OSOYOOS, CANADA
APRIL 28, 2016

# BIODIVERSITY MEETS TERROIR

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LALLEMAND

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PROCEEDINGS
OF

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# FOREWORD

Understanding the biodiversity of wine microorganisms during fermentation is essential for controlling the production of quality wine. At the ***XVIth Entretiens Scientifiques Lallemand*** in Osoyoos, British Colombia, Canada, a group of experts on wine ecology presented the latest research on this topic.

Dr. Dan Durall and Sydney Morgan from UBC in Kelowna presented their results from the last five years on Pinot Noir and Chardonnay fermenting yeasts and also identified the yeasts involved in spontaneous fermentations at commercial wineries in the Okanagan Valley wine region of Canada.

Dr. Thomas Henick-Kling presented the results of research conducted with his colleagues at Washington State University. Their studies of the grape and vineyard microbial populations in Washington State have revealed a wide diversity of fungi and bacteria. Fifty-three species were found among five fungal subphyla, including a new species of fungi that had not previously been reported in the vineyard biota, *Curvibasidium rogersii* (class of Microbotryomycetes).

Dr. Elizabeth Henaff presented a technology developed by Wineseq that identifies the relevant microbial communities throughout the winemaking process, from the soil to the bottle, and the data science to interpret the results.

Dr. Richard DeSchenzo from ETS Laboratories in California provided insight into the yeast population dynamics occurring during both inoculated and non-inoculated fermentations.

Finally, Dr. Vincent Gerbaux from IFV in Burgundy, France, presented the latest findings on non-*Saccharomyces* selection and how the transformation of a quality wine from quality grapes requires the biodiversity of microorganisms selected for winemaking, in the cleverly integrated management and shaping of a wine style.

The meeting was also an opportunity to present the Lallemand Prize to two deserving students: Gordon Walker from UCD California for his exceptional contribution to research; and Diego Bonnel, master of wine student, for his original and well-researched paper.

The ***Entretiens Scientifiques Lallemand*** 2016 on the composition and behavior of microorganisms during fermentation allowed us to expand our understanding of fermentation problems and to improve fermentation control to obtain final products with the desired sensory characteristics and style.

# CONTENTS

**BIODIVERSITY MEETS TERROIR**

# YEASTS IN WINERY FERMENTATIONS DURING FIVE YEARS OF SAMPLING

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## Introduction

Inoculation with a commercial ADY *S. cerevisiae* strain is the most common type of fermentation practised at commercial wineries in the Okanagan Valley wine region of Canada. However, spontaneous fermentations are often practised in parallel with inoculated fermentations. One draw of spontaneous fermentations is that they have been described as being more complex and full-bodied than wines fermented with a single inoculated strain (Fleet, 2008; Vilanova and Sieiro, 2006). This increased complexity can be attributed to many things, including the higher diversity of *S. cerevisiae* strains, the increased involvement of non-*Saccharomyces* species, and the potentially greater number of indigenous *S. cerevisiae* strains in spontaneous fermentations as compared with inoculated fermentations (Fleet, 2008; Vigentini et al., 2014). However, it is unclear whether at least some of these things are achieved, even accidentally, with inoculated fermentations. The objective of this study was to describe the commercial and indigenous yeast strains in both inoculated and spontaneous Pinot Noir and Chardonnay fermentations at multiple wineries in the Okanagan Valley.

## Methods

The number of wineries involved in each vintage is shown in Table 1. Fermentations were conducted in a variety of containers including 250 L oak barrels, 1500 kg macrobins, and 5300 L stainless steel tanks. If fermentations were inoculated, inoculation was performed by winery staff following manufacturer specifications. All fermentation treatments were conducted in triplicate vessels, and samples for microbial analysis were taken at multiple stages of fermentation. Each fermentation sample was diluted in series, plated on solid YEPD media, and incubated at 28°C for two days. For each sample, plates containing 30–300 yeast colonies were used. Yeast colonies were randomly chosen (between 8 and 40 colonies per sample, depending on the vintage) and were subsequently isolat-

**Table 1**. Implantation success of Pinot Noir fermentations inoculated with a variety of commercial ADY yeasts. Successful implantation is defined as the inoculated strain representing > 80% of the relative yeast abundance by the end of fermentation.

| Vintage | Number of Wineries Sampled | Number of Tanks with > 80% Inoculum/ Total Tanks Sampled | Percentage (%) of Tanks with Successful Implantation |
|---|---|---|---|
| 2009 | 1 | 0/3 | 0 |
| 2010 | 3 | 3/9 | 33 |
| 2011 | 1 | 2/3 | 67 |
| 2012 | 3 | 5/8 | 63 |
| 2013 | 4 | 9/12 | 75 |

ed onto YEPD media. DNA from each *S. cerevisiae* isolate was extracted in preparation for strain identification using a water DNA extraction method (Scholl et al., 2016). Strain identification was conducted as described either by Lange et al. (2014) or by Scholl et al. (2016). Multiplex PCR was performed on the following microsatellite loci to identify *S. cerevisiae* isolates to the strain level: C4, C8, C3, C11, YML091c, YPL009c, YOR267c, and YLR177w. These loci are mostly unlinked, with the exception of C3 and C8 (both located on Chromosome VII), and C4 and YOR267c (both located on Chromosome XV) (Legras et al., 2005; Richards et al., 2009). PCR, fragment analysis, and genetic fingerprinting were performed as outlined by Scholl et al. (2016). GenAlEx v.6.1 software was used to calculate the probability that two unrelated strains would have identical multilocus genotypes (Peakall and Smouse, 2012, 2006). This probability was determined to be one in 1.2e7 (probability of identity = 1.7e-9).

