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Title: Analysis of microbial diversity and dynamics during wine fermentation of Grenache grape variety by high-throughput barcoding sequencing

Article Type: Research paper

Keywords: wine; fermentation; high-throughput sequencing; lactic acid bacteria; yeast.

Corresponding Author: Dr. Maria del Carmen Portillo, Ph.D.

Corresponding Author's Institution: University of Rovira i Virgili

First Author: Maria del Carmen Portillo, Ph.D.

Order of Authors: Maria del Carmen Portillo, Ph.D.; Albert Mas

Abstract: Understanding the diversity and evolution of microorganisms during wine fermentation is essential for controlling its production. Previous studies have been primarily based on culture-dependent methods but recent incorporation of culture-independent molecular methods is showing a quite different view of microbial composition and diversity during the wine making process. Herein we applied barcoded pyrosequencing technology to monitor bacterial and yeast dynamics during laboratory scale spontaneous wine fermentation from Grenache variety. Members of the lactic acid bacteria (LAB) and acetic acid bacteria (AAB) were the most abundant, representing the orders Lactobacillales and Rhodospirillales more than 70% of the bacterial population. Other bacterial genera, not previously detected at the end of fermentation, were present in low proportion and their possible role remains unknown. Within the yeast community, the genera Hanseniaspora and Candida were dominant during the initial and mid fermentation while the final fermentation was mainly dominated by Candida and Saccharomyces. This study contributes to the knowledge of the microbial dynamics across spontaneous wine fermentation and presents high-throughput sequencing as a useful tool to monitor and evaluate bacterial and yeast diversity and dynamics during wine fermentation.

Maria del Carmen Portillo
Departament de Bioquímica i Biotecnologia
Facultat d'Enologia de Tarragona
Marcel·lí Domingo, 1
43007-TARRAGONA
Tel: +34-977 558688
Fax: +34-977 558232
E-mail: carmen.portillo@urv.cat

R. K. Singh
LWT Food Science and Technology

Dear Editor,

Please, find attached the submitted manuscript of our paper entitled "Analysis of microbial diversity and dynamics during wine making by high-throughput barcoding sequencing" authored by M.C. Portillo and A. Mas.

The manuscript describes the microbial population evolution during spontaneous alcoholic fermentation of Grenache grapes by applying the barcoded pyrosequencing technique. The results point acetic acid bacteria (AAB) belonging to Acetobacteriaceae and lactic acid bacteria (LAB) belonging to Bacillales as the most abundant bacterial groups detected throughout the fermentation with the predominance of the genus *Gluconobacter*. We also found that *Candida* and *Saccharomyces* imposed at the end of the fermentation and finished it successfully. This study suggests that the AAB and LAB populations present during spontaneous wine fermentation are more abundant than was previously thought. Also, high-throughput sequencing is presented as a useful technique for assessing microbial changes in wine fermentations being able to differentiate between the yeast communities of different grape varieties.

We hope you will find the manuscript acceptable for your journal.
Sincerely,

M.C. Portillo

***Detailed Response to Reviewers**

Reviewers' comments:

Reviewer #1: The study reveals the microbial dynamics across spontaneous wine fermentation and presents high-throughput sequencing as a useful tool to monitor and evaluate bacterial and yeast diversity and dynamics during wine fermentation. The paper is a good scientific addition but should not be published as a Research Article. Rather, a Short Communication would be sufficient for this work.

Thank you for the good comments. Because of the length of the manuscript, we think that a research article is more appropriate for our contribution. However, if the editor considers it necessary, we could take the effort to short substantially the manuscript to fit into the short communications requirements.

Although the approach seems to be very interesting from the scientific standpoint, some comments were made:

1. How can authors state the applicability of the method in medium and small-size companies? Is it feasible?

The reviewer makes an excellent point, the HTS techniques are not immediately applicable to the small or medium-size companies due to the high cost of the technology and the specific informatics skills requirements. However, it is possible to develop in a short term an external service to make periodic analysis to wines and to prevent microbial contaminations. In fact, there is already a pioneer company offering this kind of service (massive sequencing) to analyze vineyard microbiomes across the world: "Biome Makers".

