-coding DNA region is detectable by the hybridization of DNA probe. PFGE is a karyotyping approach based on the presence or absence of long DNA fragments in the chromosomes (Fernandez-Espinar et al., 2000; Hall et al., 2011).

Amplification of δ sequence

The difference between two *S. cerevisiae* strains due to the presence or absence of “Ty1” element in the genome of this yeast was previously demonstrated (Fernandez-Espinar et al., 2000; Hall et al., 2011). “Ty1” is a retrotransposon built up of an approximately 6 kb fragment called epsilon which is flanked by long terminal repeats (LTRs) also referred to as delta (δ) elements. The δ elements also exist as solo elements separate from the retrotransposon. The number and location of these elements varies between strains of *S. cerevisiae*. Consequently,

this feature has been used as a genetic fingerprinting tool to differentiate the strains from each other (Fernandez-Espinar et al., 2000; Hall et al., 2011).

PCR analysis of repetitive genomic DNA (microsatellites and minisatellites)

Microsatellites or simple sequence repeats (SSR) and minisatellites are repetitive regions of genomic DNA that display high degree of length variability in individual strains of the same species such as *S. cerevisiae* (Gonzalez Techera et al., 2001). The analysis of these regions has been extensively used for *S. cerevisiae* strain differentiation (Gonzalez Techera et al., 2001). The method uses a set of specific oligonucleotides such as, (GTG)₅, (GAG)₅, (GACA)₄ and M13 to amplify variable regions (Gonzalez Techera et al., 2001). Previous studies have demonstrated high discriminatory level of the microsatellite technique and its ability to identify different strains of *S. cerevisiae* (Pérez et al., 2001; Schuller et al., 2005).

Restriction analysis of Mitochondrial DNA

Restriction analysis of mitochondrial DNA (mt-DNA) is a widely used approach for characterisation of *S. cerevisiae* strains in the wine industry (Schuller et al., 2005; Valero et al., 2005). The mt-DNA is a small molecule between 60 - 80 Kb that exhibit a high degree of variation due to high mutation rates in the genome (Capece et al., 2012). This technique relies on the Gcn5-related *N*-acetyltransferases (GNATs) type enzymes that exhibit different digest patterns in different strains. The most common enzymes used for *S. cerevisiae* are **Hae** III and **Hinf** I. This method has been extensively used for characterization of wine strains due to the great degree of discrimination, high speed and low cost (Nikolaou et al., 2007; Clavijo et al., 2010, Capece et al., 2012).

As explained in the previous section, different molecular approaches have been used to genetically differentiate the *Saccharomyces* strains. These methods have been shown to yield inconsistent results (Couto et al., 1996; Siesto et al., 2013; Martínez et al., 2004). For instance, the study conducted by Siesto et al. (2013), comparing three different methods, for genetic characterisation of *S. cerevisiae* strains, clearly underlined that each technique leads to different results. For instance, while some strains showing the same delta profiles, they exhibit different mt-DNA restriction profile and electrophoretic karyotype. Hence, application of more than one method has been suggested for genetic characterisation of *Saccharomyces* strains.

2.3.5 Phenotypic characterization of *Saccharomyces cerevisiae* strains

S. cerevisiae strains present in wine fermentation may also exhibit phenotypic heterogeneity. It has been demonstrated that the strains with tight genetic relationship can exhibit significant phenotypic variability (Settanni et al., 2012). Several technological tests have been used for phenotypic characterisation of *S. cerevisiae* strains. For instance, the ability of different *S.*

cerevisiae to produce H₂S based on the level of colony blackening on Sulphite Glucose Glycerine Yeast extract, as well as the ability of strains to grow at various temperatures (13 - 25°C), ethanol (12-16%), and potassium metabisulphite (50 - 300 mg/L) are the most common technological tests for phenotypic characterization of *S. cerevisiae* strains (Settanni et al., 2012; Salinas et al., 2010; Nikolaou et al., 2007). The data indicated that the strains derived from the same fermentation with high genetic relatedness, exhibit significant differences in phenotypic characteristics (Settanni et al., 2012; Nikolaou et al., 2007; Salinas et al., 2010). For instance, the study performed by Settanni et al. (2012), based on screening the oenological characteristics of different *S. cerevisiae* strains isolated from the same fermentation, showed that out of 51 screened strains, some were characterised by low production of H₂S and also could tolerate high levels of ethanol, while the others exhibited growth in high concentrations of potassium metabisulphite. On the other hand, some strains had low levels of acetic acid production and foam formation, some capable of growing in low temperatures, and finally, different strains were shown to have different fermentation rates. It has been demonstrated that *S. cerevisiae* strains which dominate the final stage of fermentation are more ethanol tolerant in comparison with the dominant strains in the beginning and the middle of alcoholic fermentation (Torija et al., 2001; Zagorc et al., 2001). This might be due to the fact that as the fermentation proceeds, the concentration of ethanol increases and only the ethanol tolerant species can persist in the final stage of alcoholic fermentation (Torija et al., 2001). Exhibition of different phenotypic characteristics regardless of genetic relatedness has been attributed to the adaptation of different strains to the wineries and therefore the possible changes in the gene expression or the genes with the unknown function during the fermentation that can lead the modification in phenotype characterisation such as fermentation kinetics (Cavaliere et al., 2000; Zuzuarregui et al., 2006)

In conclusion, although several culture dependent and culture in-dependent techniques have been used to characterize and monitor the yeasts dynamics throughout spontaneous fermentation, there are stills many unanswerable questions and gaps regarding the microbial ecology in the vineyard and the impacts of farming systems on the microbial community associated with grape must. One of the most important concerns is regarding the origin of the non-*Saccharomyces* fermentative yeasts that has been shown to persist throughout alcoholic fermentation (Settanni et al., 2012; Cordero-Bueso et al., 2011) and also, *S. cerevisiae*. Despite of the several works that have been done on spontaneous fermentation in different agronomic systems, there is still no comprehensive data available and therefore, it is not yet possible to conclude the origin of fermentative yeasts in fermentation, the impacts of different agronomic systems on fermentative yeasts and to explain the persistent behaviour of fermentative yeasts throughout alcoholic fermentation. Thus, deep investigation regarding the yeasts communities

associated with grape must and their dynamics during the spontaneous fermentation is essential steps in wine microbiology.

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Chapter 3

Research results

Comparative analysis of fermentative yeasts during spontaneous fermentation of Cabernet Sauvignon grapes from conventional, biodynamic and integrated vineyard management systems

This manuscript will be submitted for publication in the International Journal of Food Microbiology

3.1 ABSTRACT

The focus of the current study was to evaluate yeast diversity in grape musts obtained from biodynamic (BD), conventional (CONV) and integrated (IPW) farming systems over two vintages (2012 and 2013), and to monitor the population dynamics of wine yeasts throughout spontaneous fermentation. The results demonstrated that, species and strains diversity in grape must is influenced by the farming system. A clear difference was observed in the yeast diversity and yeast composition, in the grape must obtained from the biodynamic and conventional farming system in comparison with the integrated farming system. Similar yeast dynamics trends were observed in all fermentations. The initial stage of spontaneous fermentations was dominated by the weakly fermentative yeasts followed by the dominance of strongly fermentative yeasts in the middle and final stage of alcoholic fermentation. However, different non-*Saccharomyces* species and *Saccharomyces cerevisiae* strains were dominant in the individual fermentations. In the fermentation of the must from CONV, *Hanseniaspora uvarum* and *Lachancea thermotolerans* were the dominant non-*Saccharomyces* yeasts, while *H. uvarum* and *Candida parapsilosis* were dominant in BD, and *H. uvarum* and *Wickerhamomyces anomalus* were dominant in IPW. Varying numbers of *S. cerevisiae* strains were involved in the fermentations, and a replacement/substitution of some strains was observed as the fermentations progressed. Overall, yeast diversity was slightly different in two vintages; however, the biodynamic vineyard displayed high species diversity over the two year period.

3.2 INTRODUCTION

Grapes are the primary source of the microbial communities important in the winemaking process. Grape musts exhibit complex microbial communities including bacteria, yeasts and filamentous fungi. Yeasts are the most important microorganisms in wine fermentation that partially influence wine quality (Pretorius et al., 1999). Freshly crushed grape musts usually exhibit high diversity of non-*Saccharomyces* yeast species including members of the genera *Aureobasidium*, *Hanseniaspora*, *Candida*, *Metschnikowia*, *Torulaspora* and *Pichia*. The non-*Saccharomyces* yeast population is often present in the range of 10^2 - 10^4 cfu/mL on sound grape berries (González et al., 2007; Ocón et al., 2013; Bezerra-Bussoli et al., 2013; Sun et al., 2009). In contrast, *Saccharomyces cerevisiae* is usually present below the detection level (Martini et al., 1996; Mortimer et al., 1999; Mercado et al., 2007; Di Maro et al., 2007). The population density and diversity of indigenous yeasts on grape berries is linked to numerous factors such as grape variety, (Cordero-Bueso et al., 2011), grape health (Loureiro et al., 2003; Barata et al., 2008), grape ripeness (Martins et al., 2012), climatic condition and geographic location (Bezerra-Bussoli et al., 2013), application of different chemicals (Milanović et al., 2013), different oenological practices (Andorrà et al., 2008) as well as different farming systems (Cordero-Bueso et al., 2011; Martins et al., 2012; Setati et al., 2012). The different farming systems include organic, conventional, biodynamic and integrated pest management systems. Recently, several studies focusing on the

vineyard as the initial source of grapes, have investigated the impacts of farming systems on yeast communities (Cordero-Bueso et al., 2011; Milanović et al., 2013; Tello et al., 2011; Martins et al., 2012; Setati et al., 2012). The results obtained in these studies did not reveal common trends. For instance, Cordero-Bueso et al. (2011) reported higher yeast diversity in grapes obtained from an organic farming system in comparison with a conventional farming system, whereas Milanović et al. (2013) demonstrated higher diversity and species richness in a conventional farming system than an organic farming system. Therefore, there is still no comprehensive data available to establish general consensus on the influence of farming systems on yeast communities associated with grape musts.

It has been demonstrated that the non-*Saccharomyces* species derived from the grape must can conduct and initiate the beginning stage of spontaneous fermentation (Jemec et al., 2001; Combina et al., 2005; Di Maro et al., 2007; Wang and Liu, 2013; Tello et al., 2011). The kinetics of the main yeast species have been monitored using both culture-dependent approaches as well as culture-independent methods including, application of fluorescence in situ hybridisation (Xufre et al., 2006), PCR-Denaturing Gel Gradient Electrophoresis (Renouf et al., 2007; Di Maro et al., 2007) and quantitative Real Time-PCR (Zott et al., 2011). All the methods show that as fermentation proceeds and the concentration of ethanol increases over 5-7%, the population of the non-*Saccharomyces* species gradually decreases. However, the persistence of some ethanol tolerant non-*Saccharomyces* species (e.g. *Hanseniaspora uvarum*, *Candida zemplinina*, *Issatchenkia terricola*, *Torulaspota delbrueckii* and *Lachancea thermotolerans*), throughout alcoholic fermentation has been recently demonstrated (Combina et al., 2005; Di Maro et al., 2007; Settanni et al., 2012). These yeasts have been suggested and demonstrated to influence wine quality through the production of secondary metabolites such as esters, polyols and acid alcohols (Settanni et al., 2012; Zott et al., 2008). Several studies have shown that in addition to the heterogeneous yeast communities initiating spontaneous fermentation, there is also a sequential development of non-*Saccharomyces* species and different *Saccharomyces cerevisiae* strains throughout alcoholic fermentation (Cordero-Bueso et al., 2011; Sun et al., 2009; Settanni et al., 2012). This activity is often characterized by the replacement of weakly fermentative yeasts with strongly fermentative species.

