The Diversity and Dynamics of Indigenous Yeast Communities in Grape Must from Vineyards Employing Different Agronomic Practices and their Influence on Wine Fermentation

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The current study evaluated the diversity of yeast species in Cabernet Sauvignon grape must derived from three neighbouring vineyards from a similar terroir but on which significantly different management practices are employed. The fermentation kinetics and yeast population dynamics were monitored from the beginning to the end of spontaneous fermentation. The grape musts were characterised by distinct yeast populations comprising oxidative, weakly fermentative and strongly fermentative yeasts. Different combinations of dominant non-*Saccharomyces* yeasts were observed in each must, with significantly different assortments of dominant species, including *Starmerella bacillaris* (synonym *Candida zemplinina*), *Lachancea thermotolerans*, *Hanseniaspora uvarum*, *Candida parapsilosis* and *Wickerhamomyces anomalus*. None of these yeast consortia appeared to affect the growth of *Saccharomyces cerevisiae* or inhibit the overall progress of fermentation. However, the percentage of fermentative yeasts was positively correlated with the fermentation rate. Glucose and fructose consumption rates suggested active participation of both glucophilic and fructophilic yeasts from the onset of fermentation. The data highlight two parameters, viz. initial cell concentration and yeast community composition, as important fermentation drivers and open the possibility to predict fermentation behaviour based on the initial composition of the yeast community.

INTRODUCTION

Grapes harbour a wide variety of yeasts, many of which are intimately associated with the berry skin. The diversity and density of yeasts fluctuate throughout the berry-ripening stages (Prakitchaiwattana *et al*., 2004; Renouf *et al*., 2005; 2007; Barata *et al*., 2012a) and also vary between healthy and damaged berries. Typically, the yeast population associated with healthy grapes from the berry peppercorn stage until the time of harvest is characterised by a high incidence of basidiomycetous yeasts of the genera *Cryptococcus*, *Rhodotorula* and *Rhodosporidium*, as well as the ascomycetous yeast-like fungus *Aureobasidium pullulans* (Rementeria *et al*., 2003; Renouf *et al*., 2005; Barata *et al*., 2012a; Setati *et al*., 2012; Díaz *et al*., 2013). Fermentative yeasts, on the other hand, generally are numerically under-represented on healthy grapes (Mortimer & Polsinelli, 1999; Barata *et al*., 2012a), and studies have suggested that insects such as members of the genera *Drosophila* and *Vespula* act as the primary vectors of ascomycetous fermentative yeasts such as *Saccharomyces* spp., *Saccharomycodes* spp., *Hanseniaspora uvarum*, *Candida* spp., *Pichia* spp. and *Zygoascus* spp. (Mortimer & Polsinelli, 1999; Barata *et al*., 2012b; Stefanini *et al*., 2012). Several factors, including agronomic practices, influence yeast diversity on grapes. In particular, farming practices such as the application of pesticides have been reported to affect yeast populations, including the diversity of fermentative yeast strains (Corder-Bueso *et al*., 2011; Tello *et al*., 2011; Tofalo *et al*., 2011; Setati *et al*., 2012). However, when considering the preponderance of non-fermentative yeasts in the vineyard microbiota, the impact of such practices on the fermentation microbiota has received less attention.

Today, most wine fermentations are inoculated with commercially produced active dried wine yeast strains. However, spontaneous fermentation, i.e. the fermentation by the microorganisms that are naturally present on grapes and in cellars, is again becoming more widespread, in particular because of the adoption of less interventionist practices that also are perceived as more environmentally friendly. Spontaneous fermentation, while presenting increased risks of off-flavour production and stuck fermentation, is furthermore believed to generate wines with more complex aroma and flavour, presumably due to the significant contribution of non-*Saccharomyces* yeasts (Tello *et al*., 2011).

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The general pattern of the development of microbial populations during spontaneous wine fermentation is reasonably well established. Fresh grape juice typically contains between 10^4 and 10^6 CFU/mL yeast counts, which increases to 10^8 CFU/mL during fermentation (Povhe Jemec *et al*., 2001; Combina *et al*., 2005; Di Maro *et al*., 2007). The kinetics of the main yeast species have been monitored using both culture-dependent approaches as well as culture-independent methods, including FISH (Xufre *et al*., 2006), PCR-DGGE (Di Maro *et al*., 2007; Renouf *et al*., 2007) and qRT-PCR (Zott *et al*., 2008). These studies revealed rapid and significant increases in *Saccharomyces cerevisiae* strains and sometimes a dramatic decline in non-*Saccharomyces* species. The decline appears most likely to be associated with SO_2 intolerance, sensitivity to anaerobic conditions, and intolerance of increasing levels of ethanol (Combina *et al*., 2004). However, since these traits are strain dependent, there have been reports of strains of *H. uvarum*, *Issatchenkia orientalis*, *Pichia kluyveri*, *Starmerella bacillaris* (synonym *Candida zemplinina*) (Duarte *et al*., 2012), *Torulaspora delbrueckii* and *Lachancea thermotolerans* that were able to persist until the middle and sometimes end of fermentation in significant numbers (Povhe Jemec *et al*., 2001; Sun *et al*., 2009; Bezerra-Bussoli *et al*., 2013; Milanovic *et al*., 2013; Tristezza *et al*., 2013), although *S. cerevisiae* remains the dominant yeast in these stages. The dynamics of *S. cerevisiae* strains are variable and may involve one or more strains, and the sequential substitution of different strains has also been reported (Sturm *et al*., 2006).