2. The units in the submissions are not equalized. There are g.l-1 and g/l… there are also g/l and g/L……..be consistent

The reviewer is right; we have reviewed the manuscript and corrected the inconsistences in the units.

3. What is the accuracy of the barcoding technique using the method applied in the study/?

Ion Torrent$^{(TM)}$ currently support fragments up to 430 bp with basecalling accuracy on par with other platforms like Illumina MiSeq or Pacific Biosciences.

4. Figure 1: authors should improve the quality of the image. In addition, authors should add the standard deviation of the replicates as an estimate of error.
The Figure 1 has been changed as suggested, with the standard deviation included and the quality improved.

Reviewer #2: The manuscript is about an analysis of microbial diversity and dymamics during wine fermantation of Grenache grape, one of the most important cultivars grown in Catalunya, Spain. The paper was well prepared and discussed, but needs some minor revision, as described below:

Thank you for the good comments.
L88: add (Vitis vinifera L.) after Grenache.

Added as suggested
L89: add "Catalynia, Spain" after Priorat region.

Added as suggested
L90: add information concerning the vineyard conditions, chemical properties of harvested grapes (TSS, AT, pH etc). Were the grapes processsed right after harvesting. How the 3-kg of grapes were collected inside the vineyard (sampling)?

The pH, acidity and density of the initial grape must is already indicated (lines 97-98) and the sampling information has been now expanded (lines 89-94)
L99: check "...was below 2 g l-1" The units must standarized, such as liters (L) and not (l). Check it all over the manuscript.

We have reviewed the manuscript and corrected the inconsistences in the units.

L106: check the unit G-force (4000 x g). I think that the correct way is 4000 x G, since g is used for grams.

We have checked the unit G-force and the correct way is in fact "x g"
L214-242. This paragragh is too long.

The paragraph (current lines 218-236) has been modified and shortened.
Tables and Figures: a) add at the end of each title: "... during wine fermentation of 'Grenache' grapes. b) add the meaning of d0, d1...d10 (day 0, day 1...day 10) as a footnote of each Table/Figure.

"Grenache grapes" has been added as suggested at the end of each title and the meaning of d0, d1, d2, d3 and d10 has been included as a footnote of Tables 1 and 2 and also as part of the caption of Figure 2.

Highlights:

- Bacteria and yeast on Grenache wine were analyzed by high-throughput sequencing
- Rhodospirillales and Lactobacillales were the most abundant bacterial taxa
- *Hanseniaspora*, *Candida* and *Saccharomyces* were the dominant yeasts
- High-throughput sequencing is useful to monitor microbial dynamics during fermentations

Analysis of microbial diversity and dynamics during wine fermentation of Grenache grape variety by high-throughput barcoding sequencing

Maria del Carmen Portillo*, Albert Mas

Biotecnología Enológica. Dept. Bioquímica i Biotecnologia, Facultat d'Enologia. Universitat Rovira i Virgili. C/ Marcel·lí Domingo s/n. 43007 Tarragona.

Corresponding author: Maria del Carmen Portillo

Postal address: *Dept. Bioquímica i Biotecnologia, Facultat d'Enologia. Universitat Rovira i Virgili. C/ Marcel·lí Domingo 1. 43007 Tarragona*

E-mail: carmen.portillo@urv.cat

Phone: +34 977 558688, Fax: +34 977 558232

ABSTRACT

Understanding the diversity and evolution of microorganisms during wine fermentation is essential for controlling its production. Previous studies have been primarily based on culture-dependent methods but recent incorporation of culture-independent molecular methods is showing a quite different view of microbial composition and diversity during the wine making process. Herein we applied barcoded pyrosequencing technology to monitor bacterial and yeast dynamics during laboratory scale spontaneous wine fermentation from Grenache variety. Members of the lactic acid bacteria (LAB) and acetic acid bacteria (AAB) were the most abundant, representing the orders Lactobacillales and Rhodospirillales more than 70% of the bacterial population. Other bacterial genera, not previously detected at the end of fermentation, were present in low proportion and their possible role remains unknown. Within the yeast community, the genera *Hanseniaspora* and *Candida* were dominant during the initial and mid fermentation while the final fermentation was mainly dominated by *Candida* and *Saccharomyces*. This study contributes to the knowledge of the microbial dynamics across spontaneous wine fermentation and presents high-throughput sequencing as a useful tool to monitor and evaluate bacterial and yeast diversity and dynamics during wine fermentation.

