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ORIGINAL ARTICLE

Assessment of wine microbial diversity using ARISA and cultivation-based methods

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Abstract This study investigated the microbial diversity present in grape juice and in the early stage of alcoholic fermentation. The grapes were obtained from a South African biodynamic vineyard in three consecutive vintages (2012, 2013, and 2014). Culture-dependent and culture-independent approaches were used to investigate yeast and bacterial diversity. For the culture-independent approach, Automated Ribosomal Intergenic Spacer Analysis (ARISA) was employed. Using basic microbiological analyses, 4, 12, and 15 yeast species were obtained in 2012, 2013, and 2014, respectively. In contrast, ARISA profiles revealed 13 fungal peaks for the year 2013 and 14 for 2012 and 2014. Out of 14 peaks in 2012, two peaks were identified as probable corresponding fungal Operational Taxonomical Units (OTUs) and six peaks were identified for the years 2013 and 2014. The bacterial ARISA revealed ten, seven, and 12 peaks for these three years, respectively. Furthermore, the same technique was used to assess the evolution of the fungal community in the first three days of fermentation of the 2013 grape must. The data show that the yeast population diversity declined rapidly and revealed 12, 10, and 6 peaks for days 1, 2, and 3, respectively. The study demonstrated the suitability of ARISA for studying microbial diversity and dynamics in grape must and during wine fermentation.

Key words ARISA · Microbial diversity · Yeast dynamics · Wine fermentation · Non-*Saccharomyces* yeasts · Lactic acid bacteria

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Introduction

Fermenting grape must harbors a wide range of microorganisms comprising several species of yeasts and bacteria derived from the vineyard and winery equipment and surfaces (Prakichaiwattana et al. 2004; Renouf et al. 2007; Ocón et al. 2010; Jolly et al. 2014). These diverse microbial populations and their potential interplay in the different stages of alcoholic fermentation constitute a complex biological process that contributes largely to the final chemical composition of wine and, therefore, to its sensory properties (Fleet 2003). The diversity within the wine microbial population also evolves throughout the winemaking process (Fleet 2003; Renouf et al. 2007; Jolly et al. 2014). The early phase of spontaneous alcoholic fermentation is dominated by non-*Saccharomyces* yeasts of the genera *Hanseniaspora*/*Kloeckera*, *Rhodotorula*, *Pichia*, *Candida*, *Metschnikowia*, *Debaryomyces*, *Issatchenkia*, *Kluyveromyces,* and *Starmerella*. Among these, *Hanseniaspora uvarum* usually has the highest abundance followed by different *Candida* spp. By the middle phase of alcoholic fermentation, *Saccharomyces cerevisiae* outnumbers the non-*Saccharomyces* yeasts and remains the dominant yeast species until the end of fermentation (Renouf et al. 2007; Bezzera-Bussoli et al. 2013). The bacterial population in wine is dominated by lactic acid and acetic acid bacteria (LAB and AAB). The LAB population responsible for wine malolactic fermentation mainly comprises *Oenococcus oeni* and *Lactobacillus plantarum*. Other LAB such as *Leuconostoc mesenteroides*, *Pediococcus* spp., and most of the other *Lactobacillus* spp. are present in low levels (König and Fröhlich 2009). Acetic acid bacteria mainly of the genera *Acetobacter*, *Acidomonas*, *Gluconobacter*, and *Gluconoacetobacter* are responsible for post fermentation spoilage of wine (Bartowsky and Henschke 2008). Minor populations of other bacteria such as *Chryseobacterium*, *Methylobacterium*, *Sphingomonas*,

Arcobacter, *Naxibacter*, *Ralstonia*, *Frigoribacterium*, *Pseudomonas*, *Zymobacter,* and *Acinetobacter* have also been reported to be present in must (Bokulich et al. 2012).