The kinetics and completion of fermentation are believed to be largely dependent on *S. cerevisiae*, and competition by a large number of non-*Saccharomyces* species is thought to be detrimental from a fermentation kinetic perspective. Here we evaluate the fermentation kinetics and population dynamics of three spontaneous fermentations of grape juices derived from three differently treated vineyards. The three vineyards were shown, using automated ribosomal intergenic spacer analysis (ARISA), to harbour distinct grape-associated yeast populations, although cultivation-dependent analyses could not show a clear distinction (Setati *et al*., 2012). The current study aimed to investigate whether the differences in the three vineyards were also evident in the yeast populations constituting the wine microbial consortium, and how differences in the initial yeast population composition and concentration would influence the fermentation kinetics. A better understanding of the initial diversity in grape must and the dynamics of the yeast population throughout spontaneous fermentation will help improve the management of such fermentations and potentially avoid sluggish fermentations and risks of spoilage.

MATERIALS AND METHODS

Grape sampling and wine fermentations

The grapes used in the current study were obtained from three vineyards that employ different management systems: biodynamic (33°57'39.33" S, 18°45'13.46" E, elevation 183 m), conventional (33°57'41.50" S, 18°45'11.87" E, elevation 179 m) and integrated pest management (33°57'40.65" S, 18°45'08.23" E, elevation 184 m). The conventional and biodynamic vineyards had the same Cabernet Sauvignon rootstock (R101-14), while the integrated vineyard had rootstock R110-CS23A. The layout, location and chemical treatments of the vineyards were described in detail in a previous study (Setati *et al*., 2012). Samples were collected in two consecutive years (2012 and 2013) and used for microvinification. For each vineyard, 5 kg of Cabernet Sauvignon grapes were collected and transported to the laboratory in sterile "Ziploc®" bags. The grapes were aseptically hand-destemmed and crushed within 1 h of collection. The chemical composition of the must was analysed by Fourier transform infrared (FT-IR) spectroscopy using the GrapeScan 2000 instrument (FOSS Electric, Denmark). Fifty millilitre samples were collected from the fresh must and used for yeast isolation and enumeration. The remaining must was transferred into duplicate 2 L fermentation bottles and set up for natural fermentation at 25°C to allow sufficient growth of the non-*Saccharomyces* population without negatively affecting the development of the indigenous *S. cerevisiae* strains. Alcoholic fermentation was followed by measuring the loss of weight resulting from CO_2 release. In addition, samples were withdrawn regularly for sugar analysis. The glucose and fructose concentrations were measured using the Enytec™ *Fluid* D-Glucose Id-No: 5140 and Enytec™ *Fluid* D-Fructose Id-No: 5120 (R-Biopharm AG, Germany) enzymatic kits on the Arena™ 20XT Photometric analyser (Thermo Electron, Oy, Finland). Yeast dynamics were evaluated after 12.5%, 30%, 50% and 70% sugar consumption.

Yeast enumeration and isolation

Samples were taken every two days for monitoring the yeast populations. Serial decimal dilutions were prepared in triplicate in 0.9% (w/v) NaCl solution and spread on three different media: (i) Wallerstein nutrient (WLN) agar, supplemented with 34 µg/mL chloramphenicol and 200 µg/mL biphenyl to suppress bacteria and moulds, was used for total yeast enumeration, (ii) WLN agar with 34 µg/mL chloramphenicol, 200 µg/mL biphenyl and 1 µg/mL cycloheximide was used for the enumeration of the non-*Saccharomyces* (NS) population, and (iii) YPD agar supplemented with 12% ethanol, 25 mg/L kanamycin and 150 mg/L $K_2S_2O_5$ was used for the enumeration of *Saccharomyces* species. Yeast enumeration and isolation were carried out on plates from the dilutions that contained between 30 and 300 colonies. Colonies with different characteristics (colour, texture, size, shape, margin) were isolated and purified through two rounds of streaking. A minimum of six representative colonies per colony morphology were streaked out from each plate where possible. The isolates obtained were stored in glycerol 20% (v/v) at -80°C.