Keywords: wine; fermentation; high-throughput sequencing; lactic acid bacteria; yeast;

1. Introduction

The conversion of grape must to wine is a complex biochemical process involving interactions between yeasts and bacteria. It is essential to understand the composition and behaviour of these microorganisms during fermentation in order to expand our understanding of fermentation problems, to improve fermentation control and to obtain final products with the desired organoleptic characteristics. However, most of the analyses of microbial diversity during wine fermentation have been based on culture-dependent methods that relied on the isolation of colonies for further characterization. Using culture-dependent methods, there is a high risk of misidentification of the ecology of complex microbial ecosystems (Amann, Ludwig, & Schleifer, 1995; Rantsiou et al., 2005).

Recently, several culture-independent methods based on the nucleic acids have been used to analyze the microbial diversity of grapes and wines (reviewed in Cocolin, Campolongo, Alessandria, Dolci, & Rantsiou, 2011), and these methods have complemented classical physiological techniques. For example, the technique PCR-DGGE has been widely used to study the microbial ecology of grapes and wine fermentation (Andorrà, Landi, Mas, Esteve-Zarzoso, & Guillamón, 2010; Cocolin, Bisson, & Mills, 2000; Prieto, Jara, Mas, & Romero, 2007; Renouf, Strehaiano, & Lonvaud-Funel, 2007), and qPCR assays have been developed to detect and enumerate both bacteria and yeast in wine (Andorrà, Landi, Mas, Esteve-Zarzoso, & Guillamón, 2010; González, Hierro, Poblet, Mas, & Guillamon, 2006; Hierro, Esteve-Zarzoso, Mas, & Guillamón, 2007). The use of molecular biology methods has generally supported the traditional results but, in addition, these newer techniques have identified a much higher microbial diversity than previously expected (Navarro, Mateo, Torija, & Mas, 2013). Thus, the present view of bacterial and eukaryotic species associated with grapes, must and wines is much more complex than has

been previously described. Nevertheless, the detection by DGGE is difficult for species present at population densities below 10^3 CFU/ml or two orders of magnitude lower than the most abundant members of these communities (Muyzer & Smalla, 1998; Prakitchaiwattana, Fleet, & Heard, 2004) and the detection by qPCR is limited to the quantification of the few targeted species.

Nowadays, high-throughput sequencing (HTS) technologies, such as the 454 pyrosequencing of amplicons, can be used to characterize more precisely the microbial diversity of complex environmental ecosystems, including food samples (Ercolini, 2013; Solieri, Dakal, & Giudici, 2013; Galimberti et al., 2015). For example, HTS techniques have recently been used to determine the bacterial diversity of botrytized wines (Bokulich, Joseph, Allen, Benson, & Mills, 2012) and Chinese traditional sourdough (Liu et al., 2016), to monitor seasonal changes in winery-resident microbiota (Bokulich, Ohta, Richardson, & Mills, 2013) or to analyze the microbial biogeography of grapes from a Californian region (Bokulich, Thorngate, Richardson, & Mills, 2014).

The aim of this study was to analyze bacterial and yeast community structure and dynamics during wine fermentation of Grenache variety using barcoded pyrosequencing which eliminates the limits imposed by techniques such as culturing the primary species, the limited detection of DGGE-PCR and the quantification of only targeted species by qPCR.