Currently, certain wine cellars still carry out traditional winemaking by spontaneous alcoholic fermentation with autochthonous yeasts, derived from the vineyard and continue throughout the fermentation (Bezerra-Bussoli et al., 2013). In some cases, such as in biodynamic farming, spontaneous fermentation is the only form of wine fermentation that is allowed. Since this form of fermentation relies entirely on the indigenous yeasts on grape berries, it is important to understand the impact of farming practices especially on fermentative yeasts. Thus, the focus of the current study was to evaluate the impact of farming systems on yeast diversity associated with grape

musts obtained from conventional, biodynamic and integrated vineyard and to also monitor the population dynamics of yeasts during different stages of alcoholic fermentation. The combination of yeast cultivation on agar media and different molecular techniques such as PCR-RFLP and interdelta PCR were used for yeast isolation, identification and strain differentiation.

3.3 MATERIALS AND METHODS

3.3.1 Sampling procedure

Three vineyards located in the Polkadraai region of Stellenbosch, South Africa were sampled. The vineyards are positioned on the same slope and aspects and they were established in 1994-1995. The biodynamic vineyard was treated with Kumulus (sulphur), nordox (copper oxide), striker (organic fungicide with chitosan) and lime for the protection of powdery mildew and downy mildew, from leaf-fall until full bloom. The integrated pest management vineyard uses chicken manure, inoculation of mycorrhizae and *Trichoderma* spp. into the soil for fertilization, as well as oats for cover crops. Pest management consisted of a combination of fungicides including hyperphos (mono- and dipotassium hydrogen phosphate), dithane (ethylene bisdithiocarbamate), Kumulus (80% sulphur), acrobat MZ (dimethomorph/ mancozeb), talendo (proquinazid), curzate (cymoxanil/mancozeb) and stroby (kresoximethyl); and insecticides such as vantex (pyrethroid) and delmathrin, based on IPW guidelines. In contrast, the vines in the conventional vineyard were treated with chemical fertilizers applied when necessary and the vines were consistently treated with a combination of fungicides including folpan (N-trichloromethyl)thio phthalimide), rootex (phosphorous acid), cumulus, dithane, acrobat, talendo, cungfu (copper hydroxide) and topaz (mono- and di-potassium salts of phosphorous acid).

Cabernet Sauvignon was the only variety of grapes which was analysed. Five kilogram samples of Cabernet Sauvignon grapes were picked manually during 2012 and 2013 from a Conventional (Conv), Biodynamic (BD) and Integrated (IPW) vineyard. The samples were collected aseptically, from specific rows based on the sampling design indicated in Fig. 3.1 that allowed for comprehensive assessment of yeast diversity between the three vineyards. From a sampling design perspective a vineyard included different rows in which panels (each containing 6 vines) make up a 'group'. In the present study, one bunch was collected from each group. Thus in the Conv vineyard six rows (9, 11, 13, 15, 17 and 19) were sampled, where bunches were collected between panel 3, 7 and 11. In BD vineyard seven rows (1, 4, 7, 10, 13, 16, 19) were sampled while in IPW vineyard only three rows were targeted (115, 117 and 119); here the bunches were collected from panels 1, 3, 5, 7, 9 and 11, respectively. The samples were transported to the laboratory in sterile plastic bags and processed within 1 hour after harvest. The grapes were hand de-stemmed and crushed under aseptic conditions. Fifty millilitre samples were withdrawn from the fresh must and analysed for yeast diversity and chemical composition of the must. The chemical composition of must such as glucose, fructose, volatile acidity and tartaric acid was measured,

using spectroscopy technique by Foss grape scan 2000 (Rhine Ruhr, Denmark). The remaining musts were divided into 1.5 L samples and transferred to 2 L fermentation bottles. The musts were allowed to ferment spontaneously at 25 °C.

3.3.2 Fermentations

The fermentation kinetics were monitored daily in 2013 vintage by measuring the weight of fermentation flask and also measuring the glucose/fructose concentrations during the fermentation. Glucose/fructose measurements were performed using the Enzyme robot (Arena 2000, Thermo Scientific, South Africa). Fermentations were considered as completed when the residual sugar was less than 2 g/L. In order to obtain comprehensive data regarding the yeast dynamics throughout fermentation, samples were withdrawn after 12.5%, 30%, 50% and 70% of the initial sugars were consumed.

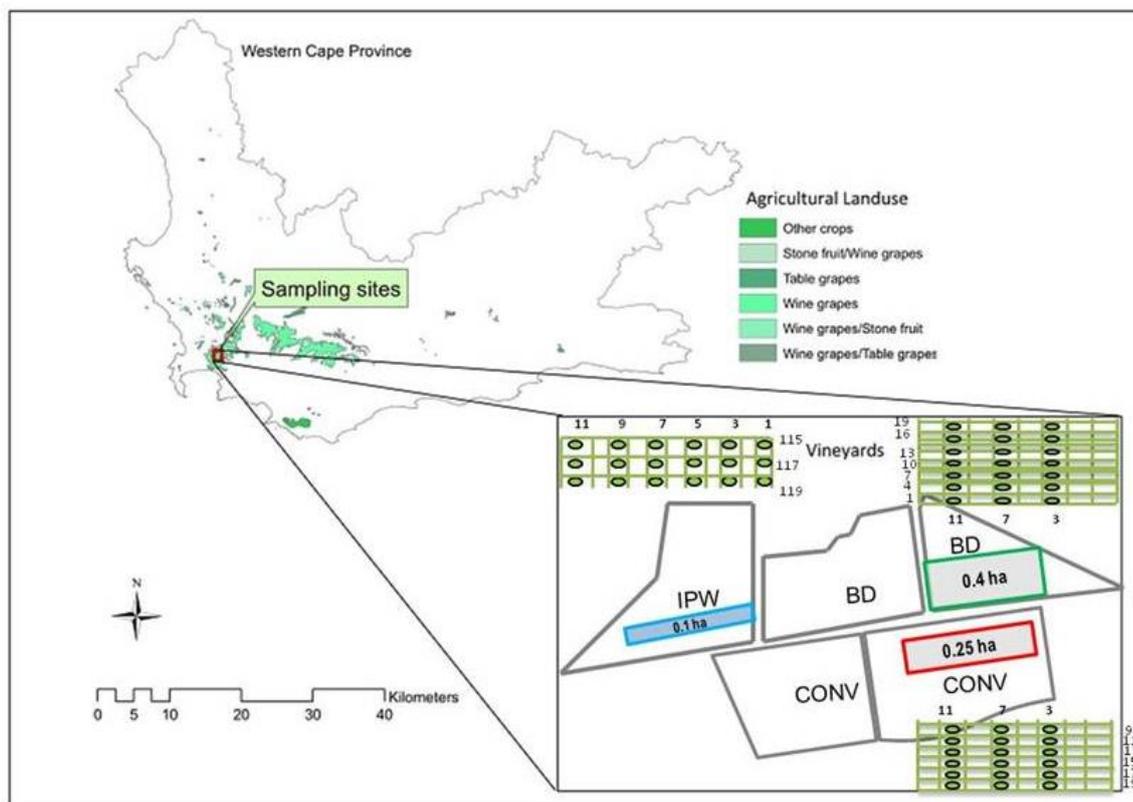


Figure 3.1 Geographic location of the study sites and layout of the vineyards. IPW = integrated production of wine; BD = biodynamic; CONV = conventional (Setati et al., 2012).

3.3.3 Yeast enumeration and yeast isolation

Aliquots (0.1 mL) of diluted samples were spread on nutrient media in duplicate. To obtain detailed diversity results, three types of media were used; Wallerstein Laboratory Nutrient agar (WLN) (BioLab, Merck, South Africa), supplemented with 34 mg/L chloramphenicol, 150 mg/L biphenyl for total yeast enumeration, WLN agar with 34mg/L chloramphenicol, 150 mg/L biphenyl and 1 mg/L cycloheximide for non-*Saccharomyces* enumeration and Yeast peptone dextrose (YPD),

supplemented with 50 mg/L chloramphenicol, 25 mg/L kanamycin and 150 mg/L metabiosulfite and 120 ml/L ethanol for *Saccharomyces* enumeration. The chloramphenicol and kanamycin, were added to the media to inhibit the growth of bacteria and acetic acid bacteria (AAB) whereas, biphenyl and cycloheximide were added to inhibit the growth of filamentous fungi and *Saccharomyces* species respectively. The plates were incubated in 30 °C for 3-5 days until growth was observed. Yeast isolation was performed using the plates comprising 30 - 300 colonies. The colonies were selected and further categorized based on the colony morphology including colour, texture, elevation, shape and size. Pure culture were obtained by repetitive streaking of the colonies on WLN agar and Yeast extract peptone dextrose agar (YEPD: composed of 10 g/L yeast extract, 20 g/L bacteriological peptone, 20 g/L dextrose and 20 g/L agar), respectively. The plates were incubated for further analysis at 30 °C for 3-5 days until the growth was observed.

3.3.4 DNA extraction

From each vineyard, a total of 140 isolates obtained from grape must and samples were subjected to the further identification using molecular techniques. In addition, 150 isolates obtained from the beginning, middle and end of the fermentation of the 2013, must samples were also identified. The genomic DNA of each isolate was extracted from the fresh yeast culture, using the protocol as described by Hoffman and Winston, (1987). Yeast cells were cultivated in 5 mL of YPD broth (BioLab, Merck, SA) for 16 h. One millilitre of samples were taken and centrifuged for 60 seconds at 5000 rpm. The pellets were re-suspended in 100 µL of STES buffer (0.2 M Tris-cl, 0.5 M Nacl, 0.1 % (w/v) SDS, 0.01 M EDTA). Hundred microliters of glass-beads, 130 µL phenol-chloroform – isoamyl alcohol (25:24:1) was added to the pellet. The tubes were vortex for 3 minutes, followed by 5 minutes centrifuge in 6000 rpm. The supernatant was transferred to fresh tubes. Chloroform extraction was performed and the DNA was precipitated using standard protocols and re-dissolved in TE buffer.