Yeast identification

For the isolation of genomic DNA, yeast cells were grown in 5 mL YPD broth. DNA was extracted from a 1 mL sample using the rapid yeast DNA extraction method (Hoffman, 2003). The ITS1-5.8S rRNA-ITS2 region was amplified by PCR using the primer set ITS1 (5'-TCCGTAGGTGAACCTCGCG-3') and ITS4 (5'-TCCTCCGCTTTATTGATATGC-3') (Esteve-Zarzoso *et al*., 1999). PCR was performed in a final volume

of 25 µL containing 0.4 mM dNTP mix, 0.25 µM of each primer, 1 U of Ex-Taq polymerase (TaKara), 1× buffer, 1 mM $MgCl_2$ and 100 ng template DNA. The PCR products were analysed on a 1% agarose gel and purified using the Zymoclean™ Gel DNA recovery kit (Zymo Research Corporation, Irvine, CA, USA) following the manufacture's instruction. Restriction fragment polymorphisms of the ITS-5.8S rRNA gene were analysed by digesting the PCR product with *Hae*III, *Hin*fI and *Cfo*I in separate reactions as described by Esteve-Zarzoso *et al*. (1999). The isolates were grouped according to distinct restriction patterns, and previously sequenced species were digested with the same enzymes and used as references to identify the current isolates.

Statistical analyses

The relative abundance of species was calculated as a proportion of a particular species in the samples based on colony counts and frequency of isolation. Yeast species diversity was determined using the Shanon Weiner index and Simpson's index, as described by Cordero-Bueso *et al*. (2011). The Shanon index for diversity was calculated as follows:

$$H' = -\sum^{s} p_i \log_2(p_i) \quad (1)$$

where *s* is the number of species and is the proportion of the species within the total population. Species dominance was determined on the basis of Simpson's index using the following equation:

$$D = \sum^{s} (p_i)^2 \quad (2)$$

RESULTS

Isolation and identification of yeasts

The chemical composition of the grape must from the three vineyards revealed differences in the sugar concentration, pH and tartaric acid concentrations. The total glucose and fructose concentrations ranged from 210 g/L to 265 g/L, pH varied between 3.35 and 3.66, while tartaric acid concentrations were varied from 2.0 to 5.4 g/L (Table 1).

The three farming systems displayed different yeast community composition in the initial musts. Since the evaluation was culture based, only yeast species that represented > 1% of the total yeast population are reported here, since all other species would likely not have any significant impacts on the system. In general, total species numbers were low, indicating the rapid die-off of many yeast species that were prominent on the grapes. The data show that, overall, the 2013 vintage displayed more diversity than the 2012 in all musts (Table 2). In total, eight, four and one yeast species were identified in the 2012 musts, whereas 12, 11 and nine species were identified in the 2013 musts obtained from the biodynamic, "conventional" and "integrated" vineyards respectively. During the 2012 vintage, *Aureobasidium pullulans* and *Kazachstania aerobia* were the two dominant yeasts in the biodynamic and conventional vineyards, whereas *Hanseniaspora uvarum* was the only yeast isolated in the integrated vineyard in 2012. In contrast, a more diverse yeast community, comprising *A. pullulans*, *H. uvarum*, *Torulaspora delbrueckii*, *Wickerhamomyces anomalus* (formerly *Pichia anomala* or *Hansenula anomala*), *Rhodotorula* spp., *Rhodosporidium diobovatum*, *Candida azyma* and *Lachancea thermotolerans* (formerly *Kluyveromyces thermotolerans*), was observed in the integrated vineyard in 2013 (Table 2). *Saccharomyces cerevisiae* could only be isolated from the initial must of the biodynamic vineyard in both vintages. *Candida parapsilosis* was also only isolated in the biodynamic vineyard. Other yeasts present included *Cryptococcus* spp., *Rhodotorula* spp. and *Issatchenkia terricola*. When the two vintages were taken together, the biodynamic vineyard exhibited the highest culturable diversity (H' = 2.32) and the lowest dominance (D = 0.11), followed by the conventional vineyard (H' = 2.23 and D = 0.17), while the integrated vineyard revealed lower diversity (H' = 1.15) and the highest dominance (D = 0.37).

General fermentation kinetics

The current study evaluated spontaneous fermentation kinetics in grape must derived from biodynamic, integrated and conventional farming systems. Two vintages were analysed and similar fermentation trends were observed. The initial population of NS yeasts varied from 10^4 to 10^6 CFU/mL, depending on the vintage, with minor variations between the farming systems. Data from the 2013 vintage are presented to demonstrate the trends observed. The must from the biodynamic vineyard had an initial total yeast population of 2.3 × 10^6 CFU/mL and the fermentation proceeded rapidly, while fermentation of the musts from the conventional and integrated vineyards had an initial yeast load of 2.35 × 10^4 and 8.43 × 10^4 CFU/mL respectively. Both fermentations displayed a short lag phase, but took

TABLE 1
Chemical composition of grape musts prepared in 2012 and 2013.