2. Materials and methods

2.1. Alcoholic fermentation and sample collection

Three kg of healthy grapes of the Grenache (*Vitis vinifera L*.) variety were collected from Ferrer Bobet vineyard from the DOC Priorat region (Catalonia, Spain) and were aseptically destemmed and hand squeezed in the laboratory. Fermentation was

91 performed in duplicate, 1.8 kg of grape must and pomace were put in 2 L bottles.
92 Alcoholic fermentation was performed at room temperature (controlled at 23 ± 1 ºC)
93 with gentle shaking (120 rpm). The initial must had a density of 1100 g/L, 4.8 g/L of
94 total acidity (expressed as tartaric acid) and pH of 3.2. The fermentation was
95 sulphited (30 ppm of SO_2 as metabisulphite). Sugar consumption was monitored daily
96 by measuring the density (g/L) of the fermenting must and by enzymatic assay
97 (Roche Applied Science; Germany). Both fermentations were allowed to proceed
98 without yeast inoculation and finished successfully in 10 days. Fermentations were
99 considered to be finished when the level of reducing sugars was below 2 g/L and
100 must density was below 1000 g/L.
101 Concentrations of acetic acid and glycerol in the samples were tested by Miura One
102 Multianalyzer (TDI, Barcelona, Spain) using the enzymatic kit from Biosystems S. A.
103 (Barcelona, Spain); and those of glucose, fructose and ethanol by enzymatic kit from
104 Roche Diagnostics (Darmstadt, Germany) at the end of fermentation. Samples of 10
105 mL each were taken aseptically from both fermentations at days 0, 1, 2, 3 and 10,
106 corresponding with initial (days 0 and 1), mid (days 2 and 3) and final fermentation
107 (day 10) (Fig. 1). The samples were centrifuged at 4000 x g and pellets were
108 immediately stored at -80ºC.

109 *2.2. DNA extraction and pyrosequencing of the 16S and 18S rRNA genes*

110 DNA of samples was extracted using the standard protocol for the QIAamp DNA Mini
111 kit (Qiagen, Hilden, Germany), including three bead-beating steps for 3 min in a
112 FastPrep-24 bead beater (MP Bio, Solon, OH) to homogenize the samples. Extracted
113 DNA was sent to LifeSequencing S.L. (Valencia, Spain) for amplification and
114 pyrosequencing analysis. Briefly, partial 16S and 18S rRNA genes were amplified
115 using the Roche 454 FLS GS Titanium sequencer at LifeSequencing S.L. with

primers provided by the company. One library barcoded with a different molecular identifier tag (Roche) was constructed per sample. The amplified DNA was quantified using Quant-IT™ PicoGreen® kit (Invitrogen) to generate an equimolecular pool for subsequent sequencing. Raw 16S and 18S rRNA gene sequence data generated from pyrosequencing were processed in QIIME (Caporaso et al., 2010). Briefly, reads were discarded if the average quality score of the read was <25, if the length of the read was <200 and any read containing one or more ambiguous base calls. Operational taxonomic units (OTUs) were assigned using QIIME's uclust-based (Edgar, 2010) open-reference OTU-picking workflow, with a threshold of 97% pairwise identity. OTU taxonomy was determined using the RDP classifier at 97% similarity (Wang et al., 2007) and retrained toward the GreenGenes bacterial 16S rRNA database (13_8 release) for 16S sequences (DeSantis et al., 2006) and toward the Silva 111 release for 18S sequences (Quast et al., 2013). Chimeric sequences were identified and removed using ChimeraSlayer (Haas et al., 2011). A final OTU table was created, excluding singletons (sequences observed just once), and sequences matching plant mitochondria or chloroplast. To avoid biases generated by differences in sequencing depth, bacterial and eukaryotic reads were rarified to an even depth of 300 sequences per sample. Those samples below that threshold following all quality filtering steps were discarded. Alpha diversity (within-sample species richness) estimates were calculated by analyzing the observed species and Chao1 indexes and the Good´s coverage.

3. Results and discussion

Replicated fermentations of Grenache must were allowed to proceed without yeast inoculation and finished successfully in 10 days (Fig.1). The values of the main parameters of the final wines were identical with 14% ethanol (v/v), 12.6 g/L glycerol

and 1.2 g/L of residual sugars (only fructose) and density 997g/L. These values were consistent with previous alcoholic fermentations of the natural grape musts performed by our laboratory and proceeded without presenting stuck or sluggish fermentations.