This knowledge of the microbial diversity associated with the grape and wine environments, as described above, has been progressively established over the past one-and-a-half centuries through an array of techniques ranging from microscopic observation to molecular biology techniques. Traditional culture-dependent methods used in conjunction with polymerase chain reaction-based (PCR-based) methods such as restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) for species and strain identification have generated valuable information on the ecology of grapes and fermenting must. However, these methods can be laborious, time-consuming, and, to some extent, unreliable. Consequently, culture-independent methods such as PCR-denaturing gradient gel electrophoresis (PCR-DGGE), quantitative real-time-PCR (qRT-PCR), and fluorescence in situ hybridization (FISH) have increasingly been employed as tools for monitoring microbial dynamics during wine fermentation. While these methods are more sensitive and provide more reliable information on species diversity and richness, they also have limitations. For instance, q-PCR and FISH mostly rely on species specific primers or probes and, therefore, only provide information on selected species, while PCR-DGGE is not quantitative, cannot detect certain yeasts at concentrations below 10^3 CFU/mL, and requires additional steps for identification of the various bands (Cocolin et al. 2000; Prakichaiwattana et al. 2004; Renouf et al. 2007). Recently, T-RFLP has been evaluated as a tool to assess the composition and species dynamics of yeast and bacteria during wine fermentation. This method allows for high-throughput data processing and semiquantitative estimation of species richness and abundant in microbial samples and is also suited for microbial communities with low to intermediate richness (i.e., ≤ 50 taxa) (Ivey and Phister 2011). Despite these advantages, the use of terminal-RFLP (T-RFLP) in wine fermentation ecology might be limited by poor resolution of yeast species. For instance, Sun and Liu (2014) reported that 15 wine yeast species generated the same terminal restriction fragment (TRF) profiles using *Hae*III-TRFs and/or *Hinf*I-TRFs, thus, necessitating the use of more enzymes. Such challenges can make this method more time-consuming as more enzymes would be required to get a better estimation of species richness. In contrast, ARISA uses the natural variability of the internal transcribed spacer (ITS) region to compare microbial communities among samples without the additional restriction digestion analyses, thus, making it less time-consuming. It has been successfully used in a number of ecological studies (Fisher and Triplett 1999; Green et al. 2004; Torzilli et al. 2006; Slabbert et al. 2010b). Recently, ARISA was also implemented for the identification of yeasts isolated from the Slovakian wine and for direct evaluation of yeast diversity in grape must and wine without cultivation (Brezna et al. 2010; Chovanova et al. 2011; Krakova et al. 2012; Zenisova et al. 2014). The authors identified the yeast isolates from different wineries by ARISA and demonstrated the suitability of the technique for rapid analysis of a large number of samples. In addition, they monitored the yeast population dynamics at the different stages of fermentation (Brezna et al. 2010). The focus of these studies was, however, limited to the yeast diversity and not to the entire wine microbial consortium. The current study employed ARISA in conjunction with culture-dependent approaches to evaluate microbial population fluctuation (for both yeasts and bacteria) over vintages in grape must. In addition, the yeast population dynamics was investigated in the early stages of fermentation.

Materials and methods

Collection of wine must

Samples of red grape must (*Vitis vinifera* L cv. Cabernet Sauvignon) from the vintages 2012, 2013, and 2014 were collected from the Reyneke biodynamic vineyard (33°57’ 39.33” S 18° 45’13.46” E elev 183 m), Stellenbosch, South Africa. The 2012 must samples were collected immediately after crushing from the commercial wine cellar, while in 2013 and 2014 grape samples were picked from the vineyard and transported in sterile zipper storage bags to our laboratory and crushed under aseptic conditions with sterilized utensils. Additionally in 2013, spontaneous fermentation was performed on the must and samples were withdrawn during the first three days to analyze the yeast dynamics.

Yeasts and bacterial enumeration and isolation

Decimal dilutions of the samples were made in 0.9 % (w/v) NaCl solution and 100 μL of both the undiluted and diluted samples were plated on different culture media for isolation of yeasts. The Wallerstein Laboratory (WL) Nutrient agar (Sigma Aldrich, Steinheim, Germany) supplemented with 34 mg/L chloramphenicol (Sigma Aldrich) and 200 mg/L biphenyl (Riedel-de Haen AG, Seelze, Germany) to prevent the growth of bacteria and moulds, respectively, was used for the isolation of yeasts. The MRS (Biolab, Merck, South Africa) media supplemented with kanamycin sulphate (Roche, Germany) (25 mg/L) to prevent acetic acid bacteria and GYC (5 % Glucose, 1 % yeast extract, and 3 % calcium carbonate) De Ley et al. (1984) media supplemented with streptomycin sulphate (25 mg/L) (Sigma Aldrich, Germany) to inhibit gram positive bacteria were used to cultivate the lactic acid bacteria (LAB) and acetic acid bacteria, respectively. One hundred milligram per liter Delvocid (DSM, The Netherlands) was added in both the MRS and the GYC media to avoid the

growth of the yeast (De Ley and Swings 1984). The plates were incubated until the growth was observed at 30 °C and 37 °C for yeast and bacteria, respectively, before enumeration. Morphologically distinguishable colonies were selected and further streaked out onto the WL agar media to obtain pure cultures. Each of the pure yeast isolates was grown in 5 mL YPD (Merck, Biolabs, Modderfontein, South Africa) broth and an aliquot was stored in 20 % (v/v) glycerol at −80 °C.