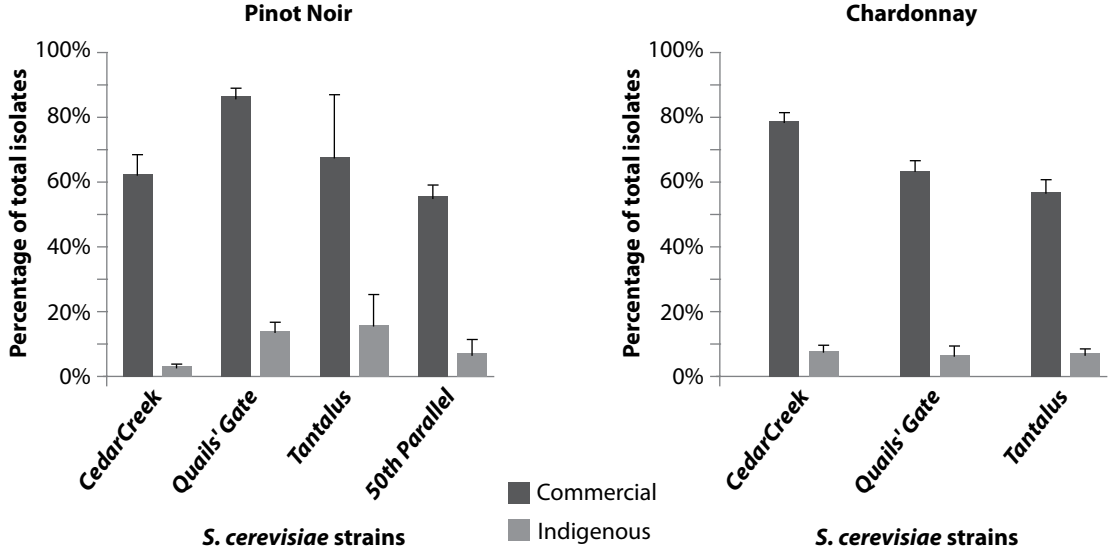


**Figure 1**. Relative percent of total *S. cerevisiae* isolates identified as either commercial or indigenous strains in spontaneous fermentations of Pinot Noir (four wineries) and Chardonnay (3 wineries). Values are means ± SE of 3 replicate fermentations. Data taken from Scholl et al. (2016).

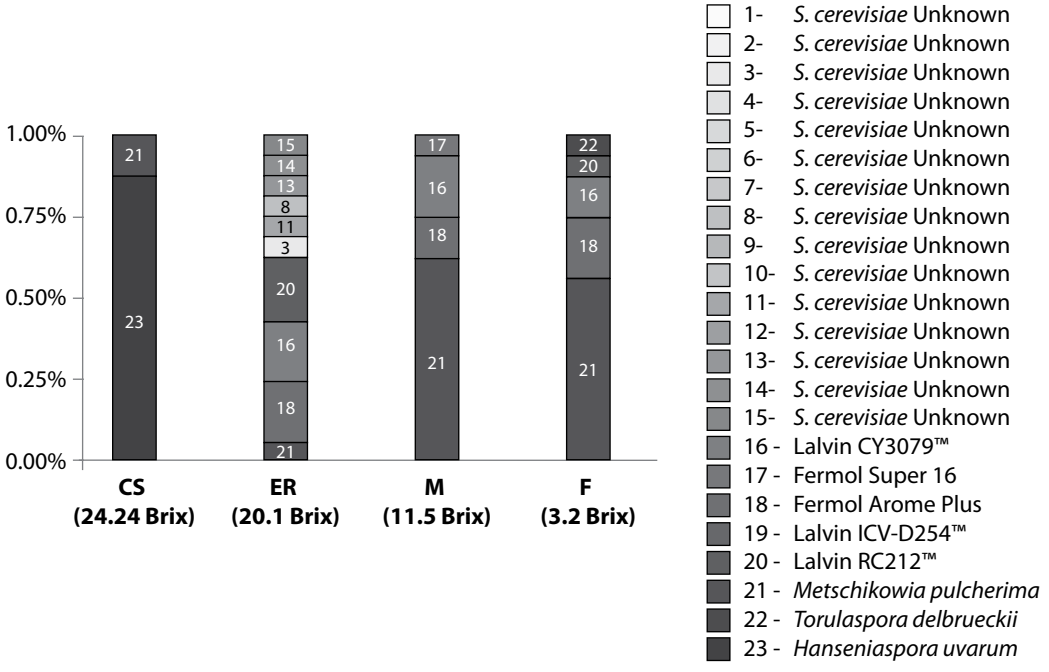


**Figure 2**. Relative abundance of *S. cerevisiae* strains isolated from Cold Soak (CS), Early (ER), Mid (M), and Final (F) stages of spontaneous Pinot Noir fermentations during the 2012 vintage. Values are means of 3 replicate fermentations.

## Results

The implantation successes of different commercial yeast strains in Pinot Noir must were evaluated over a five-year period (Table 1). Approximately 30% of the inoculated fermentations had < 80% of the inoculated strain present at the end of fermentation (Table 1), indicating that up to 30% of the fermentations studied did not have a typical successful implantation of the inoculum. The *S. cerevisiae* strains co-occurring with the inoculum were mainly commercial strains that had been previously used in the wineries as ADY inoculum. As such, the dominant strains at the end of fermentation (i.e., those comprising ≥ 10% relative abundance) were almost exclusively commercial strains. Unknown or indigenous strains were present in most fermentations, but in very low numbers (data not shown).