The amplification of ITS region of the isolate were carried out in the gene amplification PCR system 2700 thermo cycler (Applied Biosystems, Life Technologies, Johannesburg, South Africa) using ITS1 primer (5'TCC GTA GGT GAA CCTTGC GG 3') and ITS 4 (5'TCC TCC GCT TAT TGA TAT GC 3') (White et al., 1990). The amplification reaction was performed in the final volume of 25 µL comprising 1 µL DNA (diluted to 100 ng), 1U Takara Ex Taq™ DNA polymerase (TaKaRa Bio Inc., Olsu, Shiga, Japan), 1 x Taq buffer, 0.25 µM of each primer, 400 mM dNTP mix and 1 mM MgCl₂. The PCR was run under the following conditions: an initial denaturation cycle of 3 min at 94°C , followed by 40 cycles consisting of, 30 s at 94°C, 30 sec at 54°C and 45 s at 72°C and the final extension step of 7 min (Esteve-Zarzoso et al., 2001). PCR products were evaluated on 1% (w/v) agarose gel prepared in 1X Tris-Acetic acid-EDTA (TAE) buffer, stained with GelRed™. The gel was visualised under ultraviolet light. A GeneRuler™ 100 base pair (bp) plus DNA ladder (Fermentas, South Africa) served as the standard size. The PCR products were excised from the

gel and purified using the Zymoclean™ Gel DNA Recovery Kit Short Protocol (Zymo Research Corporation, Irvine, CA, USA).

3.3.5 Yeast isolates identification

PCR-RFLP was employed for the identification of the yeast isolates. The PCR products were digested in separate reactions with three restriction endonucleases *HaeIII*, *Hinfl* and *CfoI* (Thermo Scientific, Inqaba Biotechnologies, Pretoria, South Africa) as described by Querol et al. (1994). The fragments were separated on 1.2% (w/v) agarose gel, for 2 hours at 80 V. Fragment sizes were determined with the aid of the 100 bp plus DNA ladder. The gel were analysed under the UV and documented by photography. Individual banding profiles were obtained for different yeasts. The isolates were categorized based on the comparison of obtained restriction profiles. Three representatives of each group were subjected for PCR-ITS fragment sequencing at the Central Analytical Facility, Stellenbosch University.

Sequences were processed and aligned in BioEdit and basic local alignment search tool (BLAST) algorithm was used to compare the sequences with published fungal ITS-5.8S rRNA gene sequences in the national centre for biotechnology information (NCBI) Genbank database on (<http://www.ncbi.nlm.nih.gov/blast.cgi>). The identification was considered as valid when the sequences displayed 97 – 100% sequence identity to a known species.

3.3.6 *Saccharomyces* strain identification

The identities of *Saccharomyces cerevisiae* isolates were first confirmed by amplification of the ITS-5.8S rRNA gene followed by digestion of amplicons with *HaeIII*. Isolates which displayed the typical *HaeIII* digest profile of *S. cerevisiae* were then subjected to interdelta PCR for strain differentiation. The PCR amplification of δ region was performed using the protocol described by Hoff (2012). The amplification was carried out using the Bio- Rad thermal cycler, using the one set of delta primers as suggested by Legras & Karst, (2003), forward primer, delta 12 (5'-TCAACAATGGAAATCCCAAC-3') and reverse primer, delta 21 (5'-CATCTTAACACCGTATATGA-3'). The PCR mixture was prepared in the final volume of 25 μ L as described before. The PCR reaction was started by the initial denaturation (95°C for 4 min) followed by 35 cycles of (95°C for 30 s, 48°C for 30 s, 72°C for 90 s) and a final extension at 72°C for 10 min. The amplification products were separated through gel electrophoresis on 1.2% (w/v) agarose gels at 80 V (constant voltage) for 2.5 h in 1 \times TAE buffer. The gel was visualised under ultraviolet light. A Gene Ruler™ 100 base pair (bp) plus DNA ladder (Fermentas, South Africa) served as the standard size marker. The similarity between the samples was evaluated by cluster analysis using the GENE Directory Application software Version 2.01.01 (copy right 2000-2010, Ltd, Vacutec, South Africa).

3.4 RESULTS

3.4.1 Chemical analysis of grape musts

The chemical parameters of musts were determined, using the Foss wine scan (Denmark). The chemical analysis followed the same composition trend of sugar, total acidity, pH among musts samples. The musts sugar in 2012 was between 20.7-24.3 whereas the higher rate of sugar was observed for biodynamic and conventional must in 2013 vintage (Table 3.1). The similar amount of sugar and total acidity was observed for must samples in both vintages.

Table 3.1 Principal oenological parameters in the grape musts

Parameters	2012 Juice			2013 Juice		
	CONV	BD	IPW	CONV	BD	IPW
Sugar (°Brix)	20.7 (± 1.32)	23.4 (± 1.54)	24.3 (± 1.87)	25.6 (± 0.02)	25.4 (± 0.09)	23.4 (± 0.02)
pH	3.35 (± 0.16)	3.61 (± 0.21)	3.66 (± 0.12)	3.49 (± 0.01)	3.51 (± 0.01)	3.55 (± 0.17)
TA (g/L)	3.85 (± 0.51)	3.23 (± 0.75)	2.34 (± 0.62)	4.28 (± 0)	3.96 (± 0)	3.65 (± 0.07)

3.4.2 Fermentation kinetics

The alcoholic fermentation of the 3 musts proceeded to completion (Glucose, fructose < 2 g/L) of fermentation of grape must sugar to the dryness between 22-41 days. However, the fermentations have not proceeded at the same rate. Fermentation of the must from BD vineyard progressed rapidly and was completed in 24 days while the integrated and conventional took approximately 40 days to reach dryness. The initial yeast population in the must from the biodynamic vineyard started at 10^6 cfu/mL and increased to 10^8 cfu/mL after which it stabilized until the end of fermentation with a slight decrease to 10^7 cfu/mL (Figure 3.2). The non-*Saccharomyces* yeast population displayed the slight increase from 10^6 cfu/mL to 10^8 cfu/mL after 4 days of fermentation followed by the steady decline until it dropped below detection level after 90% of the sugar was consumed whereas the *Saccharomyces* population was 3% of the total population in grape must. However, its population rapidly increased to 10^7 cfu/mL in the first 2 days of fermentation. The *Saccharomyces* yeast population reached a maximum of 10^8 cfu/mL and remained stable until the end of fermentation then decreased slightly to 10^7 cfu/mL. On the other hand, conventional must exhibited the total population of 10^4 cfu/mL in which the *Saccharomyces* population was below the detection level (Figure 3.3). The total yeast population reached maximum of 10^7 cfu/mL on day 4 and remained stable until the end of alcoholic fermentation. Initial levels of total non-*Saccharomyces* spp. yeasts were 10^4 cfu/mL, but increased to 10^7 cfu/mL after 4 days of fermentation and then gradually decreased until the population was below detection level in which 90% of sugar has been consumed. Similarly, integrated must displayed a total population of 10^5

cfu/mL and the *Saccharomyces* population was below the detection level in the first two days (Figure 3.4). The non-*Saccharomyces* population started at 10^4 cfu/mL on day zero and rapidly increased to 10^7 cfu/mL on the fourth day of fermentation. The non-*Saccharomyces* population started to decline gradually from day 8 and their population was below detection level after the 16th day of fermentation, whereas the population of *Saccharomyces* species which was below detection in grape must increased to 10^7 cfu/mL in the first 4 days of fermentation and remained stable until the end of fermentation.

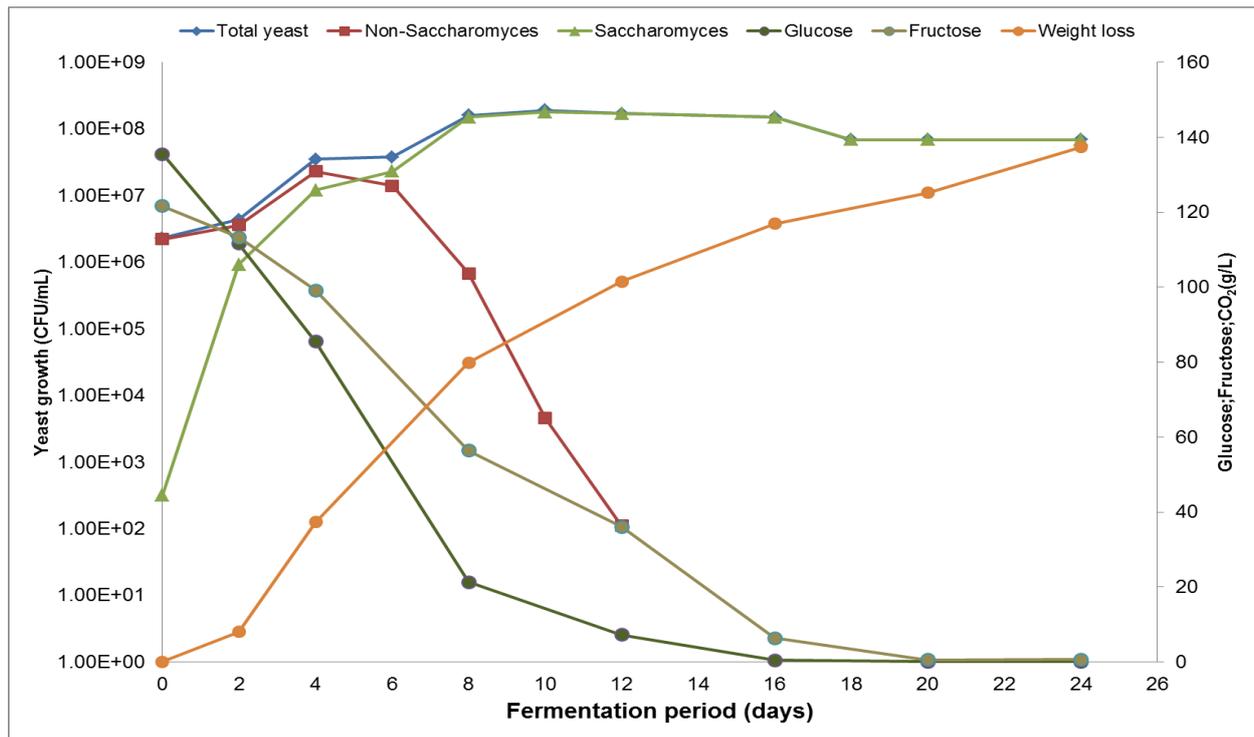


Figure 3.2 2013 Biodynamic spontaneous fermentation. Yeast population dynamics and the content of sugar reduction during fermentation.