Molecular identification of the yeast isolates

Yeast genomic DNA (gDNA) extraction was performed according to the protocol of Hoffman (2003). The PCR amplification of the ITS1-5.8S-ITS2 rRNA region was performed using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990) in a 2720 Gene Thermal cycler (Applied Biosystems, Life Technologies, Johannesburg, South Africa). The 50 μL PCR reaction contained 100–200 ng of template DNA, 0.25 μM of each of the primers, 1 μM of deoxynucleotides, 1 mM of $MgCl_2$ along with 1U of Ex Taq™ polymerase (TaKaRa Bio Inc., Olsu, Shiga, Japan) in Ex Taq polymerase buffer (1×). The thermal cycling parameters were set with an initial denaturation at 94 °C for 2 min 30 s, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s and, with an extension for 40 s at 72 °C. A final extension was performed for 10 min at 72 °C. The amplified PCR products were individually digested with restriction endonucleases *Hae*III, *Hinf*I, and *Cfo*I in three separate reactions, and the digested fragments were resolved on a 1 % agarose gel containing ethidium bromide. Then restriction fragment sizes estimated against a 100 bp DNA ladder and the banding profiles were used to categorize the yeast isolates into different groups. The PCR products of two representatives from each of the groups were sent for sequencing at the Central Analytical Facility (CAF), Stellenbosch University. The nucleotide sequences obtained from each of the isolates were compared using the BLAST (Basic local alignment search tool) algorithm with the available sequences in GeneBank at National Center for Biotechnology Information (NCBI) http://www.ncbi.nlm.nih.gov/genebank/index.html (Altschul et al. 1997). Sequences exhibiting more than 98 % identity to the previously deposited sequences were identified as such. Isolates that could not be identified from the ITS-5.8S rRNA region were further analyzed by sequencing of the domains 1 and 2 of the large ribosomal subunit (26/28S rDNA). The NLI (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') primers (Bezerra-Bussoli et al. 2013) were employed to amplify the said domains with an identical PCR reaction as mentioned above for the amplification of the ITS1-5.8S-ITS2 rRNA region. The thermal cycle parameters were set with an initial denaturation at 94 °C for 1 min, followed by 36 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, extension at 72 °C for 1.5 min, and a final 5 min extension at 72 °C (Bezerra-Bussoli et al. 2013).The yeast species relative abundance was calculated as the percent of each of the isolates present in the sample (retrieved by colony counts) to the total population of the isolates.

DNA extraction from the fermenting wine must

Twenty milliliters of the fermenting grape must sample, which were collected daily, were centrifuged at 5,000*g* for 10 min. The pellet was washed three times with EDTA-PVP solution (0.15 M NaCl, 0.1 M EDTA, 2 % (w/v) PVP) (Jara et al. 2008) and, subsequently, three times with TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA). The DNA was then extracted according to the protocol described by Wilson (1997) with the following modifications at the cell lysis step: along with proteinase K and SDS, 200 μL glass beads (diameter 0.65 mm) and 20 μL lysozyme solution (10 mg/mL) were added in the TE buffer, and this was followed by 3 min vortexing and an incubation at 37 °C for 50 min.

Fungal and bacterial Automated Ribosomal Intergenic Spacer Analysis (F-ARISA and B-ARISA)