These results were also reflected in the spontaneous fermentations that were conducted at the same wineries. The spontaneous fermentations were dominated by commercial, rather than indigenous, *S. cerevisiae* strains. This was observed at all wineries studied as well as with fermentations of different varietals (Figure 1, taken from Scholl et al., 2016). The spontaneous fermentations had a larger indigenous *S. cerevisiae* presence than the inoculated fermentations (data not shown), but the dominant strains were still commercial ADY strains used previously at their respective wineries: an example is found in Figure 2. The dominant *S. cerevisiae* strains in the Figure 2 fermentations were the commercial strains Lalvin RC212™, Lalvin ICV D254™, Fermol Arôme Plus, and Lalvin CY3079™, all of which had been used previously at the winery where the fermentations were conducted. The trend of having commercial strains dominating at the end stage of spontaneous fermentations was observed for all years sampled, but data from the 2013 vintage for both Pinot Noir (4 wineries) and Chardonnay (3 wineries) is shown as representative of this result (Figure 1).

## Industrial implications

Our finding that approximately 30% of the inoculated fermentations studied had < 80% implantation of the inoculum persisting at the end of fermentation supports the idea that under operational practices, > 80% implantation is not always achieved (Clavijo et al., 2011). Worldwide, *S. cerevisiae* strain typing of inoculated fermentations is relatively rare, because it is often assumed that the inoculum fully implants and persists to the end of the fermentation. Thus, it is not known whether this result typically occurs in all wine-producing regions. Nevertheless, in all cases where the inoculum was < 80%, the other *S. cerevisiae* strains that co-occurred with the inoculum in the fermentation were usually commercial strains that had been used previously or concurrently as inoculum at the winery. All wineries in this study had a history of using multiple commercial strains for inoculation. More research is needed to determine whether a winery that uses very few strains would have a higher rate of successful implantations and/or have fewer other strains co-occurring with the inoculant.

The finding that spontaneous fermentations were composed of predominantly commercial strains used concurrently or previously at the winery indicates that the commercial strains are likely aggressive towards indigenous *S. cerevisiae,* and potentially against spoilage yeasts, since no spoilage organisms were detected in any of the fermentations sampled over the five vintages. In a recent winery-based study conducted in the Okanagan Valley, wines produced with a diversity of yeast strains were found by an expert panel to have more complex and full-bodied sensory attributes as compared with wines that were fermented by a single *S. cerevisiae* strain (Tantikachornkiat, unpublished). The typical practice would be to use the spontaneous fermentations as a blending option with wines produced from inoculated fermentations. Spontaneous fermentations could also be useful as a bioassay tool to determine the yeast residents of the winery and, in turn, used in a way to manage those residents (Hall et al., 2011). Our results suggest that using a variety of commercial *S. cerevisiae* strains may be a way for winemakers to increase the diversity of strains involved in their fermentations, while still mitigating the risks of stuck and spoiled fermentations that can accompany spontaneous fermentations.

## References

Clavijo, A., I.L. Calderon, and P. Paneque. 2011. Effect of the use of commercial Saccharomyces strains in a newly established winery in Ronda (Malaga, Spain). *Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol.* 99: 727–731. doi:10.1007/s10482-010-9514-5.

Fleet, G.H., 2008. Wine yeasts for the future. *FEMS Yeast Res*. 8:979–995. doi:10.1111/j.1567-1364.2008.00427.x.

Hall, B., D.M. Durall, and G. Stanley. 2011. Population dynamics of Saccharomyces cerevisiae during spontaneous fermentation at a British Columbia winery. *Am. J. Enol. Vitic*. 62:66–72. doi:10.5344/ajev.2010.10054.

Lange, J.N., E. Faasse, M. Tantikachornkiat, F.S. Gustafsson, L.C. Halvorsen, A. Kluftinger, D. Ledderhof, and D.M. Dural. 2014. Implantation and persistence of yeast inoculum in Pinot noir fermentations at three Canadian wineries. *Int. J. Food Microbiol*. 180:56–61. doi:10.1016/j.ijfoodmicro.2014.04.003.

Legras, J.L., O. Ruh, D. Merdinoglu, and F. Karst. 2005. Selection of hypervariable microsatellite loci for the characterization of Saccharomyces cerevisiae strains. *Int. J. Food Microbiol*. 102:73–83. doi:10.1016/j.ijfoodmicro.2004.12.007.

Peakall, R. and P.E. Smouse. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Mol. Ecol. Notes.* 6:288–295. doi:10.1111/j.1471-8286.2005.01155.x.

Peakall, R. and P.E. Smouse. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research – an update. *Bioinformatics* 28:2537– 2539. doi:10.1093/bioinformatics/bts460.

Richards, K.D., M.R. Goddard, and R.C. Gardner. 2009. A database of microsatellite genotypes for Saccharomyces cerevisiae. *Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol.* 96:355–359. doi:10.1007/s10482-009-9346-3.

Scholl, C.M., S.C. Morgan, M.L. Stone, M. Tantikachornkiat, M. Neuner, and D.M. Durall. 2016. Composition of Saccharomyces cerevisiae strains in spontaneous fermentations of Pinot Noir and Chardonnay. *Aust. J. Grape Wine Res*. 22:384–390. doi:10.1111/ajgw.12221.

Vigentini, I., V. Fabrizio, M. Faccincani, C. Picozzi, A. Comasio, and R. Foschino. 2014. Dynamics of Saccharomyces cerevisiae populations in controlled and spontaneous fermentations for Franciacorta D.O.C.G. base wine production. *Ann. Microbiol*. 64:639–651. doi:10.1007/ s13213-013-0697-7.

Vilanova, M. and C. Sieiro. 2006. Contribution by Saccharomyces cerevisiae yeast to fermentative flavour compounds in wines from cv. Albarino. *J. Ind. Microbiol. Biotechnol*. 33:929–933. doi:10.1007/s10295-006-0162-8.