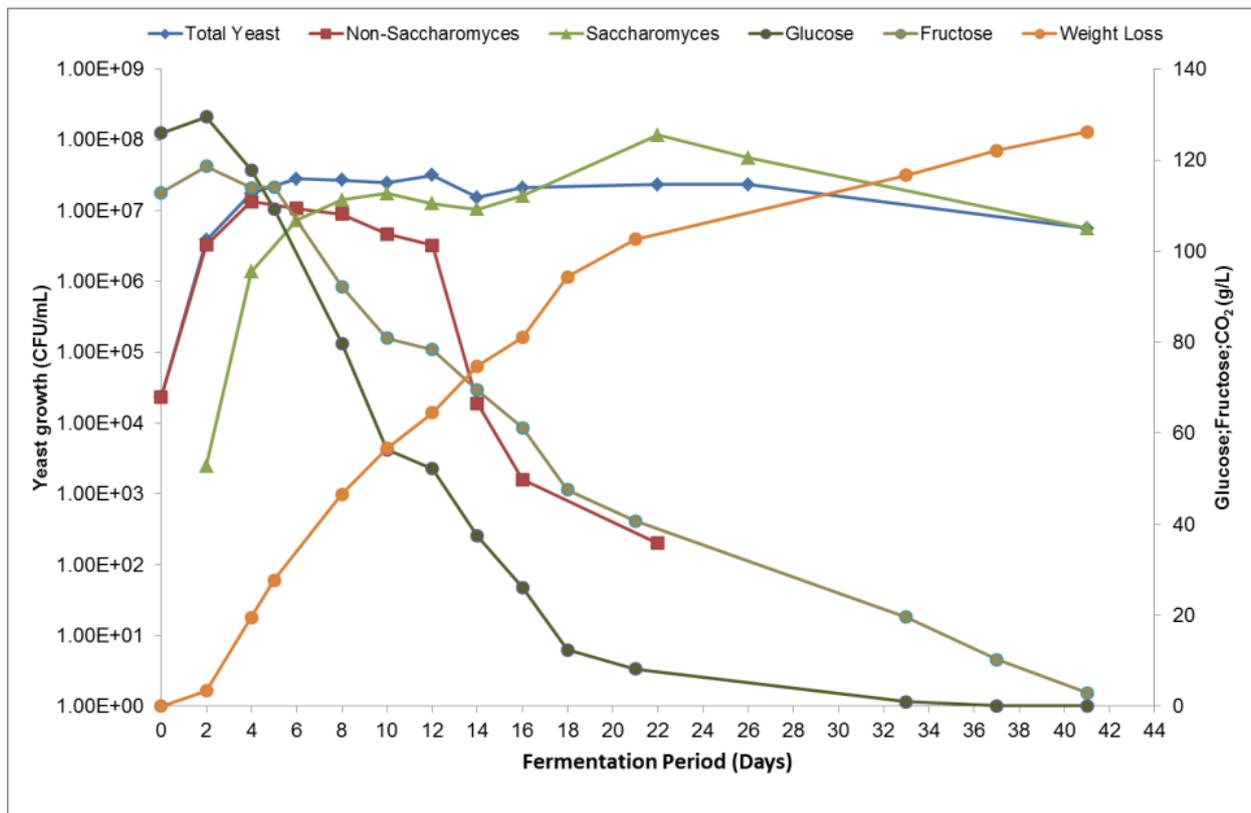


Figure 3.3 2013 Conventional spontaneous fermentation. Yeast population dynamics and the content of sugar reduction during fermentation.

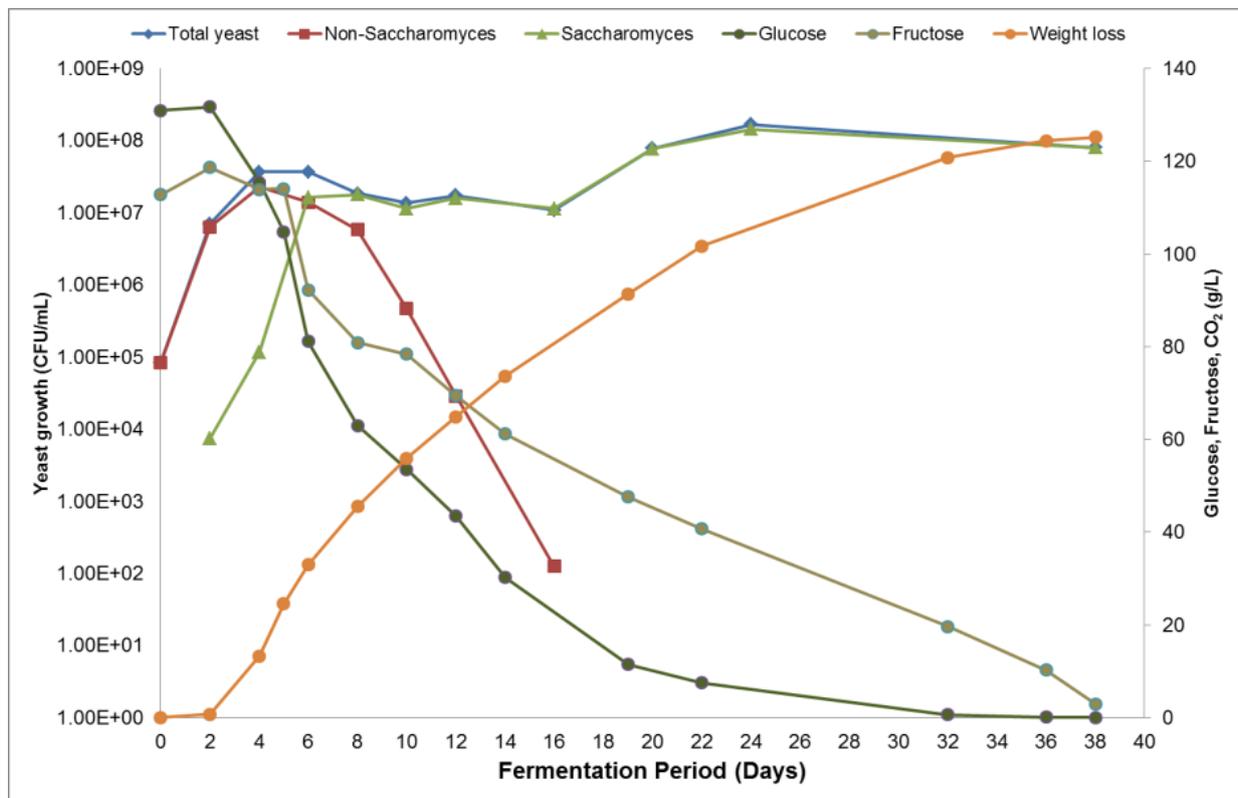


Figure 3.4 2013 Integrated spontaneous fermentation. Yeast population dynamics and the content of sugar reduction during fermentation.

3.4.3 Yeast isolation and identification

A total of 600 colonies including 510 colonies representative of the diversity found in all analyzed grape samples from WLN and 90 colonies from YPD were isolated, purified and categorized on the basis of the appearance of colony morphology on WLN. At least three cultures from each colony morphology type were subjected to molecular identification. PCR-RFLP was used for the initial grouping of the yeast isolates. For instance, biodynamic must in 2013 exhibited 12 different banding profiles, using restriction enzyme *Hinf*I. Three representatives of each group were subjected to further analysis with *Hae* III and *Cfo*I. If all the samples in the group exhibited the same banding profile (Figure 3.5), a representative of each group was submitted for sequencing. In total 8, 4 and 1 banding profiles were obtained for 2012 musts whereas, 12, 11 and 9 different groups were identified for 2013 musts obtained from the biodynamic, conventional and integrated vineyards, respectively. The identity of the organisms was confirmed by sequencing of the ITS-5.8S rRNA region. Table 3.2 shows a summary of the yeast isolates obtained and confirmed by sequencing.

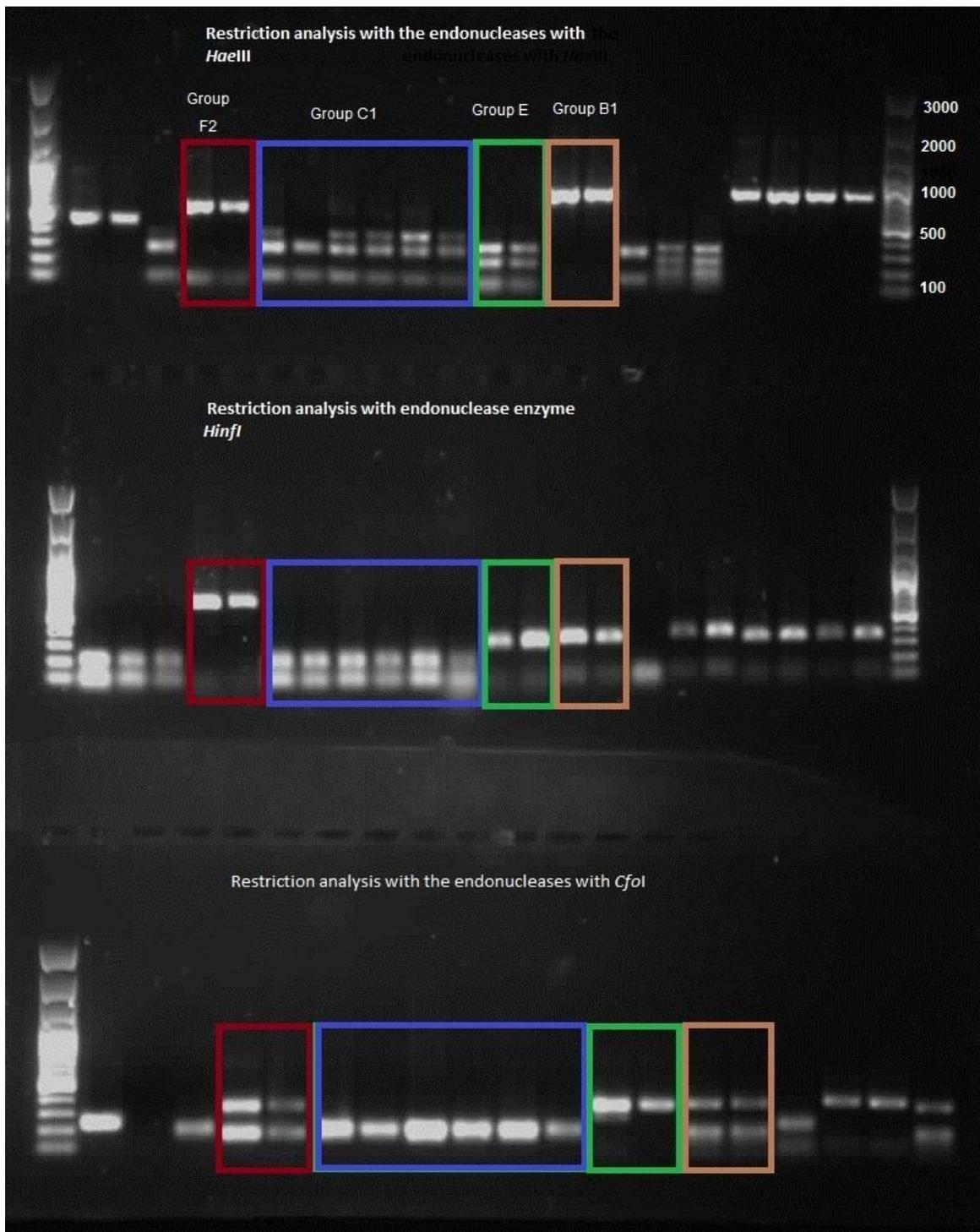


Figure 3.5 Restriction analysis with the endonuclease enzymes *Hae III* and *CfoI* and *Hinf I*

Table 3.2 Restriction fragment lengths of ITS-5.8S rRNA regions of different yeast species