We obtained a total of 132,354 sequences after the quality filtering with an average length of 531 nt. At the 97% identity level, the final OTU table contained 134 bacterial and 238 eukaryotic distinct OTUs. The Good´s coverage values were above 90 % for bacterial and between 81 and 100% for eukaryotic sequences (Table 1). These results indicated that the selected sampling effort was appropriated to reveal most bacterial and eukaryotic diversity in these samples. The exception was represented by the sequences obtained from wine fermentation samples at day 0 that were estimated to cover just 58% of the total bacterial diversity. According to the number of observed species and the estimated Chao1, the bacterial diversity was fluctuating across fermentation while eukaryotic diversity clearly tended to increase through the end of the fermentation (Table 1).

The most abundant bacterial taxonomic group during all the fermentation was the *Acetobacteraceae* representing up to 92% of the sequences (Fig. 2A). Within *Acetobacteraceae*, the dominant genus was *Gluconobacter* representing 41-81% of the bacterial reads and peaked at mid fermentation (Table 2). This is somewhat unexpected because although *Gluconobacter* have been described as common in grapes, they usually are sensitive to alcohol, and thus normally decline during alcoholic fermentation (Du Toit & Pretorius, 2002; Joyeux, Lafon-Lafourcade, & Ribereau-Gayon, 1984; González, Hierro, Poblet, Mas, & Guillamon, 2005). Du Toit & Lambrechts (2002) observed a decrease in AAB from 10^6-10^7 cfu/mL prior to yeast inoculation to 10^3-10^4 and 10^2-10^3 cfu/mL in the middle and at the end of the fermentation, respectively. However, the use of culture-independent methods

reported that the AAB population remained elevated (between 10^4-10^5 cells/mL) throughout fermentation (Andorrà, Landi, Mas, Esteve-Zarzoso, & Guillamón, 2010), which would agree with our present results. Furthermore, a previous study based on pyrosequencing reported high abundance of AAB in botrytized wine in the range of 40-80% of Rhodospirillales, mainly belonging to *Gluconobacter* (Bokulich, Joseph, Allen, Benson, & Mills, 2012). These authors attributed the high abundance of acetic acid bacteria to the nature of botrytized wine. However, on a more recent study also using massive sequencing to monitor the effect of sulfite addition over the microbial communities present in wine fermentation of Chardonnay variety, LAB and *Gluconobacter* bloomed during fermentation with concentrations below 25 µg/L of SO_2 (Bokulich, Swadener, Sakamoto, Mills, & Bisson, 2014) which supports our results on Grenache grape variety. Altogether, these results suggest that AAB are more abundant and dynamic than previously thought during low-sulfited or unsulfited wine fermentations, independently of the grape variety, but further studies on additional grape varietal should be necessary to confirm if this is a generalized fact.

We detect low proportion of *Acetobacter* (from 6 to 2%) and its abundance decreased across fermentation. This result was also unexpected because *Acetobacter* is moderately resistant to alcohol and has been described in the late stages of alcoholic fermentation (Du Toit & Lamberchts, 2002; Joyeux, Lafon-Lafourcade, & Ribereau-Gayon, 1984; González et al., 2004). In wines, *Acetobacter aceti* has been considered the main altering AAB and later studies indicated that *A. pasteurianus* was present in spoiled bottled wines (Bartowsky & Henschke, 2008). Previous HTS studies did not detect Acetobacter at all genus during Chardonnay wine fermentation (Bokulich, Swadener, Sakamoto, Mills, & Bisson, 2014), which is a clear difference with our Grenache wine fermentation results.