Parameter	Biodynamic		Conventional		Integrated	
	2012	2013	2012	2013	2012	2013
Glucose/fructose (g/L)	239 ± 0.20	264 ± 1.00	210 ± 0.40	265 ± 0.60	249 ± 0.30	239 ± 0.40
°Brix	23.4 ± 0.00	25.4 ± 0.09	20.7 ± 0.00	25.6 ± 0.02	24.3 ± 0.02	23.4 ± 0.02
Titratable acidity (g/L)	3.23 ± 0.01	3.96 ± 0.003	3.85 ± 0.02	4.28 ± 0.01	2.37 ± 0.04	3.65 ± 0.07
pH	3.61 ± 0.001	3.50 ± 0.013	3.35 ± 0.02	3.49 ± 0.02	3.66 ± 0.002	3.55 ± 0.02
Tartaric acid (g/L)	3.10 ± 0.05	4.80 ± 0.01	2.30 ± 0.09	5.40 ± 0.01	3.20 ± 0.03	5.00 ± 0.02
Malic acid (g/L)	0.90 ± 0.005	0.70 ± 0.17	1.40 ± 0.10	0.30 ± 0.03	nd	0.55 ± 0.02
Volatile acidity (g/L)	0.4 ± 0.001	0.41 ± 0.02	0.31 ± 0.02	0.32 ± 0.02	0.32 ± 0.01	0.27 ± 0.01

nd: not detected

TABLE 2
Percentage yeast species distribution in grape must from biodynamic, conventional and integrated vineyard in two consecutive years.

	BIODYNAMIC		CONVENTIONAL		INTEGRATED	
	2012	**2013**	**2012**	**2013**	**2012**	**2013**
Kazachstania aerobia	27	-	48	-	-	-
Aureobasidium pullulans	32	6	28	12	-	36
Hanseniaspora uvarum	3	20	21	18	100	23
Hanseniaspora vineae	-	2	-	-	-	-
Hanseniaspora guilliermondii	-	1	-	-	-	-
Issatchenkia terricola	-	6	3	4	-	-
Lachancea thermotolerans	-	7	-	15	-	5
Metschnikowia pulcherrima	7	19	-	20	-	-
Metschnikowia chrysoperlae	13	-	-	-	-	-
Phaemoniella prunicola	8	-	-	-	-	-
Starmerella bacillaris	-	22	-	16	-	-
Candida glabrata	7	-	-	-	-	-
Candida azyma	-	-	-	5	-	5
Candinda pomicola	-	2	-	-	-	-
Candida apicola	-	-	-	2	-	-
Cryptococcus bhutanensis	-	5	-	-	-	-
Cryptococcus carnescens	-	-	-	3	-	-
Rhodosporidium diobovatum	-	-	-	2	-	6
Rhodotorula nothofagi	-	-	-	-	-	3
Rhodotorula glutinis	-	-	-	3	-	5
Torulaspora delbrueckii	-	-	-	-	-	5
Wickerhamomyces anomalus	-	-	-	-	-	12
Candida parapsilosis	-	7	-	-	-	-
Saccharomyces cerevisiae	3	3	-	-	-	-

almost twice the amount of time to complete compared to the must from the biodynamic vineyard. The initial total yeast population in the must from the biodynamic vineyard increased from 10^6 to 10^8 CFU/mL, after which it stabilised until the end of fermentation, with a slight decrease to 10^7 CFU/mL (Fig. 1). The non-*Saccharomyces* yeast population displayed a notable increase from 10^6 to 10^8 CFU/mL after four days of fermentation, followed by a steady decline until it dropped below detection level after 90% of the sugar was consumed, whereas the *Saccharomyces* population increased rapidly to 10^7 CFU/mL in the first two days of fermentation, reaching a maximum of 10^8 CFU/mL and remaining stable until the end of fermentation. Similar trends were observed in the fermentation of the must from the conventional and integrated vineyards, even though the initial yeast population levels were lower and the fermentation took longer. In these two fermentations, the *Saccharomyces* population could only be detected two days after the onset of fermentation.

Yeast dynamics throughout fermentation

The oxidative yeasts, including *A. pullulans*, *Rhodotorula* spp., *R. diobovatum* and *Cryptococcus* spp., which accounted for 5, 8 and 14% of the total initial yeast population in the must from the biodynamic, conventional and integrated vineyard respectively, dropped below detection when fermentation commenced (Fig. 2). The must from the biodynamic vineyard displayed 12 different species at the onset of fermentation; however, after 12.5% of the sugar had been consumed the number decreased to five species, comprising *Candida parapsilosis* (32%), *S. bacillaris* (19.9%), *H. uvarum* (25.4%), *M. pulcherrima* (2.5%) and *S. cerevisiae* (20.2%). Similarly, in the must from the conventional vineyard the yeast diversity declined from 11 species to six species, *viz*. *H. uvarum* (30.4%), *L. thermotolerans* (22.7%), *S. bacillaris* (18%), *M. pulcherrima* (12.5%), *C. azyma* (7.1%) and *S. cerevisiae* (9.3%), in the first five days of fermentation. Nine different species were found in the must from the integrated vineyard. The number of species declined to five after 12.5% of the sugar had been consumed. *H. uvarum* (66%) was the most dominant species, while *W. anomalus* (14%), *T. delbrueckii* (8.9%), *C. azyma* (4.6%) and *S. cerevisiae* (6.5%) were present at lower percentages. *H. uvarum*, which was present in all the fermentations, persisted until the middle of fermentation. In contrast, *L. thermotolerans* only persisted in the must from the conventional vineyard, where its initial level was higher (15% of the total population). This yeast increased up to 2×10^6 CFU/mL and then declined to 3×10^3 CFU/mL by late fermentation. *S. bacillaris* was detected