# A LOOK INTO THE MICROBIAL POPULATIONS OF VINEYARDS IN THE STATE OF WASHINGTON AND THEIR PERSISTENCE DURING WINE FERMENTATION

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Recent studies of microbial populations of grapes and vineyards in the State of Washington (WA), USA, revealed a wide diversity of fungi and bacteria, including a new fungus species, *Curvibasidium rogersii,* which belongs to the class of *Microbotryomycetes* (Bourret at al., 2012). In a recent study we employed next-generation sequencing (NGS), a culture-independent method, to monitor the temporal succession of the prokaryotic population during the conventional and non-conventional native yeast fermentation process of grapes farmed in WA (Piao et al., 2014). The sequencing data, based on the V1-V3 region of the 16S rRNA gene, indicated distinct prokaryotic profiles during the two fermentation techniques. These studies aim to expand our understanding of how native yeast and bacteria interact in wine fermentation, how these populations influence regional and grape varietal flavours, and to what extent native microorganisms persist in wine fermentation and aging.

## Introduction

Wine fermentation is a succession of populations of various yeast and bacteria, starting with the population brought into the winery on the grapes and combined with the populations in the winery. Depending on the winemaking conditions used during the fermentation process, various yeast and bacteria, including aerobic and non-fermentative microorganisms, can grow to significant numbers before onset of alcoholic fermentation. Good fermentation management aims to minimize the impact of aerobic, potential spoilage yeast and bacteria. Alcoholic fermentation should be dominated by fermentative yeast, mostly *Saccharomyces* sp. that were either selected from the native population or added by the winemaker with a starter culture (Henick-Kling et al., 1998). Traditionally, malolactic fermentation (MLF) follows alcoholic fermentation, primarily depending on pH, alcohol content, and the temperature of the wine. MLF might start within one to two weeks of completion of alcoholic fermentation or several months later, when cellars in traditional winemaking areas warm in spring. MLF is carried out by bacteria populations consisting of various lactic acid bacteria, such as *Lactobacillus* sp., *Pediococcus* sp., and *Oenococcus oeni*. To avoid off-flavours, it is best when alcoholic fermentation is followed by MLF during which *Oenococcus oeni* predominate. With the addition of starter cultures, MLF can now also be conducted with a high degree of success as co-fermentation during alcoholic fermentation. Finally, the microbial population present during wine aging in barrels and tanks has a significant impact on wine flavour. Ideally, only fermentative yeasts like *S. cerevisiae* and remaining *O. oeni* bacteria will impact the wine flavour during this phase of the winemaking process. All other microorganisms should be suppressed at this stage of wine development (aging).

## Microbial Ecology of Grapes

Sanitation is a crucial tool in winemaking for creating wines without detracting off-flavours. This starts with the sanitary status of the grapes. Fruit damaged by mould and other microbes, birds, or insects can harbour large amounts of spoilage microorganisms and, in extreme cases, noticeable spoilage aromas. It is more difficult to de-

**Table 1.** Yeast population on grapes (% of total population). Based on various studies using culture-dependent methods

| On grapes: | |
|---|---|
| *Saccharomyces cerevisiae* (0.3–3.0 %) | |
| *Hanseniaspora uvarum* (50.9–89.1) | |
| *Metschnikowia pulcherima* (0.5–2.7) | |
| *Rhodotorula* (0–26.1) | *Brettanomyces bruxellensis* (0–0.4) |
| *Candida glabrata* (4.0–7.2) | *Hyphopichia butonii* (0–0.3) |
| *Zygosaccharomyces* (1.0–3.9) | *Kluyveromyces* (0.2–0.2) |
| *Candida zeylanoides* (1.0–2.3) | *Williopsis sat.* (0–0.2) |
| *Debaryomyces* (0.6–2.1) | *Kryptocokkus* (0–0.2) |
| *Pichia kluveri* (0.4–1.4) | Other *Saccharomyces* (0.1–0.1) |
| *Candida* (0.5–0.9) | Unidentified yeasts (0.1–0.2) |
| *Lipomyces* (0–0.5) | |

| In grape must: | |
|---|---|
| *Kloeckera apiculata (Hanseniaspora)* 50–90% | |
| *Rhodotorula* 0–26% | |
| *Candida stellata, C. pulcherrima, C. glabrata, C. zeylanoides* 5–10% | |
| *Metschnikowia* 0.5–3% | |
| *Pichia kluveri (membranefaciens)* 0.4–1.4% | |
| *Kluyveromyces* 0.2% | |
| *Hyphopichia butonii, Lipomycys* 0–0.3% | |
| *Cryptococcus, Williopsis sat.,* 0–0.2% | |
| Other non-identified yeasts 0.1–0.2% | *Saccharomyces cerevisiae (*0.3–3%) *Brettanomyces (*0–0.4%) |

tect fruit with barely visible signs of infection, which can also harbour large amounts of spoilage microorganisms. A study by Gadoury et al., (2007) described this condition, known as diffuse powdery mildew infection. Successful guidance of native microorganisms in wine fermentation starts with careful monitoring of the microbial populations on the fruit. Verification of the sanitary status (microbial load) of the fruit begins in the vineyard and should include some analysis of the microbial load. To do this successfully, we need to develop new tools for analyzing microbial populations in the vineyard and on the fruit entering the winery. The "perfect" fruit for a wine not only has the "right" chemistry for desired flavours and stability, but also the "right" microbial population to help express the desired flavours. Today, we are only just beginning to understand the flavour impact of the microbial populations of the fruit.

Table 1 gives a general overview of yeast on grapes and in grape must. This complex microbial population becomes even more complex when we use non-culture dependent methods for detection and quantification of these populations. We know little about how all these yeasts and bacteria interact during the various stages of fermentation, or how their sensory impact affects the final wine flavour.