193 Sequences belonging to the genera *Gluconacetobacter* or *Asaia* were also rare in our
194 analysis (Table 2). Several species previously classified within *Gluconacetobacter*
195 have been reclassified as members of the new genus *Komagataeibacter* (Yamada et
196 al., 2012), and some of these have been observed in some alcoholic fermentations,
197 albeit in low proportions (González et al., 2004). Other *Acetobacteraceae* were also
198 present at very low percentages, although they could not be unambiguously assigned
199 to specific genera (Table 2).
200 At the end of fermentation, we observed a decrease in AAB sequences (Table 2; Fig.
201 2A) at the same time that an increase of LAB of the order Lactobacillales (Fig. 2A).
202 Andorrà, Landi, Mas, Guillamón, & Esteve-Zarzoso (2008) described an increase of
203 LAB population close to 10^4 cells/mL at the end of Carignan wine fermentation using
204 qPCR technique and Bokulich, Swadener, Sakamoto, Mills, & Bisson (2014) reported
205 more than 60% of sequences obtained by HTS related to *Lactobacillaceae* at the 10^{th}
206 day of Chardonnay wine fermentation, both results for non-inoculated and SO_2 free
207 fermentations. The observed increase of LAB at the end of alcoholic fermentation on
208 our Grenache wine could be expected to generate the appropriate conditions for the
209 subsequent malolactic fermentation (although, as in most spontaneous
210 fermentations, it did not proceed).
211 We could detect other minor bacterial taxonomic groups during the fermentations
212 (Fig. 2A), some of them, like genera *Orbus* and *Wolbachia,* were detected until the
213 end of the alcoholic fermentation although their role remains unknown and they have
214 not been detected in previous studies.
215 The most abundant eukaryotic genera were *Hanseniaspora, Candida* and
216 *Saccharomyces*, with a clear succession during fermentation (Fig. 2B). *Issatchenkia*
217 was relevant in the must too. These genera have been frequently isolated on
218 spontaneous fermentations (Clemente-Jimenez, Mingorance-Cazorla, Martínez-

Rodríguez, Las Heras-Vázquez, & Rodríguez-Vico, 2004). At the initial fermentation, *Hanseniaspora* and *Issatchenkia* were dominant, though their abundance decreased sharply across fermentation and *Candida* represented the most abundant genus from the 24 h to mid fermentation (52-87%). At the end of the fermentation, *Saccharomyces* was the dominant genus together with *Candida* (48 and 40% respectively). These results were in agreement with previous studies from our group using PCR-DGGE and qPCR on spontaneous fermentations and describing the abundance of *Hanseniaspora* at the beginning of the fermentation and *Candida* and *Saccharomyces* at the end of alcoholic fermentation (Andorrà, Landi, Mas, Guillamón, & Esteve-Zarzoso, 2008; Andorrà, Landi, Mas, Esteve-Zarzoso, & Guillamón, 2010). However, Bokulich, Swadener, Sakamoto, Mills, & Bisson (2014) described by HTS techniques *Kluyveromyces* as the dominant yeast at early stage of Chardonnay grapes fermentation before being displaced by *Hanseniaspora* and then *S. cerevisiae*, which clearly differed from our results for Grenache variety. Although *Kluyveromyces* is often isolated in fermenting musts, in our previous studies on the ecology of Grenache and other grape varieties from Priorat wine region, its presence was very marginal (Constantí, Poblet, Arola, Mas, & Guillamón, 1997; Torija, Rozès, Poblet, Guillamón, & Mas, 2001). Our results would be somewhat comparable to partial results from David et al. (2014), who monitored by HTS technique the yeast diversity across wine fermentation from Chardonnay grapes grown under different treatments. These authors found *Hanseniaspora* as the most abundant yeast at the beginning of the fermentation on the conventional treatment and *Candida* was predominant at mid fermentation. Nevertheless, our results contribute further to yeast dynamics during wine fermentation because David et al. (2014) did not analyze the final stage of the fermentation in which *Saccharomyces* could have finally dominated.

The current study analyzed microbial DNA, thus detecting both living and dead cells and other methods should be used to detect only live cells. However, the succession of yeasts observed in all fermentations and the emergence of low-abundance bacterial organisms in the late fermentation suggest microbial growth and dynamic.