The fungal community was evaluated by amplification of the ITS1-5.8S rRNA-ITS2 region of the metagenomic DNA using a caboxy-fluorescein labeled forward primer (ITS1-6FAM) and ITS4 while the bacterial community was analyzed by amplifying the ITS region (i.e., located between the 16S and 23S rRNA genes) with the 6FAM-ITSF (5'-GTCGTAACAAGGTAGCCGTA-3') and ITS-Reub (5'-GCCAAGGCATCCACC-3') primers (Cardinale et al. 2004). The PCR amplification was done in triplicates using the same conditions described for isolation of yeast isolates. The labeled PCR products were separated by capillary electrophoresis on an ABI 3010×I Genetic analyzer (Applied Biosystems) at the Central Analytical Facility, Stellenbosch University. Both the labeled fungal and bacterial PCR-amplicons were resolved with ROX 1.1 size standards, which varied from 75 bp – 1121 bp (Slabbert et al. 2010a). The raw data were converted to electropherograms and further analyzed in Genemapper 4.1 (Applied Biosystems). Only the fragments with a size that waslarger than 0.5 % (>50 fluorescence units) of the total fluorescence were considered for analysis. A bin size of 3 bp below 700 bp and 5 bp above 700 bp was employed to minimize the inaccuracies in the ARISA analysis (Slabbert et al. 2010b). The average abundance of each of the individual peaks was calculated and represented as a percentage of the total number of peak heights displayed in the sample.

Diversity analysis

The fungal, yeast and bacterial species richness and diversity for each of the years were calculated using the Menhinick's index (I) and the Shannon-Weinner index (H) (Danilov and Ekelund 2001), respectively.

Results

The current study employed ARISA to evaluate the diversity of yeast and bacteria in Cabernet sauvignon grape juice over a period of three consecutive years. In parallel, a culture-based approach was followed to monitor the diversity and dynamics of the yeast population in grape juice and during fermentation.

Yeast community composition

The total yeast population concentrations in the 2012, 2013, and 2014 fresh grape must were 3×10^6 CFU/mL, 1×10^6 CFU/mL, and 1.56×10^6 CFU/mL, respectively. Based on visual differences in colony characteristics, a total of 130, 84, and 275 yeast colonies were isolated from the 2012, 2013, and 2014 samples, respectively. In parallel, ARISA analysis of the fungal communities was performed using the total DNA extracted directly from the grape juice samples. The electropherograms generated peaks ranging from 100 bp and 900 bp (data not shown).

Following the amplification of the fungal ITS regions, ARISA revealed 14 peaks in the 2012 sample, the 579 bp peak being the most abundant and accounting for 26 %, followed by peaks 546 bp, 749 bp, 799 bp, and 842 bp, which accounted for 9-17 % of the total fungal population. Peak 629 bp and 652 bp were present as 5.05 % and 5.54 %, respectively, while the remainder of the peaks represented less than 2 % of the total fungal population (Fig. 1a) In contrast, only four yeast species were obtained through cultivation: *Lodderomyces elongisporus* was identified as the dominant species (33 %), followed by *Rhodotorula mucilaginosa* (26.67 %) *Saccharomyces cerevisiae* (23.33 %), and *Candida spandovensis* (17 %) (Fig. 1b).

During the 2013 harvest, 12 yeast species were identified in comparison to 13 peaks revealed by ARISA. *Starmerella bacillaris* (synonym *Candida zemplinina*) was found to be the dominant yeast species (21.88 %) followed by *Hanseniaspora uvarum* (19.79 %), and *Metschnikowia pulcherrima* (18.75 %). Minor yeast species including *Cryptococcus bhutanensis*, *Aureobasidium pullulans*, *Issatchenkia terricola*, *Candida parapsilosis*, and *Lachancea thermotolerans* were only present at lower abundance (Fig. 1b). ARISA revealed that the 592 bp peak was the most dominant (30.17 %), followed by the 561 bp peak with an abundance of 24.41 %. Conversely, peak 685 bp exhibited the lowest (0.66 %) abundance. Four peaks (553 bp, 582 bp, 586 bp, and 611 bp) represented between 5-9 % of the total peaks present in the grape juice, while the remainder of the peaks were below 3.5 % (Fig. 1a).

In 2014, 15 yeast species were isolated and identified from grape juice. Similar to 2013, *Candida* spp. dominated the yeast population with *Starmerella bacillaris* as the dominant species (50.32 %) followed by *Candida apicola* (11.60 %). *Rhodotorula glutinis*, *Hanseniaspora uvarum*, *Aureobasidium pullulans*, *Kazachstania aerobia,* and *Zygosacchoromyces bisporus* accounted for 3-8 % of the population, while the rest of the yeast species were below 3 % (Fig. 1b). Fourteen ARISA-peaks were observed for the 2014 grape juice sample with 553 bp as the dominant peak (17.94 %) followed by peak 472 bp, 561 bp, 586 bp, 592 bp, and 763 bp with an abundance-range of 8 %-14 % while peak 696 bp was the least abundant at 0.60 % (Fig. 1a).