It is important to remember that non-*Saccharomyces* yeasts are always present in inoculated and in non-inoculated fermentations and may play important roles as spoilage organisms or by making positive contributions to finished wine. Figure 1 shows the impact of $SO_2$ addition on the growth of *Saccharomyces* sp. and non-*Saccharomyces* yeast in a Chardonnay must. Data from this study and others show that non-*Saccharomyces* yeasts persist throughout alcoholic fermentation and can represent a large part of the population at early and mid stages of fermentation. In this study, only the addition of 50 mg/L of $SO_2$ significantly suppressed the population of non-*Saccharomyces*.

In reality, the non-*Saccharomyces* yeast population is much more complex. A study presented by Henick-Kling et al. (1998) shows the dynamics of various yeasts during wine fermentation with 0, 20, and 50 mg/L $SO_2$ added at beginning, middle, and late stages of fermentation. It clearly demonstrates how the yeast population shifts with different additions of $SO_2$ and through different stages of fermentation.

We also should not forget that in all fermentation, with or without added starter cultures, several strains of *Saccharomyces cerevisiae* may be present depending on what other yeasts are present and on the stage of fermentation (Figure 2).

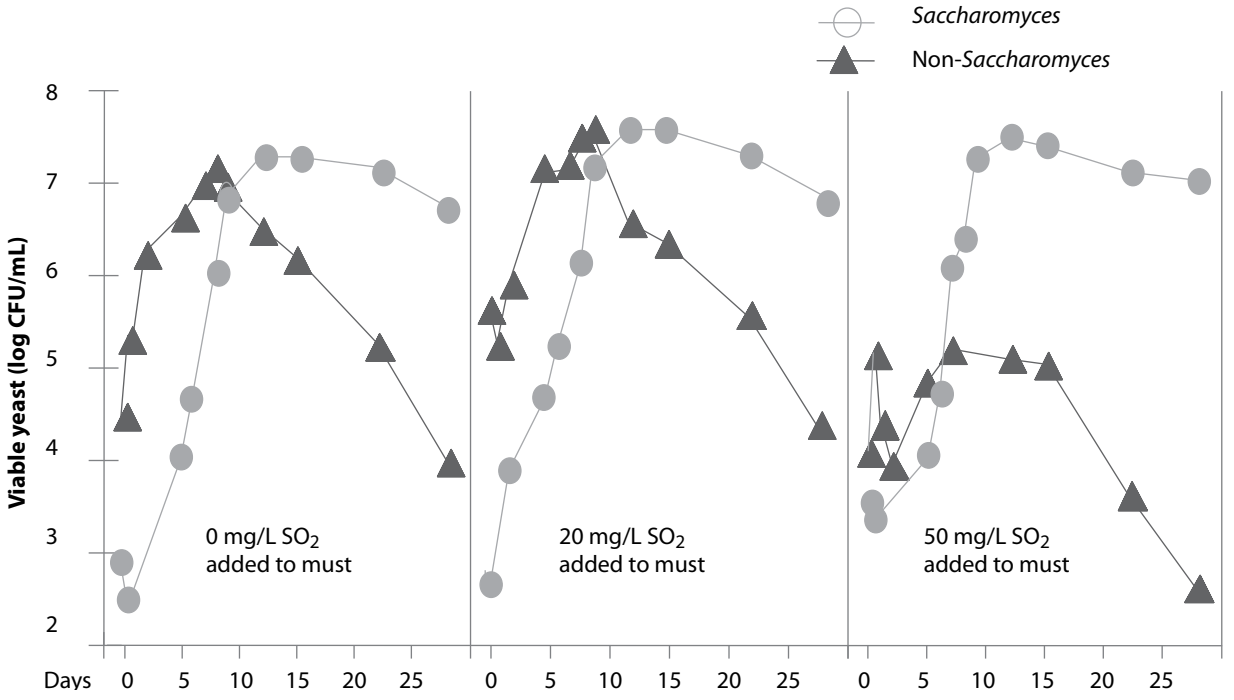


**Figure 1.** Effect of $SO_2$ additions on growth of indigenous yeast

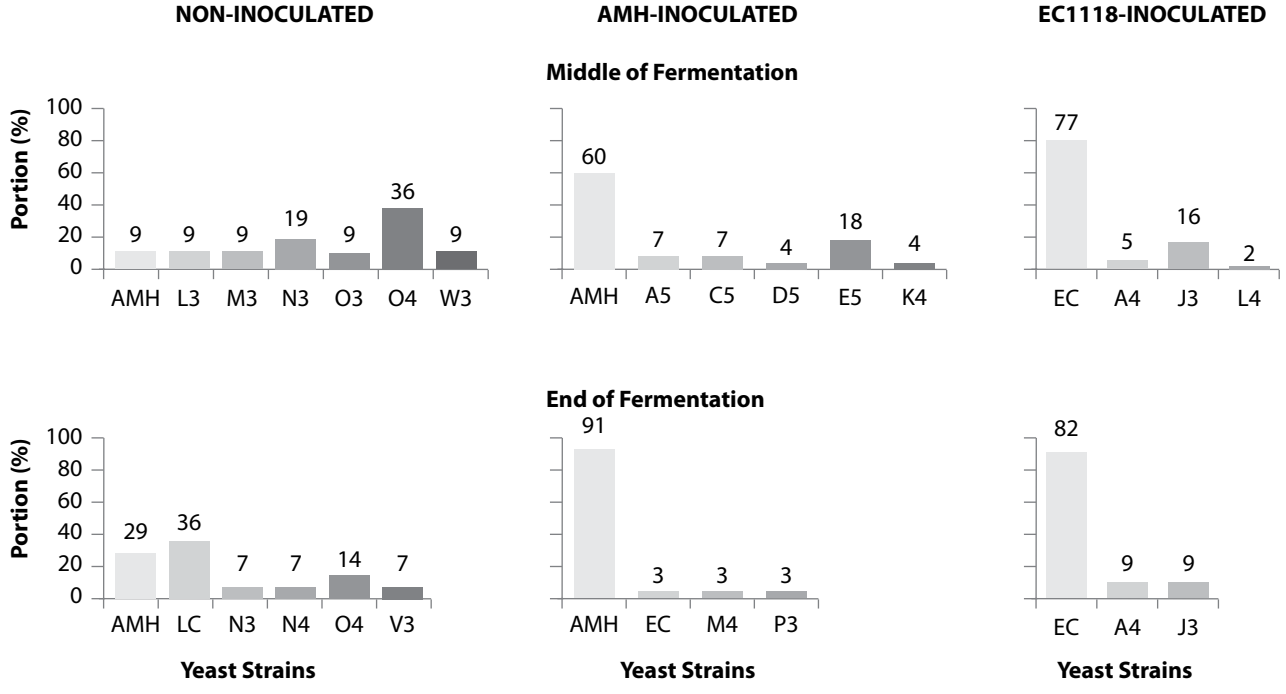


**Figure 2.** Diversity and succession of *Saccharomyces* yeast in wine fermentation

## MICROBIAL POPULATIONS IN VINEYARDS IN WASHINGTON STATE

In a recent study authors found a wide diversity of fungi in vineyards located in the State of Washington (Bourret et al., 2013; Bourret et al., 2012). *Aureobasidium pullulans* represented three phylogenetically distinct subspecific lineages. Seventeen of the 53 fungal species identified in this study were previously unreported on wine grapes, and eighteen were unreported in North America.

Several strains appear to represent non-described species, including the recently described *Curvibasidium rogersii*

The wide diversity of fungi with 53 species was distributed among five subphyla:

*Saccharomycotina,* 13 species in the genera *Candida, Hanseniaspora, Metschnikowia, Meyerozyma, Pichia, Wickerhamomyces and Yamadazyma*

*Metschnikowia pulcherrima* displaying considerable diversity.

*Pucciniomycotina* 12 species, in *Curvibasidium, Rhodosporidium, Rhodotorula, Sporidiobolus* and *Sporobolomyces*. Five phylogenetically distinct species in the subphylum could not be assigned to any described species.

*Ustilaginomycotina* were placed in *Pseudozyma* except for a single strain determined to be *Rhodotorula bacarum*.

*Agaricomycotina,* 17 species in the genera *Cryptococcus, Cystofilobasidium, Hannaella, Holtermanniella* and *Mrakiella*.

Seven species of yeast-like *Pezizomycotina* were found, representing classes *Leotiomycetes, Dothideomycetes* and *Sordariomycetes*.

(Bourret at al., 2013; Bourret et al., 2012).

The complexity of these interactions continues when we look at the bacteria populations of grapes and wine fermentations. A simple list of bacteria involved in grape fermentation based on culture-dependent techniques is given in Table 2. More recent investigations using culture-independent methods for detection and quantification show much more complex populations (Piao et al., 2015).

**Table 2.** Bacteria on grapes and in wine

| | |
|---|---|