Colony morphotypes on WLN medium	ITS-5.8S rRNA amplicon size	ITS-5.8S rRNA restriction fragment sizes			Species ^a	% Identity
		<i>HaeIII</i>	<i>HinfI</i>	<i>CfoI</i>		
A	600	150, 450	290, 180, 130	190, 180, 100	<i>Aureobasidium pullulans</i>	100
B1	770	690	340, 200, 170	320, 315, 105	<i>Hanseniaspora uvarum</i>	100
B2	810	810	370, 205, 175, 75	335, 115	<i>Hanseniaspora guilliermondii</i>	98
B3	780	300, 100	200, 180	200, 90	<i>Hanseniaspora vineae</i>	98
C	390	285,100	200,190	210,100	<i>Metschnikowia pulcherrima</i>	100
C2	370	280	100, 220, 310	210, 100	<i>Metschnikowia crysoperlae</i>	98
D1	800	675	360,270	400,395	<i>Candida glabrata</i>	98
D2	500	500	240, 125	205, 175	<i>Candida apicola</i>	98
D3	475	475	235, 235	210, 110	<i>Candida zemplinina</i>	100
D4	550	410, 115	260, 290	300, 250	<i>Candida parapsilosis</i>	98
D5	460	460	250	150, 240	<i>Candida azyma</i>	98
D6	480	100, 500	200, 320	600	<i>Candida pomicola</i>	97
E	720	340, 220, 85	315	315, 290	<i>Lachancea thermotolerans</i>	99
F	630	500, 70, 60	350, 280	330, 300	<i>Cryptococcus</i>	99

					<i>bhutanensis</i>	
F2	600	170, 400	250, 350	100, 320, 400	<i>Cryptococcus carnescens</i>	99
G	800	800	100, 230	100, 300, 400	<i>Rhodosporidium diobovatum</i>	100

Table 3.2 (cont.)

Colony morphotypes on WLN medium	ITS-5.8S rRNA amplicon size	ITS-5.8S rRNA restriction fragment sizes			Species ^a	% Identity
		<i>HaeIII</i>	<i>HinfI</i>	<i>CfoI</i>		
H	650	100, 450	150, 200, 280	300	<i>Rhodotorula nothofagi</i>	97
H2	640	430, 210	340, 225, 75	320, 240, 80	<i>Rhodotorula glutinis</i>	98
I	650	700	310	650	<i>Wickerhamomyces anomalus</i>	96
J	845	845	410, 380	320, 210, 130, 100	<i>Torulasporea delbrueckii</i>	97
K	750	750	200,300,400	50,100,200,400	<i>Kazachstania aerobia</i>	98
L	780	300	150, 200, 250, 350	220, 250, 300	<i>Phaeomoniella prunicola</i>	100
M	430	290,130	225,105,105	120,95,78,71,58	<i>Issatchenkia terricola</i>	99
N	840	300,220,175,125	360,360,120	380,340	<i>Saccharomyces cerevisiae</i>	100

^a Yeast species were confirmed by sequence analysis of the 5.8S-ITS region

During the 2012 vintage, *Aureobasidium pullulans* was the most abundant species (31.6%) followed by *Kazachstania aerobia* (26.6%) and *Metschnikowia crysoberlae* (13.3%), while *Phaeomoniella prunicola*, *Metschnikowia pulcherrima*, *Hanseniaspora uvarum* and *S. cerevisiae* were present in lower amounts in the must from the biodynamic vineyard (Figure 3.6). In contrast, the species of the genus *Hanseniaspora* (e.g. *H. uvarum*, *H. guilliermondii* and *H. vineae*) were dominant in the 2013 must accounting for 23.6% of the population followed by *C. zemplinina* (21.7%) and *M. pulcherrima* (19.2%), while members of the genera *Aureobasidium*, *Candida*, *Issatchenkia*, *Lachancea* and *Cryptococcus* were minor. In the conventional vineyard, four different non-*Saccharomyces* species were isolated from the 2012 must. *K. aerobia* accounted for almost half of the yeast population (48.5%) followed by *A. pullulans* (28.3%), *H. uvarum* (20%) and *Issatchenkia terricola* (3.33%). In the 2013 vintage *M. pulcherrima* (20%), *H. uvarum* (17.5%), *C. zemplinina* (15.9%), *L. thermotolerans* (15.3%) and *A. pullulans* (12.7%) were dominant in the must, while *Candida azyma*, *I. terricola*, *Rhodotorula* sp., *Rhodospiridium* sp., and *Cryptococcus* sp., were present in lower amounts (Figure 3.6). *H. uvarum* was the only yeast isolated from the must from the integrated vineyard in 2012 whereas higher diversity was observed from the 2013 vintage. *A. pullulans* (34.6%) and *H. uvarum* (21.5%) were the most abundant species followed by *Torulasporea delbrueckii* (12.3%), while *Wickerhamomyces anomalus*, *Rhodotorula* sp., *Rhodospiridium diobovatum*, *C. azyma* and *L. thermotolerans* together constituted less than 30% of the population (Figure 3.6).

The ecological diversity indices were calculated based on the number of identified species for the musts obtained from different vineyards (Table 3.3). The biodynamic must displayed the highest species evenness (Pielou's index) and the lowest species dominance (D) whereas, integrated vineyard exhibited the lowest species evenness (Pielou's index) and highest species dominance (D).

Table 3.3 Ecological diversity indices demonstrating total cultivable yeast diversity in the biodynamic, conventional and integrated vineyards.

Ecological index parameters	Biodynamic	Conventional	Integrated
Species richness (Menhinick's index)	1.26	1.01	0.75
Species Diversity (Shannon Weiner index)	2.32	2.05	1.34
Species diversity (Simpson index)	7.86	6.38	2.68
Species dominance (D)	0.13	0.16	0.37
Species evenness (Pielou's index)	0.86	0.82	0.61

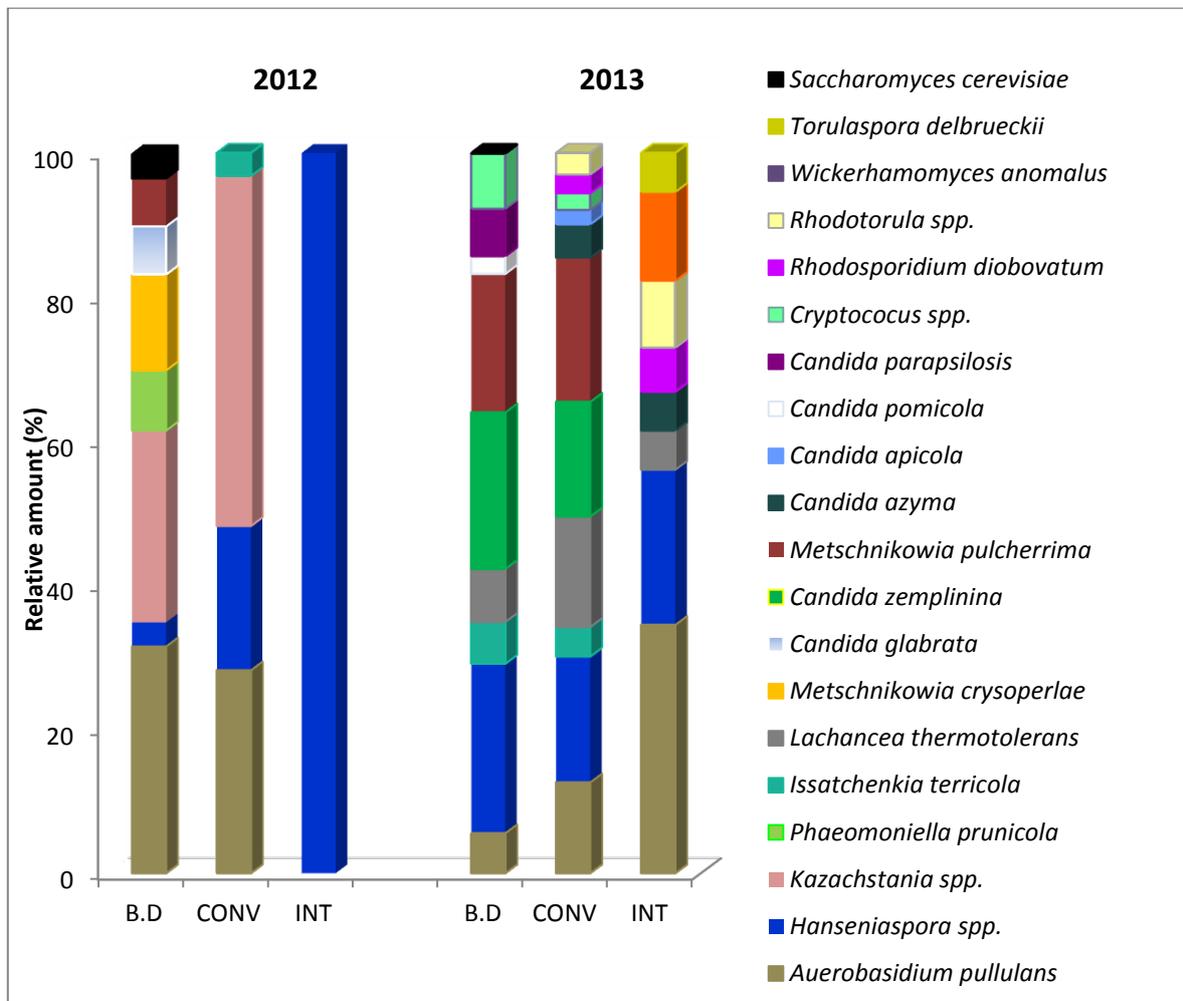


Figure 3.6 Yeast diversity and occurrence during in grape must .

3.4.4 Yeast population dynamics during spontaneous fermentation

The grape musts of all three farming systems were dominated by common non-*Saccharomyces* species such as, *H. uvarum*, *M. pulcherrima* and *A. pullulans*. As the fermentations proceeded the non-*Saccharomyces* species decreased and the ethanol tolerant *Saccharomyces* species were dominant and completed the fermentations. It is worth mentioning that only the yeast population dynamics of 2013 are available.