Bacterial community analysis

The MRS and the GYC medium plates that were employed to identify the lactic acid bacteria and acetic acid bacteria respectively showed extremely low colony counts (<30 colonies) even with the undiluted samples. Therefore, it was decided not to consider them for further analysis and the bacterial community composition was only analyzed using ARISA over the 3-year period. Ten peaks were identified in the 2012 grape juice sample where peak 592 bp was the most abundant (16.92 %) followed by 557 bp, 525 bp, 505 bp, 517 bp, 530 bp, and 633 bp, which showed an abundance of 16.48 %, 13.63 %, 14.09 %, 10.69 %, 8.78 %, and 7.16 %, respectively. The remainder of the peaks exhibited abundance below 5.50 % (Fig. 1c). In contrast, seven peaks were identified in the 2013 grape juice sample. The peaks 700 bp (30.31 %), 723 bp (25.37 %), and 433 bp (20.06 %) were the most abundant whereas the 517 bp was least abundant (2.30 %), although in 2012 it accounted for>10 % of the total peaks (Fig. 1c).

The 2014 grape juice sample displayed was dominated by 679 bp at 23.63 % whereas the 803 bp fragment had the lowest abundance (1.90 %). Similar to 2013, 433 bp was present in high levels (18.70 %). Other dominant peaks were 479 bp, 580 bp, and 587 bp, which accounted for 7 %-13 % of the total peaks (Fig. 1c). The 2012 fungal and bacterial populations exhibited the highest species richness (Menhinick's index; I) whereas the 2014 represents the highest species diversities (Shannon Weinner index; H) for both the populations. The cultivation-based study showed the highest yeast species richness (I) and diversities (H) for 2014 and 2013, respectively. Both the species richness and diversity indexes showed a gradual reduction from day 1 to day 3 of the 2013 fermenting samples (Table 1).