| **Acetic acid bacteria:** | |
| | *Acetobacter, Gluconobacter* |
| Lactic acid bacteria: | |
| *Lactobacillus plantarum* | |
| *Lactobacillus brevis* | |
| *Pediococcus* sp. | |
| *Oenococcus oeni* | |

The studies by Bourret et al. (2013) and Bokulich et al. (2012) used direct sequencing of the V1-V3 region of the 16S rRNA gene to monitor the bacterial community and its temporal succession during the fermentation of wine grapes. The Riesling grapes in the study by Piao et al. (2015) were organically grown grapes fermented in two different ways, organically and conventionally. The conventional fermented grapes received a 38 mg/L $SO_2$ addition to must and a 56 mg/L $SO_2$ addition to the Pied de Cuve (native starter culture). In addition, the Pied de Cuve received DAP as well as a complex nutrient mix and bentonite.

The organically fermented must did not receive any $SO_2$ or bentonite additions, and only received autolyzed yeast for nutrients.

The temperature profile and fermentation rate in both fermentations were the same, while pH was slightly lower in the organically fermented must (approx. pH 3.0 vs. 3.2). The wines underwent no MLF.

Principal component analysis of 16S rRNA data from microbiomes associated with grape must during the fermentation process showed a strong differentiation of the bacterial populations in the conventionally and the organically fermented musts starting at day 2 of fermentation all the way to day 16 of fermentation.

Phylogenetic analysis of the two wines showed a more diverse microbial community developing in the conventional wine with more different bacteria and with greater presence of individual bacteria. In both wines the diversity increased from day 0 to the end of alcoholic fermentation at 16 and 12 days for the organic wine and the conventional wine, respectively (Piao et al., 2015). Fifteen phyla (contributing ≥ 1 of the reads) were present during the fermentation process of the two grape musts. Nine of the 15 phyla observed were found in musts from both fermentation techniques (i.e., *Proteobacteria*, *Cyanobacteria, Bacteroidetes, Firmicutes, Actinobacteria, Acidobacteria, Spirochaetes, Verrucomicrobia*, and *Fusobacteria*). Some phyla were unique to one or the other of the wines. *Nitrospirae, Planctomycetes*, and *Tenericutes* were detected solely in the samples from organically fermented must while *Fibrobacteres* and members of the candidate phylum WYO were detected only in the conventionally produced wine must. Proteobacteria were the dominant group in both fermentations, initially constituting about 90% to 98% of the total bacteria population and declining to about 75% and 60%, respectively, in the organic wine and the conventional wine. This population shift was mainly due to a decrease in the population of *Gammaproteobacteria* and strong increases in the population of *Alphaproteobacteria* and *Deltaproteobacteria*. In the organically fermented wine, *Alphaproteobacteria* even became the dominant class, representing 57% of the total population at day 15. A similar reduction of the population of *Proteobacteria* from the must stage through alcoholic fermentation was also observed by Bokulich et al. (2012). While the population of *Proteobacteria* decreased, the population of *Bacteroidetes, Fermicutes*, and *Actinobacteria* increased, especially in the conventional fermentation. The conventionally fermented wine showed a larger diversity of genera across all samples, with 42 of 96 genera only found in the conventional wine and 33 of 96 genera