in the must from the biodynamic and conventional vineyard and displayed similar dynamics in both fermentations. The cell concentration started with an increase up to 2.5×10^6 CFU/mL, followed by a decline during the tumultuous phase of fermentation. *C. azyma*, present in the conventional and integrated must, maintained the same concentration in the early stages of fermentation and then declined after 12.5% (w/v) of the sugar had been consumed (Fig. 2). The must from the biodynamic vineyard displayed a high incidence of *C. parapsilosis*, and this yeast remained dominant throughout fermentation, while *W. anomalus* was dominant in the integrated must, second to *H. uvarum*. In contrast, *T. delbrueckii*, which was only found in the integrated must, did not show any increase. In all the fermentations,

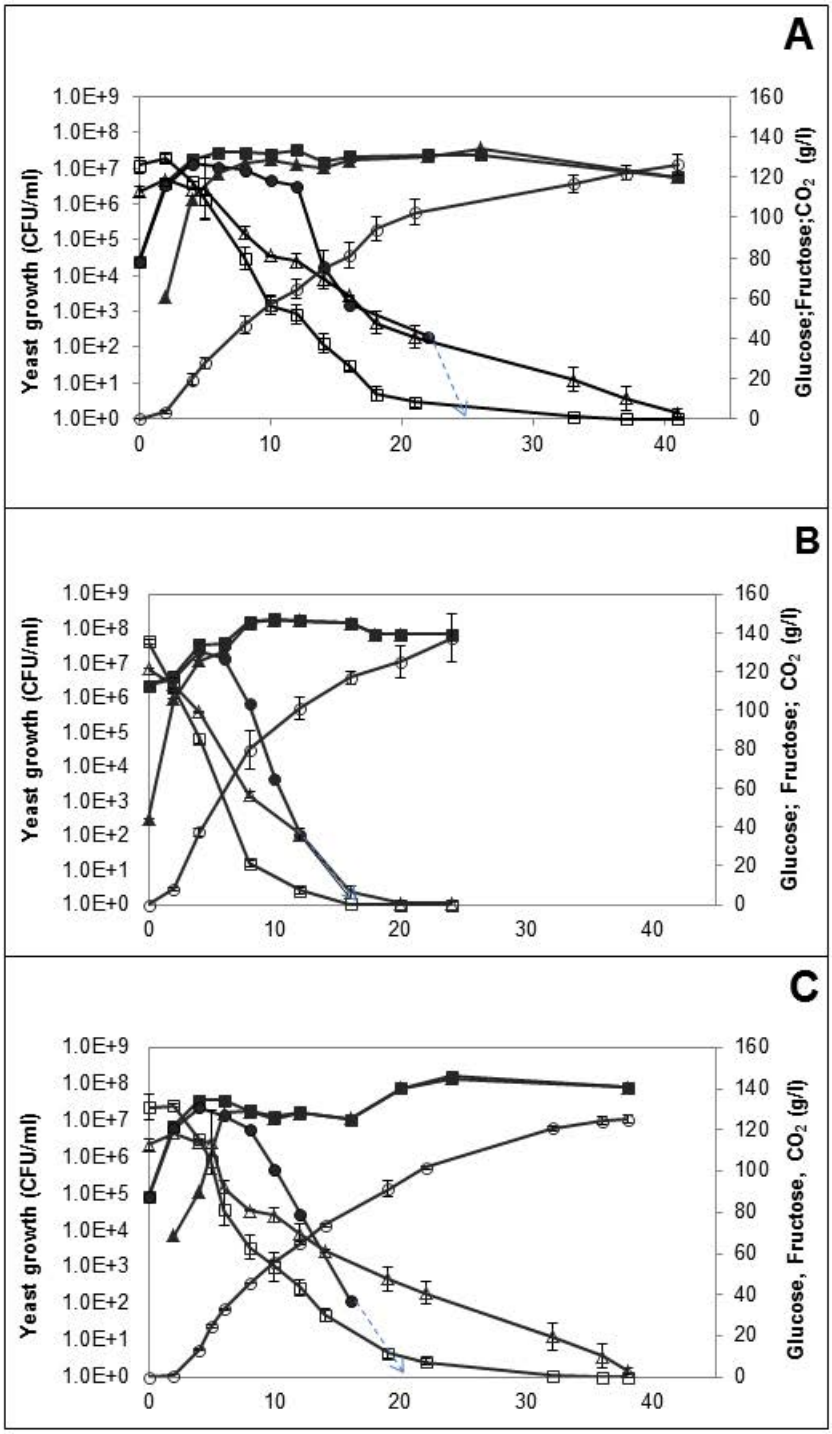

FIGURE 1

Fermentation kinetics demonstrating the yeast growth, CO_2 evolution and sugar consumption during spontaneous fermentation of the three musts from the conventional (A), biodynamic (B) and integrated (C) vineyard. The dashed arrow shows that the yeast population was below detection. Error bars indicate standard deviations of the means of duplicate measurements of three biological repeats.

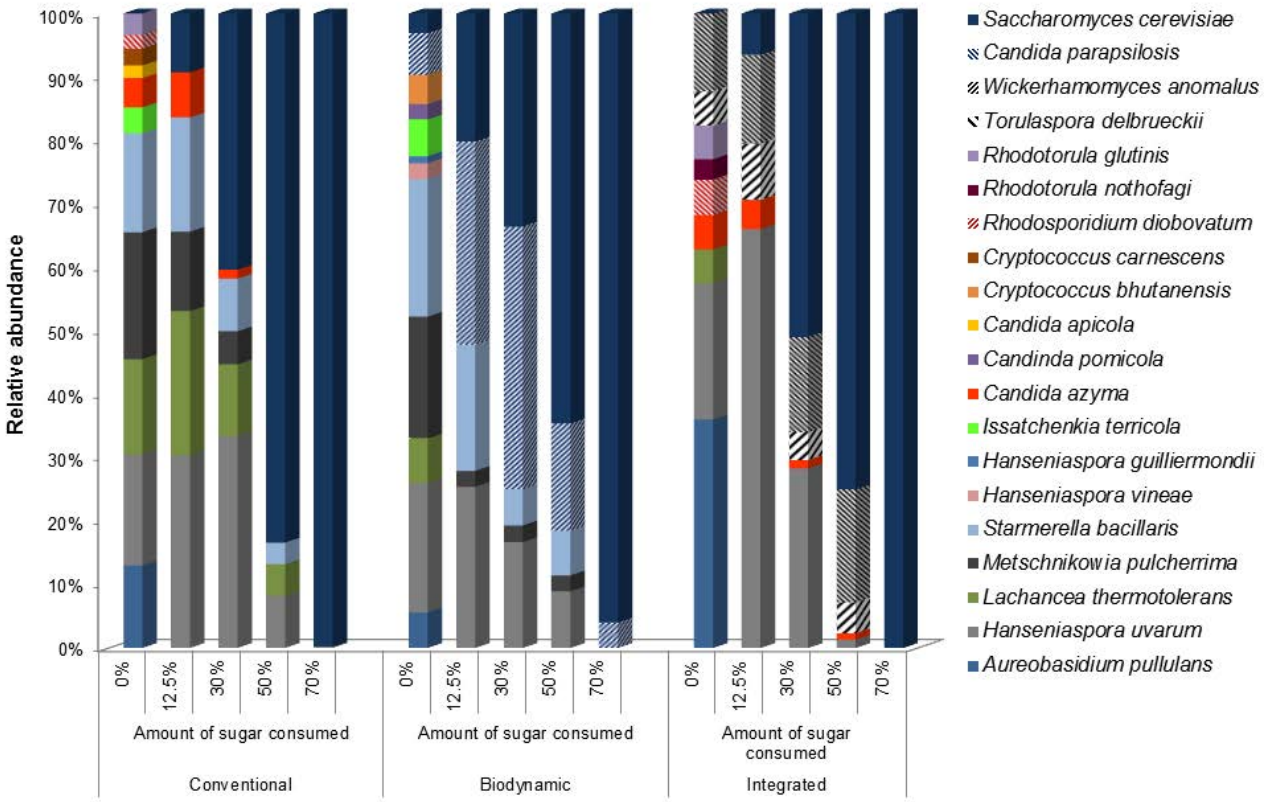

FIGURE 2
The occurrence and evolution of yeast species at different stages of spontaneous fermentation.