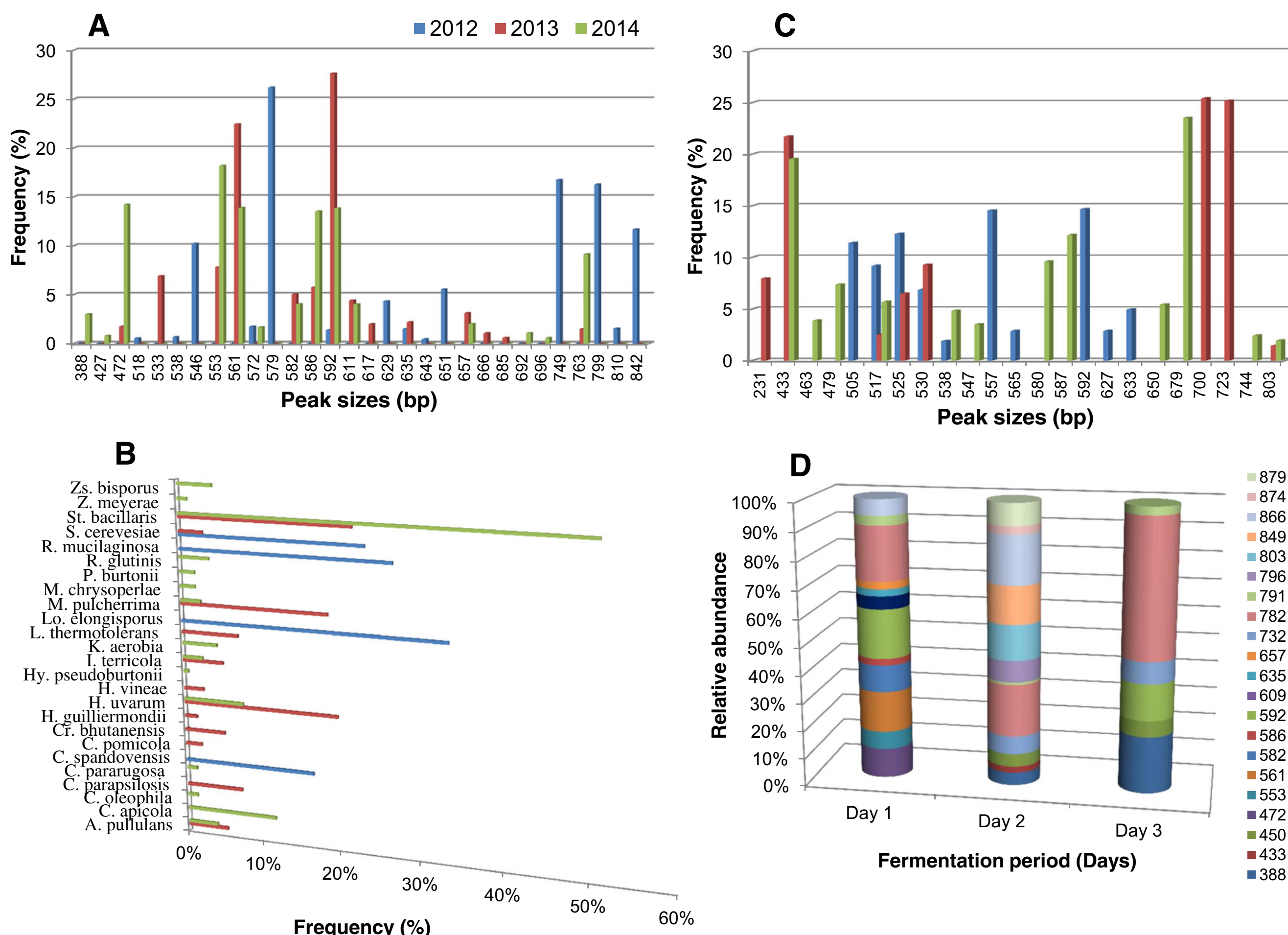

Fig. 1 Representation of the fungal, yeast, and bacterial diversity. The histograms exhibit the (**a**) fungal diversity by ARISA, (**b**) yeast diversity by PCR-RFLP, and (**c**) bacterial diversity by ARISA of the grape juice sample for the years 2012, 2013, and 2014, respectively. The values on the top of the vertical bars represent the relative peak sizes/species abundance of the sample. (**d**) The stacked columns represent the fungal population dynamics on the onset of fermentation for the 2013 grape juice sample. The days 1, 2, and 3 represent three consecutive days of fermentation after the day 0. Each vertical rectangle of the stacked columns represents the relative fungal peak sizes abundance (values shown immediate next to the vertical rectangles)

Population dynamics study of the fungal community

The use of ARISA as a tool to monitor yeast population dynamics was tested by monitoring the fungal population in the first three consecutive days of spontaneous fermentation in 2013. Our data revealed a decline in diversity from 13 peaks in the initial must to 12, ten and six the next consecutive days. The 592 bp peak, which was dominant (30.17 %), declined rapidly while peaks 782 bp, 791 bp, and 866 bp, which were not detected on the day 0, increased in abundance and remained present throughout the fermentation (Fig. 1d). Similarly, peaks 450 bp and 732 bp only appeared from day 2 of fermentation (Fig. 1d).

Discussion

The objective of the current study was to investigate the microbial consortium in the fermenting wine must over three consecutive years employing both cultivation-dependent and cultivation-independent approaches. The yeast population dynamics was also monitored over the first few days of fermentation in one specific year in order to assess the rate at which the yeast population changed. The yeast load (about 10^6 CFU/mL) of the grape juice obtained in our study was similar to those reported previously (Cocolin et al. 2000). Cultivation-based analysis showed that the yeast community comprised *Saccharomyces cerevisiae* and non-*Saccharomyces* yeasts including *Issatchenkia terricola*, *Pichia burtonii*, *Hanseniaspora uvarum*, *H. vineae*, *H. guilliermondii*, *Candida apicola*, *C. oleophila*, *Starmerella bacillaris*, *Candida pomicola*, *C. parapsilosis*, *C. pararugosa*, *C. spandovensis*, *Cryptococcus bhutanensis*, *Metschnikowia chrysoperlae*, *M. pulcherrima*, *Aureobasidium pullulans*, *Rhodotorula glutinis*, *R. mucilaginosa*, *Hyphophichia pseudoburtonii*, *Lodderomyces elongisporus*, *Zygoascus meyerae*, *Lachancea thermotolerans*, *Zygosaccharomyces bisporus*, and

Table 1 Ecological diversity indices demonstrating the fungal, cultivable yeast and bacterial diversity from the years 2012, 2013 and 2014