only found in the organically produced wine. Overall, there was also greater genus diversity in the conventional wine (76 genera) than in the organic wine (54 genera). *Gluconobacter* sp. were detected in both wines. However there was a pronounced difference in the abundance of these bacteria between the two. In the organically fermented wine, it represented 8.67% of the population at day 0 versus 0.47% in the conventionally fermented wine. These populations increased in both and represented 49% of the population at the end of alcoholic fermentation in the organic as compared to only 5–7% of the population in the conventional fermentation. These bacteria can have a significant impact on the sensory quality of wines, with various acetic acid esters impacting the final wine aroma. This study also demonstrates the risk of running wine fermentations without or with only low additions of $SO_2$ as well as no starter cultures, additions which can allow *Gluconobacter* populations to increase significantly, potentially harming the wine flavour. Also, *Gluconobacteria* (and *Acetobacter*) populations in wine fermentations might be underestimated by culture-based microbial detection systems! These bacteria are notoriously difficult to isolate and cultivate from grape and wine samples.

## CONCLUSION

Next-generation sequencing is a culture-independent method that offers great insight into the microbial populations of vineyards and wine fermentations. It offers a much richer picture of microbial populations than that obtained by plating or microscopy. Unfortunately, very few such studies on grapes and wines have been completed so far and we largely lack the metabolic and transcriptomic data accompanying these population dynamics to be able to assess the sensory impacts of these population shifts. The first look offered by this study and others is exciting and should stimulate more work to better understand microbial populations and their sensory impact on wine flavour profiles. With these new tools of microbial analysis and better understanding of their sensory impact, winemakers will be better able to guide native and added populations from yeast and bacteria starter cultures for desired flavour outcomes.

## References

Bokulich, N.A., C.M. Joseph, G. Allen, A.K. Benson, and D.A. Mills. 2012. Next-generation sequencing reveals significant bacterial diversity of botrytized wine. *PLoS ONE*. 7:e36357. doi:10.1371/journal.pone.0036357.

Bourret, T.B., G.G. Grove, G.J. Vandemark, T. Henick-Kling, and D.A. Glawe. 2013. Diversity and molecular determination of wild yeasts in a central Washington State vineyard. *N. Am. Fungi*. 8(15):1–32.

Bourret, T.B., C.G. Edwards, T. Henick-Kling, and D.A. Glawe. 2012. *Curvibasidium rogersii*, a new yeast species in the Microbotryomycetes. *N. Am. Fungi*. 7(12):1–8.

Gadoury, D.M., R.C. Seem, W.F. Wilcox, T. Henick-Kling, L. Conterno, and A. Ficke. 2007. Effects of Diffuse Colonization of Grape Berries by Uncinula necator on Bunch Rots, Berry Microflora, and Juice and Wine Quality. *Phytopathology*. 97:1356–1365.

Henick-Kling, T., W. Edinger, P. Daniel, and P. Monk. 1998. Selective effects of sulfur dioxide and yeast starter culture addition on indigenous yeast populations and sensory characteristics of the wine. *J. Appl. Microbiol*. 84:865–876.

Piao, H., E. Hawley, S. Kopf, R. Descenzo, S. Sealock, T. Henick-Kling, and M. Hess. 2015. Insights into the Bacterial Community and its Temporal Succession during the Fermentation of Wine Grapes. *Front. Microbiol*. 6:809.

# PRECISION OENOLOGY: COMPREHENSIVE WINE TERROIR ANALYSIS WITH WINESEQ

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## INTRODUCTION

In winemaking, terroir is the set of environmental factors, including climate, geography and soil, which contribute to the identity of a wine from a given region. The microbial component of the environment is an essential factor; indeed, yeast and bacterial communities associated with ripe fruit are regionally differentiated (Bokulich et al., 2013), and there is a correlation between regional microbial signatures and differential wine phenotypes (Knight et al., 2015).

As a result of their metabolic properties, this microbial consortium is responsible for many of the organoleptic characteristics of wine (Belda et al., 2016). Recently it has been shown that the microbiome of a vineyard determines, through spontaneous fermentation, much of the chemical composition and many of the sensory properties of the wines produced there (Bokulich et al., 2016). The soil has been identified as a key source of the vine-associated microbiome preharvest (Zarraonaindia et al., 2015). The microbiome of the soil thus holds the potential to define a wine terroir.

Throughout the winemaking process, winemakers are faced with numerous decisions about everything from the growing conditions of their vines (choice of land, pruning, irrigation) and the time of harvest to post-harvest processing (cultured or ambient yeast, maceration time, fermentation temperature, micro-oxygenation, barrel oak, etc). All of these decisions alter the contribution of the initial microbial communities to the final product, and thus alter the expression of the terroir. Currently the choice is between two extremes: either producing wine that is terroir-driven, unique to and dependent on the naturally occurring microbial communities and often less predictable, or on the other hand more predictable and controlled winemaking with added cultured yeast resulting in wine that often loses the emblematic signature of its terroir. In this context, the comprehensive understanding and control of microbial terroir in the vineyard through agricultural practices allows the winemaker greater control over the influence of a particular terroir expressing itself in a wine.