The must from the biodynamic vineyard displayed 12 different species at the onset of fermentation, however, in the beginning stage of fermentation (12.5% of sugar consumption), the number decreased to 5 species comprising, *Candida parapsilosis*, *C. zemplinina*, *H. uvarum*, *M. pulcherrima* and *S. cerevisiae*. As the fermentation proceeded the population of *S. cerevisiae* increased rapidly from 10^2 cfu/mL to 10^7 cfu/mL and accounted for 20% of population after 25% of sugar consumed, whereas the population of *C. parapsilosis*, *C. zemplinina*, *H. uvarum*, *M. pulcherrima* decreased to 16.9%, 7%, 8.9%, and 2.5%, respectively (Figure 3.7). The late stages of fermentation were dominated by *S. cerevisiae* (96%), however, *C. parapsilosis* still accounted for 4% of the population after 70% of the sugars were consumed. On the other hand, eleven different

species initiated the fermentation in the must from the conventional farming system followed by the decrease in the first 5 days of fermentation to 6 different species dominated by *H. uvarum*, whereas *L. thermotolerans*, *C. zemplinina*, *M. pulcherrima*, *C. azyma* and *S. cerevisiae* were present in lower amounts. The middle of fermentation (25-50% sugar consumption) was mainly dominated by ethanol tolerant yeast *S. cerevisiae* at the level of 83.4% of the total yeast population followed by the minor isolates of *H. uvarum*, *L. thermotolerans* and *C. zemplinina* (Figure 3.7). In the must from the integrated farming systems 9 different species were found. The number of the species declined to 6 after 12.5% of sugar consumption. *H. uvarum* (66%) was the most dominant species, however, *Wickerhamomyces anomalus* (14%), *T. delbrueckii* (8.9%) and *C. azyma* (4.6%) were present in lower percentages. The middle of fermentation was mainly dominated by *S. cerevisiae* at more than two thirds of the total population (73.3%). However; *W. anomalus*, *T. delbrueckii* and *C. azyma* remained persistent (Figure 3.7).

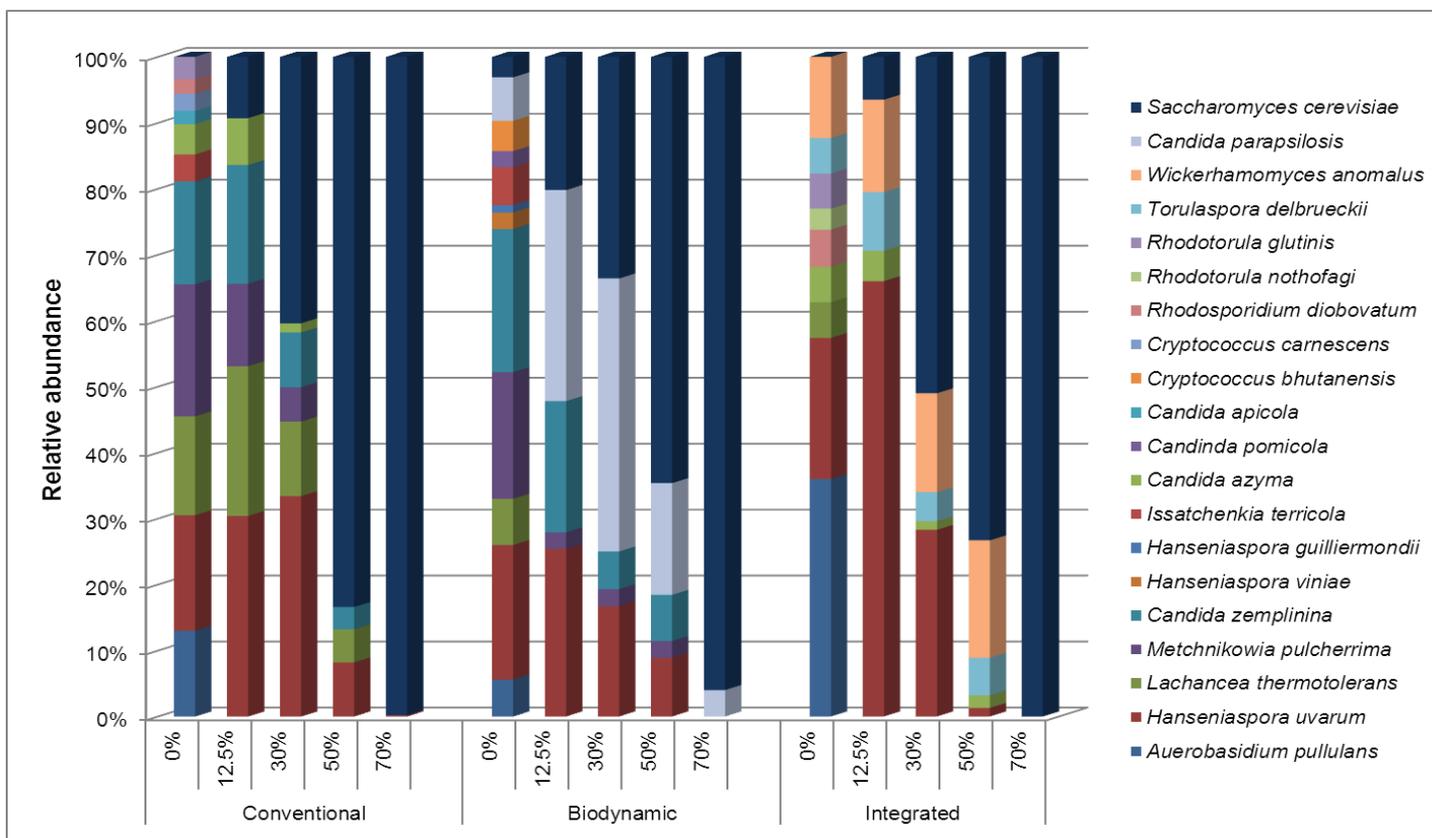


Figure 3.7 Distribution of yeast species (%) during spontaneous fermentations of three grape musts at different sugar consumption levels (2013 vintage).

3.4.5 Genetic characterization of *Saccharomyces cerevisiae* isolates

Delta PCR analysis of *S. cerevisiae* isolates from must till the end of alcoholic fermentation clearly demonstrated the replacement of some strains by others. Therefore, successional development of different strains of *S. cerevisiae* was observed throughout alcoholic fermentations. Delta PCR analyses revealed 15, 10 and 18 different profiles in the conventional, biodynamic and integrated

fermentations, respectively. Some strains were detected only in the beginning, middle or end of fermentation while others persisted from beginning until the end of fermentation. For instance, in the conventional profile II and III could only be isolated at the beginning of fermentation while profiles VII, VIII, IX were only present in the middle of fermentation (Table 3.3). Profile I, IV and V were present in all stages of fermentation although profile I was isolated as the dominant strain in the end of fermentation.

Ten different profiles could be detected in biodynamic fermentation. However, only profile I was present throughout the whole alcoholic fermentation process and was the only dominant profile at the end of alcoholic fermentation (Table 3.4). Profiles II, IV, V and VI could only be isolated in the beginning of fermentation whereas profiles VII and VIII were only present in the middle of fermentation. Eighteen different profiles were detected in the integrated fermentation. Of these 12 (profiles I to XII) were present in the beginning (Table 3.5). However, only profiles, II, V, VI and II, VII were persistent until the middle of fermentation. Profiles, XIII, XIV and XV were detected only in the middle of fermentation. The end of fermentation was mainly dominated by profiles V and VI whereas profiles I, XI, XIV and XV were present in the lower amount (Table 3.5). Profiles XVI, XVII, XVIII were only detected at the end of fermentation.

Table 3.3 Distribution of *Saccharomyces cerevisiae* strains (%) during different stages of 2013 conventional spontaneous fermentation including: Beginning (BF), Middle (MF), End (EF).

Conventional BF		Conventional MF	Conventional EF
Banding profile	Number of isolates (total)	Number of isolates (total)	Number of isolates (total)
I-conv	10/30	9/30	11/27
II-conv	6/30	0/30	0
III-conv	2/30	0/30	0
IV-conv	6/30	1/30	2/27
V-conv	4/30	2/30	3/27
VI-conv	2/30	3/30	0
VII-conv	0	5/30	0
VIII-conv	0	3/30	0
IX-conv	0	2/30	0
X-conv	0	1/30	1/27
XI-conv	0	1/30	0
XII-conv	0	1/30	3/27

XIII-conv	0	1/30	2/27
XIV-conv	0	1/30	4/27
XV-conv	0	0	1/27

Table 3.4 Distribution of *Saccharomyces cerevisiae* strains (%) during different stages of 2013 biodynamic spontaneous fermentation including: Beginning (BF), Middle (MF), End (EF)

Biodynamic BF		Biodynamic MF	Biodynamic EF
Banding profile	Number of isolates (total)	Number of isolates (total)	Number of isolates (total)
I-BD	10/20	15/18	17/17
II-BD	5/20	0	0
III-BD	2/20	1/18	0
IV-BD	1/20	0	0
V-BD	1/20	0	0
VI-BD	1/20	0	0
VII-BD		1/18	0
VIII-BD		1/18	0
IX-BD			0
X-BD			0

Table 3.5 Distribution of *Saccharomyces cerevisiae* strains (%) during different stages of 2013 integrated spontaneous fermentation including: Beginning (BF), Middle (MF), End (EF)

Integrated BF		Integrated MF	Integrated EF
Banding profile	Number of isolates (total)	Number of isolates (total)	Number of isolates (total)
I-IPW	7/25	0/27	3/24
II-IPW	1/25	2/27	0/24
III-IPW	1/25	0/27	0/24
IV-IPW	1/25	0/27	0/24
V-IPW	7/25	8/27	5/24
VI-IPW	2/25	10/27	4/24
VII-IPW	1/25	0/27	0/24
VIII-IPW	1/25	2/27	0/24

IX-IPW	1/25	0/27	0/24
X-IPW	1/25	0/27	0/24
XI-IPW	1/25	0/27	3/24
XII-IPW	1/25	0/27	0/24
XIII-IPW	0	1/27	0/24
XIV-IPW	0	2/27	2/24
XV-IPW	0	2/27	2/24
XVI-IPW	0	0	2/24
XVII-IPW	0	0	2/24
XVIII-IPW	0	0	1/24

3.5 DISCUSSION

The current study evaluated the diversity of yeasts associated with Cabernet Sauvignon grape must obtained from biodynamic, conventional and integrated farming systems. The dynamics of the yeast population throughout spontaneous fermentation were also monitored. The yeast population in the grape musts obtained from biodynamic, conventional and integrated farming system in two vintages (2012 and 2013) ranged from 10^2 - 10^4 cfu/mL, which is in line with what has been reported in other studies (Loureiro et al., 2003; Barata et al., 2008). Over the 2012 and 2013 harvest, a diverse group of yeasts were isolated from the must samples and throughout fermentations. The yeast population in grape musts mainly comprised non-*Saccharomyces* yeast species, whereas *S. cerevisiae* was below detection level in conventional and integrated musts and was only isolated in the biodynamic must representing 3% of the total yeast population in 2013. The scarce isolation of *S. cerevisiae* in the grape musts has been reported by several authors, confirming that this species is not dominant in the farming systems and if present, it only occurs at approximately 10 – 100 cfu/g berries or less (Martini et al., 1996; Mortimer et al., 1999; Combina et al., 2005; Mercado et al., 2007; Di Maro et al., 2007; Guzzon et al., 2011).

In 2012, *H. uvarum* was the sole yeast isolated in the must from the integrated farming system, while 8 and 5 species were isolated from biodynamic and conventional farming systems, respectively. In general, fermentative yeast species including *H. uvarum*, *Kazachstania* sp., *Metschnikowia pulcherrima*, *Candida* spp., *Lachancea thermotolerans*, *Torulaspora delbrueckii*, and *Issatchenkia terricola* were the most common isolates in must samples. The sole isolation of *H. uvarum* in the integrated farming system was unusual; however, this yeast is often the most dominant in grape must and in some cases, it has been reported to account for 75% of the total population (Bezerra-Bussoli et al., 2013). Therefore it is possible that the other species were significantly lower on the grape surface relative to *H. uvarum* and therefore diluted below detection.