Year	Investigations		Techniques used	Species richness (Menhinick's index; I)	Species diversity (Shannon Weinner index; H)
2012	Fungal population		ARISA	0.13	2.10
			Cultivation-based	0.73	1.36
	Bacterial population		ARISA	0.17	2.17
2013	Fungal population		ARISA	0.08	1.97
			Cultivation-based	0.87	2.16
	Bacterial population		ARISA	0.04	1.69
	Fungal population dynamics	Day 1	ARISA	0.05	2.25
		Day 2		0.05	2.05
		Day 3		0.04	1.35
2014	Fungal population		ARISA	0.07	2.36
			Cultivation-based	1.21	1.87
	Bacterial population		ARISA	0.10	2.22

Kazachstania aerobia. Some of these species such as *Candida parapsilosis* and *A. pullulans* have already been shown to be part of the microbiota in this vineyard (Setati et al. 2012). Overall, the yeast diversity in the grape musts evaluated in the current study included typical wine yeasts as reviewed by Jolly et al. (2014).

Our study revealed similar yeast diversity between the culture-based study and the ARISA for three consecutive years (2012, 2013, and 2014). However, exact correlation between the isolates and the peaks could not be established. The 2013 and 2014 samples produced similar ARISA profiles with nine peaks of fungal ITS regions shared between the two years. An attempt was made to correlate the sizes of the peaks with those of the known ITS sequences of the yeast isolates (Table 2) with the assumption that it could possibly provide us with some information on the yeast species present in the grape juice and fermenting must sample. The 388-bp peak (closely related to *M. pulcherrima*, abundance frequency 2-19 %) identified from 2013 and 2014 samples was also detected at a comparable population frequency (2-21 %) on plates for both years (Fig. 1a-d and Table 2). The 427-bp and 582-bp peaks (possibly *I. terricola*, and *A. pullulans*, respectively) also showed similar abundance levels between the ARISA and culture-based method. The 472-bp peak (possibly *Starmerella bacillaris*) identified in both the 2013 and 2014 grape juice sample was also reflected in the culture-based studies. However, large discrepancies in its population abundance were observed between ARISA and PCR-RFLP for both years. In contrast, peak 611-bp could represent several yeast species, which have ITS regions of similar sizes as, for instance, *Zygoascus meyerae* and *R. mucilaginosa* (Table 2). Similar discrepancies were observed regarding the presence or absence of *H. vineae* and *S. cerevisiae* as well as their relative abundance in the ARISA profiles compared to culture-based

Table 2 Tentative identification of fungal ARISA peaks through possible correlations between fungal ARISA peak sizes and yeast isolates' ITS-5.8S rRNA-ITS2 PCR amplicons. The symbol (−) implies undetectable level

Yeast isolate	ITS-5.8S rRNA-ITS2 PCR amplicons (bp)	Peak sizes derived from ARISA (bp)
Metschnikowia chrysoperlae	375	-
Metschnikowia pulcherrima	390	388
Candida pararugosa	414	-
Hyphopichia pseudoburtonii	416	-
Issatchenkia terricola	421	427
Pichia burtonii	444	-
Starmerella bacillaris	475	472
Candida pomicola	480	-
Candida spandovensis	480	-
Candida parapsilosis	522	
Lodderomyces elongisporus	550	553
Aureobasidium pullulans	580	582
Rhodotorula glutinis	606	-
Zygoascus meyerae	609	611
Rhodotorula mucilaginosa	610	611
Candida apicola	458	450
Candida oleophila	628	629
Cryptococcus bhutanensis	630	635
Lachancea thermotolerans	675	
Zygosaccharomyces bisporus	741	-
Kazachstania aerobia	751	749
Hanseniaspora uvarum	747	749
Hanseniaspora vineae	738	732
Hanseniaspora guilliermondii	749	749
Saccharomyces cerevisiae	840	842

analysis. No tentative identification could be established for the 553-bp, 586-bp, 592-bp, 657-bp, and 763-bp peak sizes. These peaks could represent filamentous fungi or other yeasts that could not be isolated. Such discrepancies between the culture-independent and culture-dependent analyses can be expected since both approaches are biased. Failure to retrieve certain yeast isolates could be due to the rapid growth of the other competing yeasts and similarities between colony morphologies resulting in poor isolation. ARISA, like other PCR-based methods, could be affected by DNA extraction efficiencies in mixed populations. Brezna et al. (2010) reported similar challenges relating to ambiguous identification of some peaks as well as overlapping peaks preventing confident taxonomic assignment of the peaks. Ultimately, proper taxonomic identification of the peak sizes will require the establishment of a database consisting of cultivable grape and wine fungal ARISA profiles.