We are fully aware that these results are based in a limited number of analyses and, as the fermentations were spontaneous, proceeded without an external control of the microbiota present during the process. Previous studies have described the high sensitivity of LAB to SO_2 addition (Andorrà, Landi, Mas, Guillamón, & Esteve-Zarzoso, 2008) and that AAB bloomed at the end of Chardonnay wine fermentation just under low sulfite concentrations (Bokulich, Swadener, Sakamoto, Mills, & Bisson, 2014). However, the data obtained regarding the yeast succession (Non *Saccharomyces* followed by *Saccharomyces cerevisiae* after mid fermentation) and the good performance of the fermentation (alcoholic fermentation finished in 10 days with low levels of residual sugars) makes us to consider the present fermentation as representative of spontaneous fermentations of Grenache grapes. Hence, our results show that, in spontaneous fermentation of Grenache variety, AAB are dominant until the end of the fermentation when LAB population becomes abundant. Furthermore, the initial yeast population on must was composed mainly by *Hanseniaspora* and *Issatchenkia* though they were quickly displaced by *Candida* and later *Saccharomyces.* The techniques used in this study nicely reflected the dynamics of bacterial and yeast communities during wine fermentation of Grenache variety in more detail than previous used molecular techniques and these results confirmed the capability of the naturally originated microbial communities to self-regulate the species involved in the wine making process.

4. Conclusions

Next-generation sequencing was used to reveal microbial population dynamics during spontaneous fermentation of Grenache grapes. We described the persistence of *Gluconobacter* species during alcoholic fermentation suggesting that the AAB and LAB populations present during spontaneous wine fermentation are more abundant than previously thought. *Candida* and *Saccharomyces* imposed at the end of the fermentation and finished it successfully, which is different of what a previous HTS study have described for Chardonnay varietal. Additional work could be necessary to analyze more grape varieties and compare the obtained results. Our data suggest HTS techniques are useful for assessing microbial changes in wine fermentations of different grape varieties or under different treatments and this information could be used by winemakers to drive fermentation processes and to set up the most suitable environmental conditions to enhance wine characteristics.

Acknowledgements

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Figure captions

Figure 1 Evolution of wine fermentations of 'Grenache' grapes measured as density (g/L). Error bars indicated the standard deviation of the replicates.

Figure 2 Relative abundance of the most abundant bacterial (A) and eukaryotic (B) taxonomic groups across spontaneous wine fermentation process of 'Grenache' grapes. d0, d1, d2, d3 and d10 mean day 0, day 2, day 3 and day 10 respectively. “Other Bacteria” includes the genera *Propionibacterium*, *Bifidobacterium*, *Clostridium*, *Blastococcus*, *Enterococcus*, *Sporolactobacillus*, *Asaia*, *Enterobacter*, *Subdoligranulum*, *Janibacter*, *Acidovorax*, *Acinetobacter*, *Pelomonas*, *Streptococcus*, *Pseudobutyrivibrio*, *Staphylococcus*, *Stenotrophomonas*, *Gemmatimonas*, *Brevibacterium*, *Symbiobacterium*, *Faecalibacterium*, *Sphingomonas*, *Streptomyces*, *Corynebacterium*, *Microbacterium*, *Micrococcus*, *Prevotella*, *Lysinibacillus* and *Gemmatimonas*. “Other Saccharomycetales” include *Ambrosiozyma*, *Pichia*, *Wickerhamomyces*, *Saturnispora* and *Saccharomycopsis*.

Table 1 Alpha diversity index and Good´s coverage calculated for 300 bacterial (A) or 300 eukaryotic (B) sequences per sample and averaged for replicated samples during wine fermentation of Grenache grapes.

A)

	Chao1	Observed OTUs	Good´s coverage
d0[a]	50.3	20	0.58
d1	65.9	52	0.95
d2	105.3	58	0.92
d3	50.1	37	0.95
d10	83	53	0.96

B)

	Chao1	Observed OTUs	Good´s coverage
d0	17	12	0.81
d1	33	22	0.84
d2	24.6	19	0.87
d3	52.3	26	0.95
d10	60.1	41	0.94

[a] d0, d1, d2, d3 and d10 mean day 0, day 2, day 3 and day 10 respectively

Table 2: Relative abundance of *Acetobacteraceae* genera across days 0 to 10 of spontaneous wine fermentation of Grenache grapes.

	d0[a]	**d1**	**d2**	**d3**	**d10**
Gluconobacter	0.45	0.80	0.81	0.68	0.41
Gluconacetobacter	0.15	0.05	0.05	0.08	0.01
Acetobacter	0.06	0.04	0.05	0.02	0.03
Asaia	0.00	0.01	0.01	0.00	0.00
Other *Acetobacteraceae*	0.00	0.01	0.00	0.00	0.00