S. cerevisiae developed rapidly and was the dominant yeast by the middle of fermentation.

DISCUSSION

The current study evaluated the diversity of yeasts associated with Cabernet Sauvignon grapes obtained from biodynamic, conventional and integrated pest management farming systems. The dynamics of the yeast population during natural fermentation were monitored. The yeast population over two vintages (2012 and 2013) was 10^4 to 10^6 CFU/mL, as typically found in grape must. The non-*Saccharomyces* species completely dominated the grape must yeast community, with *A. pullulans*, *H. uvarum*, *M. pulcherrima* and *L. thermotolerans* being common across the three vineyards. *A. pullulans* was previously shown to be the most dominant yeast species on the surface of undamaged grapes in the three vineyards, with other yeasts, such as *Issatchenkia terricola*, *Cryptococcus carnescens*, *Rhodosporidium diobovatum*, *Rhodotorula nothofagi*, *Rhodotorula glutinis* and *Kazachstania aerobia*, present only in lower concentrations (Setati *et al*., 2012). Members of the genus *Kazachstania*, which is closely related to *Saccharomyces*, are not commonly found in yeast isolates from grape must or wine. However, the first species to be described in this genus, *Kazachstania viticola*, was isolated from grapes. Subsequently, another species, *Kazachstania hellenica*, was isolated from fermenting must (Nisiotou & Nychas, 2008), confirming the association of this genus with the vineyard and wine fermentation *milieu*. The impact that these yeasts have on wine quality remains to be elucidated. Although the isolation of these yeasts in the three vineyards seems inconsistent, it can be suggested that they are resident members of the yeast community in these vineyards and that their successful isolation depends on their level of abundance at the time of harvest. The fermentation data show that *A. pullulans*, *Rhodotorula* spp., *Cryptococcus* spp. and *R. diobovatum* are the first group of yeasts to decline when fermentation commences, probably due to their sensitivity to the prevailing anaerobic conditions. However, other studies have shown that some species of *Rhodotorula*, e.g. *R. mucilaginosa*, can persist throughout fermentation (Díaz *et al*., 2013). *S. cerevisiae* was either below detection or present only in low levels, as is evident in the biodynamic must sample, in which it accounted for 3 to 5% of the total yeast population. This representation is consistent with other studies, which have confirmed that this species is not dominant in the vineyard and usually occurs at approximately 10 to 100 CFU/g berries or less (Fleet, 2003).

Vintage variation in yeast diversity was evident in the current study. Indeed, in each must, whether from the same vineyard at different vintages or from different vineyards for a given vintage, different non-*Saccharomyces* yeasts persisted at higher levels for a significant part of the fermentation process. For instance, in the integrated vineyard, *H. uvarum* was the only species retrieved from the 2012 grape must, while nine different species were found in 2013. This could

imply that the other species, although present on the grape surface, might have been present at significantly lower levels than *H. uvarum*. In contrast, *S. bacillaris* accounted for more than 20% of the population in the 2013 must from the biodynamic and conventional vineyards, while it was not detected in 2012. This yeast has most commonly been associated with botrytised grapes or high-sugar grape musts (Tofalo *et al*., 2012). Incidentally, the 2013 musts from the two vineyards contained higher sugar levels (260 g/L compared to 239 and 210 g/L in the 2012 musts from the biodynamic and conventional vineyards, respectively). Therefore, the high levels of *S. bacillaris* could be a consequence of the presence of riper berries during harvesting and crushing. Overall, a higher representation of fermentative yeasts was evident in the 2013 musts from the three vineyards compared to the 2012 samples. The discrepancy in yeast diversity in consecutive years is not unusual and has been reported by other researchers, sometimes attributed to changes in climatic conditions, berry damage and berry ripeness (Combina *et al*., 2005; Díaz *et al*., 2013). In a recent study, Vigentini *et al.* (2015) used the Shanon Wiener index (*H'*) and multivariate data analysis to demonstrate that vintage rather than *terroir* had a more significant impact on yeast diversity associated with grapes. The data from the current study also show that the biodynamic vineyard consistently exhibited higher yeast species richness and diversity compared to the other farming practices. Although no studies have been done on the fermentation of grape must from biodynamic farms, previous studies have shown that grape must derived from organic vineyards tends to exhibit higher species and strain richness than that from conventional farms (Cordero-Bueso *et al*., 2011; Tello *et al*., 2011; Tofalo *et al*., 2011). These studies reported high biodiversity as reflected in higher *H'* values and low dominance (*D*) in organic vineyards compared to conventional vineyards.