On the other hand, in the 2013 vintage the yeast population in the must from the integrated farming system was more diverse, including *Candida zemplinina*, *Wickerhamomyces anomalus*, *Torulasporea delbrueckii*, *Lachancea thermotolerans* and oxidative yeasts of the genera *Aureobasidium*, *Cryptococcus* and red pigmented species (*Rhodosporidium* and *Rhodotorula*). In contrast, the biodynamic must comprised 86.77% weakly fermentative species (e.g. *Candida*, *Hanseniaspora* and *Metschnikowia*) and 13.23% of oxidative yeasts (e.g. *A. pullulans* and *Cryptococcus* spp.) whereas, the conventional must comprised 79.4% weakly fermentative yeasts (e.g. *Hanseniaspora*, *Candida* and *Metschnikowia*) and 20.6% oxidative yeasts (*Cryptococcus*, *Rhodotorula* and *Rhodosporidium*). *A. pullulans*, *H. uvarum* and *M. pulcherrima* were the most common isolates in both vintages. The frequent isolation of these yeasts in grape musts has been reported by several authors (Settanni et al., 2012; Cordero-Bueso et al., 2011; Sun et al., 2009). A high diversity of *Candida* species such as *C. azyma*, *C. parapsilosis*, *C. pomicola*, *C. apicola*, *C. glabrata* and *C. zemplinina* was isolated during both vintages and in particular in 2013 must. *C. zemplinina* has been most commonly isolated from botrytized grape must or in the juice with high sugar concentration (Torija et al., 2001; Sun et al., 2009; Settanni et al., 2012; Milanović et al., 2013). Therefore, the high incidence of this species in the 2013 grape musts from the three farming systems could be indicative of the presence of overripe berries in the musts. *C. azyma* was isolated from conventional and integrated must whereas, *C. parapsilosis* could be only detected in biodynamic must in 2013 vintage. Although *C. azyma* has been previously associated with the sugar cane and lichens and bees from Convolvulaceae, its presence on Bangalore blue and Cabernet grape varieties has been reported (Chavan et al., 2009). Regarding the differences observed in the yeast composition and yeast concentration, the slight tendency toward higher yeast diversity was revealed in 2013 must in comparison with 2012 must. The discrepancies in yeast diversity might be due to the changes in the climatic condition in 2012 and 2013 vintage that have been suggested by previous authors (Li et al., 2010; Bezerra-Bussoli et al., 2013). The discrepancy in yeast diversity in 2 consecutive years is not unusual and has been reported by other researchers (Torija et al., 2001; Sun et al., 2009; Settanni et al., 2012).

The results of the current study demonstrated that the musts obtained from different farming systems, share certain common yeast species such as *Hanseniaspora*, *Aureobasidium* and *Metschnikowia* which are permanently present in the grape must regardless of the farming system (Combina et al., 2005; Cordero-Bueso et al., 2011; Sun et al., 2009). In spite of these common isolates, the differences of occurrence of individual minor species in each farming system were also observed. For instance, *Phaeoemoniella prunicola*, *C. glabrata*, *C. parapsilosis*, *C. pomicola*, *M. cryosperlae* and *S. cerevisiae* were only isolated from biodynamic farming system while *W. anomalus*, *T. delbrueckii* and *Rhodotorula nothofagi* were present only in the integrated must and also a high diversity (20%) of red pigmented basidiomycetous yeasts of the genera *Rhodotorula* and *Rhodosporidium* were isolated from integrated must in 2013 vintage.

The species of the genus *Hanseniaspora*, mainly *H. uvarum* have been widely reported as the most frequent species (50-75%) of the total population of grape must that can reach up to 10^7 - 10^8 cfu/mL, in the low altitudes and warm climatic conditions (Pretorius et al., 1999; Romancino et al., 2008; Settani et al., 2012; Bezerra-Bussoli et al., 2013). In addition, *Hanseniaspora guilliermondii* and *Hanseniaspora vineae* were also isolated in the musts of warm region (Xufre et al., 2006; Di Maro et al., 2006; Nisiotou et al., 2007; Romancino et al., 2008; Settanni et al., 2012). Cadez et al. (2002) clearly demonstrated the high growth adaptation level in different strains of *H. uvarum* and *H. guilliermondii* at 34°C and 37°C, respectively. The frequent isolation of the species of the genus *Metschnikowia*, mainly *M. pulcherrima* (10-13%) was also reported by previous authors (Nguyen et al., 1998; Settanni et al., 2012). The high isolation of this species was attributed to their inhibitory effect against various yeast species (Nguyen et al., 1998; Settanni et al., 2012). The sole isolation of *S. cerevisiae* and higher isolation of *Candida* species in biodynamic farming system might be due to the absence of synthetic fungicides (Cordero-Bueso et al., 2011; Setati et al., 2012). However, the higher isolation of these species might also be due to the indirect transition by insects. Overall, the biodynamic musts displayed higher diversity compared to conventional and integrated must in both vintages. However, the diversity does not only depend on the number of the species found in each must (richness), but also on the dominance (D) and the abundance of each species. Regarding the diversity obtained from different musts, no significant difference was observed in Species evenness (Pielou's index) and Species dominance (D) between conventional and biodynamic must, however, the differences are significant in comparison with integrated must. Therefore, the biodynamic must exhibited the highest diversity and lowest dominance whereas, the integrated must exhibited the lowest diversity and highest dominance. Similarly, Tello et al. (2011) and Cordero-Bueso et al. (2011) reported the higher diversity and lower dominance of organic farming system compared to conventional farming system. In contrast, Milanović et al. (2013), demonstrated the higher diversity in comparison with organic farming system.

The three must fermentations displayed similar trends and dynamics. However, fermentation of the must from the biodynamic farming system proceeded at a faster rate in comparison with the other two fermentations. This might be due to the higher initial total yeast population which also comprised a higher percentage of fermentative yeasts as well as the rapid development of the *S. cerevisiae* population. Oxidative basidiomycetous yeasts as well as the ascomycetous yeast-like fungus *A. pullulans* were present in all the must samples, however, none of these yeasts could persist beyond the initial stage of fermentation probably due to the reduction in oxygen levels as the fermentation begins. In all cases, only the strongly fermentative yeast, *S. cerevisiae* and fermentative species such as *C. zemplinina*, *C. azyma*, *C. parapsilosis*, *H. uvarum*, *L. thermotolerans*, *M. pulcherrima*, *T. delbrueckii* and *W. anomalus*, could persist through the various stages of spontaneous fermentation. Similar to observations made in other studies (Combina et al.,

2005; Cordero-Bueso et al., 2011; Sun et al., 2009), the beginning of all three fermentations was dominated by weakly fermentative yeasts of the genera *Candida* and *Hanseniaspora* at the level of 79.8%, 90.7 and 93.5 for the musts obtained from the biodynamic, conventional and integrated farming systems, respectively. On the other hand, by the middle of the fermentation, the heterogeneity of the yeast species decreased in favour of the strongly fermentative yeast (*S. cerevisiae*) probably due to the sensitivity of some non-*Saccharomyces* species to increasing ethanol levels and the low amount of oxygen (Combina et al., 2005; Cordero-Bueso et al., 2011). By the middle of the fermentation *S. cerevisiae* accounted for 64.5 %, 73.3% and 83.4% of the total yeast population for biodynamic, integrated and conventional, respectively. Interestingly, *L. thermotolerans* which was present in all three musts could only persist until the middle stage of fermentation of the must from the conventional farming system. The persistence of this species in conventional fermentation might be in part due to the higher population of this species in the conventional must in comparison with biodynamic and integrated must. In addition, the yeast-yeast interactions might have also played a role in the dynamics of yeasts present in fermentation since the community of yeasts present in each must is slightly different. For instance, the species such as *W. anomalus* that has been previously reported to produce killer toxins (Yong Sun et al., 2012; Sabel et al., 2013) might have suppressed the growth of *L. thermotolerans* in the must from the integrated farming system, while in the biodynamic must fermentation, the rapid development of *S. cerevisiae* and *C. parapsilosis* might have resulted in competition for nutrients. Similarly, *T. delbrueckii*, which has been reported to have a strong fermentative activity and tolerate up to 10% (v/v) ethanol, (Xufre et al., 2006; Cordero-Bueso et al., 2011) could not persist until the end of fermentation and only maintained low levels until the middle of fermentation in the must from the integrated farming system. *C. azyma* and *C. parapsilosis* are not common wine yeasts and therefore there is no information available regarding their presence in wine fermentation. However, their presence in the fermentation of other substrates such as olives has been reported (Badotti et al., 2013; Aponte et al., 2010). Further investigation of the fermentative traits of these yeasts could help explain their dynamics and role in wine fermentation.

Regarding the dynamics of *S. cerevisiae* during spontaneous fermentations, the scarce isolation of this species in grape must was in line with what has been reported by previous authors (Combina et al., 2005; Mercado et al., 2007; Di Maro et al., 2007; Guzzon et al., 2011). A high strain diversity (10-15 strains per fermentation) and the sequential substitution of different *S. cerevisiae* strains were observed throughout all three spontaneous fermentations that was reported previously (Mercado et al., 2007; Tello et al., 2011; Milanović et al., 2013). Nevertheless, *S. cerevisiae* strains displayed different dominance behaviour in each fermentation. In total ten different strains were isolated from biodynamic fermentation while six strains initiated the fermentation. However, the diversity of strains decreased as the fermentation proceeded and one strain (profile I-BD) dominated the final stage of fermentation. The decrease in the strains diversity and the dominance of a single strain in final stage of fermentation was previously reported by Hall et al. (2011) and

Perrone et al. (2013). In total, fifteen and eighteen *S. cerevisiae* strains were isolated from conventional and integrated fermentation respectively. However profile I-CONV and profiles V-IPW and VI-IPW occurred most frequently throughout fermentations and dominated the final stage of fermentation. Overall, the integrated fermentation displayed higher diversity compared to conventional and biodynamic fermentation that has never been investigated before. Similarly, Milanović et al. (2013) demonstrated higher *S. cerevisiae* strain diversity in conventional farming system in comparison with organic farming system whereas, contrarily, Cordero-Bueso et al. (2011) and Tello et al. (2011) reported the more abundance of *S. cerevisiae* strains in organic farming system compared to conventional farming system. The higher diversity of *S. cerevisiae* strains in integrated and conventional fermentation might be due to the indirect transmission of this species by insects and vectors in the farming system, however, there is still a controversy regarding the source of the *S. cerevisiae* strains during the fermentation and it needs further investigation.