The current study also evaluated the yeast dynamics at the onset of fermentation as the behavior of the yeast population at this stage is an important determinant of fermentation tempo and ARISA. Our data show that some peaks, which were initially detected in must, disappear while new ones appear. This is consistent with the tumultuous nature of the onset of fermentation especially in spontaneous fermentation. Previous studies have mainly focused on yeast dynamics in three phases and show that by the middle of fermentation the yeast population stabilizes and is characterized by a clear dominance of weakly fermentative yeasts such as *Hanseniaspora* spp. and strongly fermentative yeasts especially *S. cerevisiae* (Brezna et al. 2010; Krakova et al. 2012; Zenisova et al. 2014).

Each year revealed a unique bacterial ARISA profile. Common wine bacterial species whose genomes have been sequenced were selected to generate an ITS database that was used to tentatively identify the peaks. *In silico* analysis of the genomes of *Oenococcus oeni*, *Lactobacillus brevis*, *Lactobacillus plantarum*, *Pediococcus pentosaceus*, *Lactococcus lactis*, *Enterobacter cloacae*, *Leuconostoc mesenteroides*, *Lactobacillus salivarius*, *Enterococcus faecium*, and *Gluconobacter oxydans* genomes showed that the size of their ITS regions varies from 200–700 bp (Table 3). The ARISA of the 2014 grape juice sample exhibited peak sizes of 433 bp (18.70 % abundance), 479 bp (7.76 %) and 744 bp (2.77 %), which were close to the size of the *Enterococcus faecium*, *O. oeni*, and *G. oxydans* ITS regions, respectively. The 433-bp peak (20.06 %) was also present in the year 2013. Also, the 2012 and 2013 ARISA exhibited peak sizes of 505 bp (14.09 %) and 231 bp (7.64 %), respectively. These could be related to *Lactobacillus brevis* (504 bp) and *P. pentosaceus* (229 bp) ITS regions. It was observed that a 517-bp peak with variable abundance was consistently present in all the 3 years of sampling but could not be clearly identified. No probable correlation could be established, *in silico*, with the remaining peak sizes since many of the common wine eubacterial genomes have not been sequenced.

Table 3 Tentative identification of fungal ARISA peaks through possible correlations between bacterial ARISA-peak sizes and bacterial ITS region retrieved by *in silico* analysis. The symbol (–) implies undetectable level

Bacterial isolate	ITS amplicons (bp)	Peak's size derived from ARISA (bp)
Lactobacillus plantarum	206	-
Pediococcus pentosaceus	229	231
Lactococcus lactis	305	-
Enterobacter cloacae	341	-
Leuconostoc mesenteroides	376	-
Lactobacillus salivarius	409	-
Enterococcus faecium	430	433
Oenococcus oeni	471	479
Lactobacillus brevis	504	505
Gluconobacter oxydans	739	744

Large discrepancies were noted between the three vintages studied. Fluctuations in the microbial communities associated with the grape berry surface and present in grape must are common and can be attributed to various factors including berry ripeness levels, berry damage, and climatic conditions. In addition, the detection of the different microorganisms in ARISA may be influenced not only by the relative abundance levels, cell lysis, and DNA extraction efficiency (Rastogi and Sani 2011). Furthermore, the population dynamics studied with different culture independent techniques like DGGE (Renouf et al. 2007) and FISH (Xufre et al. 2006) have shown similar trends as in our study.

Our current data confirm that ARISA can be employed as a tool to monitor microbial community structures in grape juice and their dynamics throughout fermentation. The data reveal similar trends as shown with other methods such as DGGE and FISH, with a rapid decline of the yeast population in the first few days of fermentation. However, since ARISA is semiautomated, it allows for quantitative estimation of the relative abundance of different peaks, and with the support of a robust database it can also allow for taxonomic identification of the peaks. This method is relatively fast, less time-consuming, and is more sensitive than DGGE. Future studies could also focus on retrieving, when necessary, the ITS nucleotide sequences from ARISA by eluting out each DNA fragment from the capillaries to allow direct identification from ARISA spectra.

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