The fermentation dynamics in the 2013 fermentations of the three musts were very similar. However, the fermentation of the must from the biodynamic vineyard proceeded at a faster rate in comparison to the other two fermentations. The rapid rate of fermentation coincided with the high initial cell concentration (10^6 CFU/mL) and a higher percentage (62%) of fermentative yeasts comprising six species, viz. *H. uvarum*, *Hanseniaspora vineae*, *Hanseniaspora guilliermondii*, *S. bacillaris*, *L. thermotolerans* and *S. cerevisiae*. The high fermentation tempo undoubtedly also was due to the rapid increase in *S. cerevisiae*. The must from the integrated vineyard, which contained approximately 45% fermentative yeasts and an initial cell concentration of 10^5 CFU/mL, was second to finish fermentation, while the must from the conventional vineyard, with the lowest initial cell concentration (10^4 CFU/mL) and a lower percentage (33%) of fermentative yeasts, took longer to ferment. This data confirm that community composition and cell concentration are important fermentation drivers, and that the percentage of fermentative yeasts compared to non-fermentative yeasts might be a good predictor of future fermentation performance. Similar observations have been made in other studies (Combina *et al*., 2005; Sun *et al*., 2009; Cordero-Bueso *et al*., 2011). 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 vineyards respectively. The species heterogeneity declined by the middle of fermentation, although different species were observed in the three fermentations. The must from the biodynamic vineyard displayed a high incidence of *C. parapsilosis*, and this yeast remained dominant throughout fermentation. *C. parapsilosis* was also previously shown to associate with the grapes from the biodynamic vineyard (Setati *et al*., 2012), and although it is not a common wine yeast, its presence in grape must and persistence during wine fermentation has been reported (Zott *et al*., 2008; Clavijo *et al*., 2010). This yeast has weaker glycolytic enzyme activity compared to *S. cerevisiae* and does not outcompete *S. cerevisiae* during wine fermentation. The fermentation kinetics in the must from the biodynamic vineyard also show that fructose consumption was relatively fast. This must had the highest initial level of *S. bacillaris*, which is fructophilic and persisted until 50% of the sugar was consumed. The data suggest that the two *Candida* species contributed positively to the fermentation kinetics, as their persistence did not retard the fermentation rate.

In the must from the integrated vineyard, *Wickerhamomyces anomalus* was the second dominant yeast after *H. uvarum*. This yeast has previously been associated with grape must and was shown to persist until the end of fermentation (Renouf *et al*., 2007; Díaz *et al*., 2013). In addition, some strains of this yeast can tolerate up to 12.5% (v/v) ethanol and are known to produce killer toxins (Walker, 2011; Sabel *et al*., 2014). This could allow *W. anomalus* to compete against the other yeasts in the same environment. However, this would depend on whether the killer toxins are active under wine fermentation conditions. It was observed in the current study that the cell concentration of *W. anomalus* only increased marginally throughout fermentation, suggesting that its growth is severely hampered by the lack of oxygen. This yeast generally shows low growth rates (0.056 h^{-1}) and biomass yields (0.11 g/g glucose) under anaerobic conditions (Walker, 2011). Therefore, the slight population increase was probably induced by the brief introduction of oxygen during sampling. Interestingly, *L. thermotolerans* and *T. delbrueckii* are good fermentative yeasts and some strains are now available commercially as starter cultures. Both species, however, did not persist until the end of fermentation. The decline of these yeasts during wine fermentation has been attributed to their high biosynthetic oxygen requirement (Hansen *et al*., 2001; Hanl *et al*., 2005). However, *L. thermotolerans*, which was present in all three fermentations, behaved differently across the fermentations, suggesting that yeast-yeast interactions as well as the initial cell density play a significant role in its dynamics. For instance, in the conventional must, in which the initial cell density was higher, *L. thermotolerans* survived until the middle of fermentation, whereas in the other two fermentations it died off in the early fermentation stage. This indicates that a high initial cell concentration is crucial to allow this yeast to establish itself during fermentation. It also is possible that the growth of this yeast in the integrated and biodynamic must fermentations may have been inhibited by competing yeasts. For instance, in the biodynamic must,

rapid nutrient utilisation by *C. parapsilosis* and *S. cerevisiae*, coupled with oxygen limitation and ethanol build-up, could be responsible for the suppression of *L. thermotolerans*. Similarly, *T. delbrueckii*, which has been reported to have a strong fermentative activity and to tolerate up to 10% (v/v) ethanol (Cordero-Bueso *et al*., 2011; Xufre *et al*., 2006), could not persist until the end of fermentation and only maintained low levels until the middle of fermentation in the must from the integrated vineyard.

Overall, non-*Saccharomyces* yeast species with known potential to affect the sensory quality of wine persisted in high numbers for a significant part of the fermentation. However, these species were different for each of the musts that were studied here, indicating the highly variable nature of natural fermentation. Importantly, and in all cases, persisting species were already well represented in the initial must, indicating that knowledge of the initial mycobiome would be an important management tool for winemakers in order to enhance or suppress existing dominant species. In addition, the data support the idea that a high biodiversity, including a mix of fermentative and non-fermentative glucophilic and fructophilic yeasts is beneficial to overall fermentative activity. The data also show that the ratio of fermentative to oxidative yeasts in the initial must is a crucial factor, since a low initial concentration of fermentative yeasts leads to a slow fermentation tempo. The dominance and persistence of non-*Saccharomyces* yeast species depended strongly on the initial cell concentration of a given species, the sensitivity to oxygen limitation and the ethanol concentration. Furthermore, the ability of *S. cerevisiae* to establish itself and dominate the fermentation appears not to be affected fundamentally by a low initial cell concentration and the presence of a diverse range of competing yeast consortia.

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