[a] d0, d1, d2, d3 and d10 mean day 0, day 2, day 3 and day 10 respectively

Figure 1

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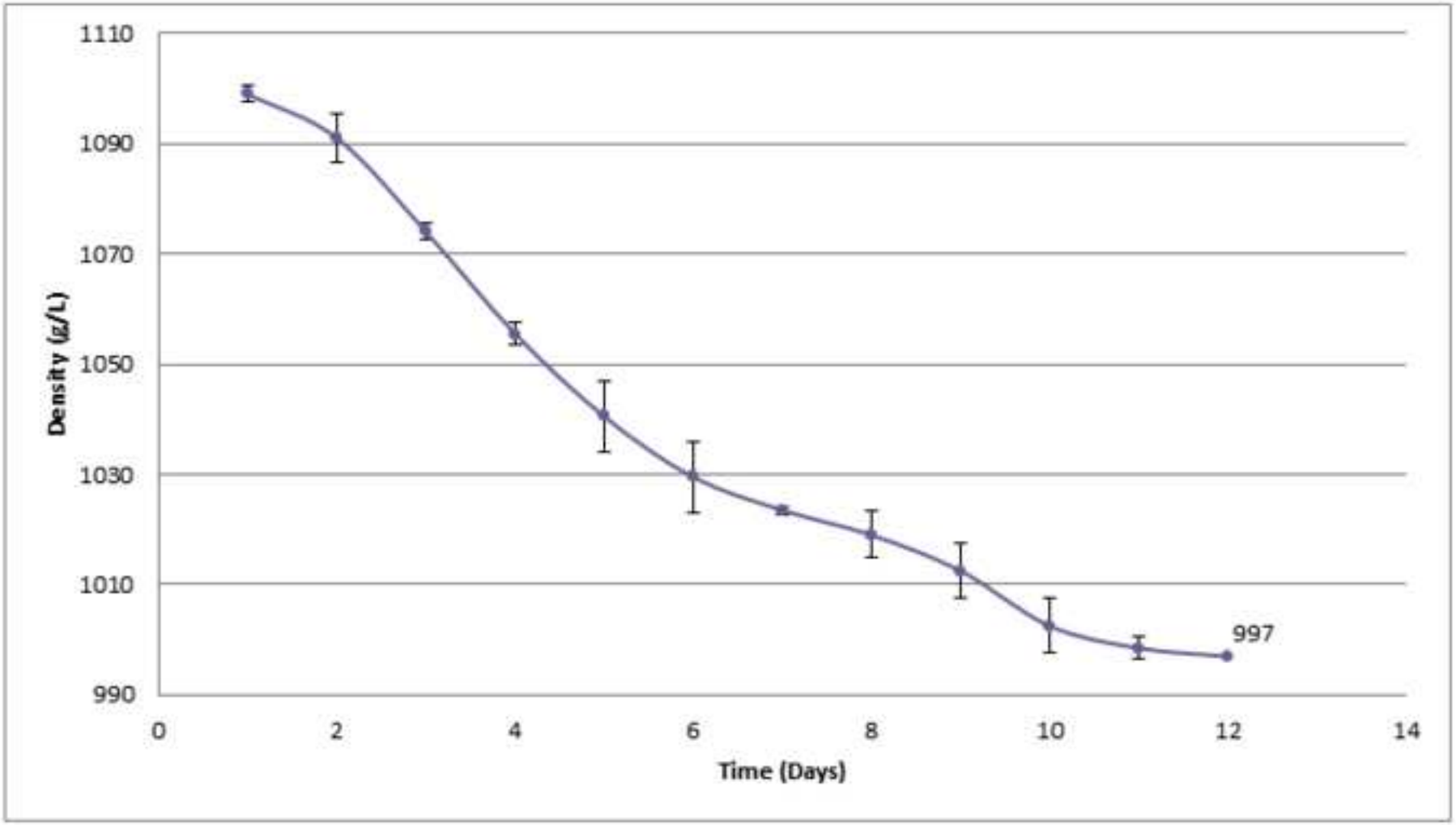

Figure 2

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