3.6 CONCLUSION

The current study clearly demonstrated the significant participation and sequential replacement of non-*Saccharomyces* yeasts during the spontaneous fermentation of grape musts with the unique presence of specific species in each farming system. This study also showed that yeast species and strains in grape must are strongly influenced by the farming system used to grow the grape in the farming system. We observed that biodynamic farming system presented the highest degree of diversity in comparison with the other two. The use of agrochemicals might have negative influence on the abundance of yeast species and yeast diversity. Thus the biodynamic farm looks like the best reservoir of indigenous yeast biodiversity. It is worth to mention that since culture-dependent methods used in the current study may cause a bias in microbiota diversity, culture-independent methods such as direct metagenomic analyses might reveal more detailed information. However, since previous studies have shown that PCR-based culture-independent methods may also impose some bias, the combination of culture-dependent and culture-independent methods would be the best approach for in-depth diversity analysis.

The knowledge regarding the microbial ecology in the farming system and the influence of different farming systems and treatments has several gaps even though this topic has been investigated recently. This study is presented as the first approach to compare the indigenous yeast ecology in the grape must and during fermentation of musts from three farming systems and will make significant contribution to our understanding of wine yeast diversity and dynamics as well as the impact of viticultural practices on the grape microbial communities.

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Chapter 4

General Discussion and Conclusions

4.1 DISCUSSION

Wine has been produced traditionally through spontaneous fermentation which results from the activities of the indigenous microorganisms present on the grape berry surface. When it comes to wine quality, it is critical to know the present microorganisms and their role during the spontaneous fermentation. Yeasts are the most relevant microorganisms responsible for alcoholic fermentation that have been shown to have an impact in the sensory quality of wine due to the production of secondary metabolites such as glycerol, higher alcohols and esters (Fleet, 1993; Torija et al., 2001; Salvadó et al., 2011). The focus of the current study was on the yeast ecology of musts obtained from conventional, biodynamic and integrated vineyard and the impacts of farming practices on the yeast diversity in grape must. The dynamics of the yeast species were also monitored throughout spontaneous fermentation. A combination of basic microbiological analyses as well as molecular approaches such as PCR-RFLP and interdelta PCR amplification were used to isolate, identify and monitor the yeasts evolution from must until the final stage of alcoholic fermentation. The yeast population in the grape musts obtained from biodynamic, conventional and integrated vineyard in two vintages (2012 and 2013) exhibited a diverse population, including non-*Saccharomyces* species (e.g. *H. uvarum*, *A. pullulans*, *M. pulcherrima*, *C. zemplinina* and *I. terricola*) and different *S. cerevisiae* strains. In line with previous studies, the *S. cerevisiae* strains were below detection level in integrated and conventional musts (Mercado et al., 2007; Di Maro et al., 2007; Guzzon et al., 2011). However, this species was isolated in low frequencies in biodynamic musts. There are currently no other studies on yeast diversity in biodynamic vineyards, thus it is not possible to confirm whether this consistent isolation of *S. cerevisiae* from the musts obtained from the biodynamic vineyard is typical of such farming practices or not. However, it can be suggested that since *S. cerevisiae* has been shown to be carried by insect vectors such as bees and wasps, its prevalence in the biodynamic vineyard is indicative of the wider diversity in such farming systems since the use of pesticides is prohibited. *A. pullulans*, *H. uvarum* and *M. pulcherrima* were the most common isolates in both vintages. The frequent isolation of these yeasts in grape musts has been reported by several authors (Settanni et al., 2012; Cordero-Bueso et al., 2011; Sun et al., 2009). The isolation of *A. pullulans* on the unripe grape berry surface and in the grape must have been reported previously (Settanni et al., 2012). The capability of this yeast to produce exopolysaccharides and form biofilms as well as survive in poor nutrient environment has been shown by previous authors (Settanni et al., 2012; Renouf et al., 2005). In contrast, *M. pulcherrima* is thought to persist in grape must due to its ability to inhibit the growth of the other yeasts (yeast-yeast interactions) by producing antimicrobial compounds such as killer toxins (Settanni et al. 2012). The high diversity of *Candida* species (e.g. *C. glabrata*, *C. parapsilosis*, and *C. zemplinina*) was observed in both vintages from grape musts and in particular in biodynamic vineyard. The compatibility of *Candida* species with the available nutrients on grape berry surface and also better adaptation of this species with the common treatments in the biodynamic system, such as lack of synthetic pesticides and fungicides, might explain the high isolation of this species

in biodynamic vineyard. Interestingly, the slight tendency toward higher yeast diversity was revealed in 2013 must in comparison with 2012 must. The discrepancy in yeast diversity in 2 consecutive years is not unusual and has been reported by other researchers (Torija et al., 2001; Sun et al., 2009; Settanni et al., 2012). Such variations have been previously attributed to changes in climatic conditions (Li et al., 2010; Bezerra-Bussoli et al., 2013). The musts obtained from biodynamic vineyard, exhibited the highest species evenness (Pielou's index) and the lowest species dominance (D), in comparison with the conventional and integrated musts whereas, the integrated must displayed the lowest species evenness and highest species dominance in both vintages. The higher yeast diversity obtained in the biodynamic must might be attributed to the higher initial population of total yeasts (mainly weakly fermentative yeasts), and also the isolation of strongly fermentative yeast (*S. cerevisiae*) in biodynamic must whereas, the absence of *S. cerevisiae* and the presence of oxidative yeasts (50%) in integrated must might explain the low diversity obtained in integrated must. Regarding the sole isolation of weakly fermentative yeast (*H. uvarum*) in 2012 integrated must, the high population of this species that probably did not allow the isolation of minor species on the culture media might explain the lowest diversity obtained in integrated must in 2012. The musts obtained from 3 vineyards were fermented until the dryness of grape sugar, between 24-41 days. Similar fermentation trend was observed among the musts. However, biodynamic fermentation had the higher fermentation rate in comparison with integrated and conventional fermentation. The initial stage of all fermentation was dominated by weakly fermentative yeasts of the genera *Candida* and *Hanseniaspora*. However, as the fermentation proceeded the population increased in the favour of *S. cerevisiae* and only non-*Saccharomyces* species such as *H. uvarum*, *C. zemplinina*, *T. delbrueckii* and *C. parapsilosis* that has been shown to tolerate more than 5-7% ethanol could persist in the middle of fermentation. The anaerobic conditions, increase in ethanol concentration and temperature was suggested by Salvadó et al. (2011) as the reason of the dominance of *S. cerevisiae* and the gradual disappearance of non-*Saccharomyces* yeasts during the spontaneous fermentation. The strongly fermentative yeast *S. cerevisiae* dominated the final stage of fermentations. However, the presence of *C. parapsilosis* (4% of total yeast population) in biodynamic fermentation was observed. Biodynamic must showed a short exponential phase and rapid fermentation (24 days) in 2013 vintage. We evaluated the tight interrelationship between the yeast composition as well as the initial total yeast population associated with the grape must and the success of the fermentation, as suggested by Díaz et al. (2013). The lower population of oxidative yeasts, the higher total initial population comprising the weakly fermentative yeasts, as well as the strongly fermentative yeasts *S. cerevisiae* in biodynamic must can explain the higher rate of fermentation compared to fermentation of the musts from the integrated and conventional vineyards. Díaz et al. (2013) clearly demonstrated that the presence of *S. cerevisiae* at the onset of fermentation resulted in higher fermentation rate whereas when oxidative and weakly fermentative yeasts such as *R. mucilaginosa* and *P. anomala* were predominant during the fermentation a longer lag phase was observed. These authors also

suggested that a longer lag phase provides an opportunity for non-*Saccharomyces* species to suppress the growth of beneficial yeasts and produce toxic compounds. Interestingly, *L. thermotolerans* which was present in all three musts could only persist until the middle stage of fermentation of the must from the conventional vineyard. The persistence of this species in conventional fermentation might be due to the higher population of this species in the conventional must or the yeast-yeast interactions (killer activity) as suggested by Yong Sun et al. (2012) and Sabel et al. (2013). Similarly, the ethanol tolerant, strongly fermentative yeast, *T. delbrueckii*, could not persist until the end of fermentation and only maintained low levels until the middle of fermentation in the must from the integrated. It is possible that the growth of this yeast was suppressed by the presence of *Wickerhamomyces anomalus* which has been shown to produce killer toxins that have a wide antifungal activity spectrum (Yong Sun et al., 2012; Sabel et al., 2013).

In line with the previous studies, the heterogeneous diversity (10-15 strains per fermentation) and sequential substitution of *S. cerevisiae* strains was observed throughout all spontaneous fermentations (Mercado et al., 2007; Tello et al., 2011; Milanović et al., 2013). The higher diversity of *S. cerevisiae* strains was observed in the integrated vineyard (18 strains) compared to conventional (15 strains) vineyard, whereas the biodynamic vineyard displayed the lowest diversity (10 strains). However, the dominance of one or two strains in the final stage of all fermentations was observed. The decrease in the strains diversity and the dominance of a single strain in final stage of fermentation was previously reported by Hall et al. (2011) and Perrone et al. (2013). Probably, the *S. cerevisiae* strains that can adapt better to the high ethanol and poor nutrition condition (low sugar) of the middle and final stage of fermentation dominated and completed the fermentation. The high degree of diversity in integrated must might be due to the indirect transmission of *S. cerevisiae* strains by insects and vectors in the vineyard.

Overall, the culture dependent, molecular techniques used in this study, allowed characterizing the non-*Saccharomyces* and *Saccharomyces* yeasts associated with grape musts obtained from, conventional, biodynamic and integrated vineyard and monitor their population dynamics throughout spontaneous fermentation. It is worth mentioning that the techniques used in this study, similar to all the other techniques have some bias and therefore poses a risk of misrepresenting the diversity of species and strains which were below detection level and could not grow under the cultivation conditions used. On the other hand, it has been reported that the culture in-dependent techniques such as PCR-DGGE and qPCR (Di Maro et al., 2007; Cocolin et al., 2011; Salvadó et al., 2011) also show some bias. Therefore, the combination of culture dependent and culture in-dependent techniques can provide the accurate tools to characterize and monitor the yeasts dynamics in wine fermentation.

4.2 CONCLUSIONS AND FUTURE PROSPECTS

In this study, we evaluated the impacts of farming systems on yeast diversity associated with the grape must and monitor the evolution of yeast dynamics throughout spontaneous fermentations. The isolation of different non-*Saccharomyces* species (e.g. *C. parapsilosis*, *C. azyma*, *T. delbrueckii*, and *L. thermotolerans*) and several *Saccharomyces* strains from different stages of fermentation and the difference in the behaviour of common species during fermentation was an important finding which revealed the significance of initial cell concentrations and yeast-yeast interactions during fermentation. The positive contribution of the non-*Saccharomyces* species, such as *T. delbrueckii* on the wine flavour has been reported previously (Ciani and Maccarelli, 1998; Renault et al., 2009). Therefore, the isolation of indigenous non-*Saccharomyces* species, during the spontaneous fermentation is a critical step for further analysis. The use of *T. delbrueckii* in mixed fermentation with *S. cerevisiae*, in order to minimise the acetic acid production has been suggested previously by Bely et al. (2008) and Ciani et al. (2006). Therefore, further investigation on the oenological characteristics of the non-*Saccharomyces* species found in this study, might reveal potential starter cultures to use in the wine industry.

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