Here we present WineSeq®, a methodology to identify the relevant microbial communities throughout the winemaking process from soil to bottle, and the data science used to interpret the results. We empower winemakers with the knowledge of the microbial dimension of their vineyard's terroir so they can maximize its potential, shape and craft their individual wine's properties, and anticipate problems earlier in order to have time to intervene.

### WineSeq Technology and applications

The main aim of the WineSeq® project is to characterize the microbiome of different wine regions around the world by studying the microbial composition of vineyard soils. These soil-associated microbial consortia have been described as the origin of subsequent spontaneous fermentative microorganisms, which is why we also are interested in the study of the role they play in shaping the expression of the terroir of the wines from a particular region.

Applying next-generation sequencing (NGS) technology we have developed an intelligent platform for analyzing and interpreting metagenomic information from an oenological point of view. The WineSeq® platform not only works with raw metagenomic data, but also allows us to contextualize the microbial information of a sample in relation to the general microbiome patterns of a particular region. It makes it possible to compare a vineyard/cellar microbiome with others microbiomes both near and far, highlighting particularities and uniqueness. It allows for comprehensive and objective testing of the effects of innovative agricultural practices, and also enables early detection of potential microbial risks to vine health and quality wine production.

WineSeq® was developed through a broad metagenomics study that included the soils of 40 distinct vineyards in 14 countries and involved the deep sequencing of 1,500 unique samples. This work made possible the development of a dynamic database linking microbiome information with the characteristics of terroir (geography, soil science, weather, agronomical practices and grape variety, among others). We also developed a computer-learning algorithm for the integration and comparison of new samples in a global context, highlighting aspects such as commonalities and peculiarities that could become advantages or disadvantages for winemaking. The result can be summarized as the WineSeq Index.

The WineSeq Index is a representation of all the metagenomic information of "relative abundance" on the different microbial species, weighted using the information compiled in the database. The WineSeq Index measures the global frequency/rarity with which certain species appear in similar samples in relation to their oenological importance, providing an objective value for each microbial species identified in a given sample. The Index shows the potential risk or benefit of the different species of oenological interest found in the sample, turning classical metagenomic information into interesting and accessible data for vine growers and winemakers.

Figure 1 shows an example of the results obtained in our metagenomic analysis of a vineyard microbiome, comparing raw data of relative abundance on various species (Figure 1A) with the results obtained by analyzing these data with the WineSeq® platform, and factoring in their relevance and importance for vine health (Figure 1B) and wine production (Figure 1C), which is represented by their WineSeq Index score. This allows for visualization of vine health–related species (Figure 1B), different fungus (*Erysiphe necator*, *Cadophora luteo-olivacea* etc.) and bacteria species (nitrogen fixing bacteria: *Pseudomonas* sp.) with a relevant role at this stage, and highlights (Figure 1C) microorganisms such as fermenting yeasts or lactic acid bacteria as relevant species in wine production.

Additionally, the WineSeq project has developed a powerful portal for data visualization. This portal allows for the comparison of different vineyards along multiple axes, including health status and risks and microbiological potential (Figure 2). Figure 2a shows the distribution of four distinct but geographically close vineyards with different soil types and viticulture characteristics. The diagrams in Figure 2b show an estimation of the health status of these four vineyards. In light of these results, we can reasonably assume that vineyard "1D" has better microbiological potential than the other three, whose samples show a higher proportion of detrimental microorganisms, as is also highlighted in the sample "1C".

Finally we also sought to study the relationship between the microbial consortia of vineyard soils and their impact throughout the entire winemaking process. For that purpose we examined 50 different complete processes (from vineyard to bottle). By systematically studying the evolution of the microbial composition of these samples during the winemaking process, we were able to model the dynamic behaviour of different microbial species during wine production, making it possible to anticipate their potential influence on everything from soil and grape samples to the later fermentation and barrel-aging stages. In time we anticipate that the microbial fingerprint of the soil will be used to predict certain organoleptic characteristics of wine resulting from that soil.

Figure 3 shows a real-world application of this technology being used to detect potential detrimental or enhancing species for winemaking at the prefermentative stages. With this information, winemakers can decide which oenological practices to employ, based on the potential risks and benefits of the naturally occurring microbial fingerprint. It is now possible to decide a priori based on objective sample information whether to inoculate a production or to develop spontaneous fermentations based

on the microbial fingerprint of the sample. This application of WineSeq reduces the risks associated with spontaneous or natural fermentations by providing information on the potential of the sample.

WineSeq provides broad knowledge about the microbial aspects of terroir and allows this information to be used to improve all winemaking processes from soil to bottle by helping us understand the microbial fingerprint and its influence on both vine health and fermentations.

## References


Belda, I., J. Ruiz, A. Alastruey-Izquierdo, E. Navascues, D. Marquina, and A. Santos. 2016. Unraveling the Enzymatic Basis of Wine "Flavorome": A Phylo-Functional Study of Wine Related Yeast Species. *Front Microbiol.* 7:12.

Benavent-Gil, Y., C. Berbegal, O. Lucio, I. Pardo, and S. Ferrer, 2016. A new fear in wine: isolation of *Staphylococcus epidermidis* histamine producer. *Food Cont.* 62: 142–149.

Bokulich, N.A., T.S. Collins, C. Masarweh, G. Allen, H. Heymann, S.E. Ebeler, and D.A. Mills. 2016. Associations among Wine Grape Microbiome, Metabolome, and Fermentation Behavior Suggest Microbial Contribution to Regional Wine Characteristics. *mBio* 7(3):e00631-16.

Liu, Y., S. Rousseaux, R. Tourdot-Marechal, M. Sadoudi, R. Gougeon, P. Schmitt-Kopplin, and H. Alexandre. 2015. Wine microbiome, a dynamic world of microbial interactions. *Crit. Rev. Food Sci. Nutr.* doi:10.1080/10408398.2014.983591.

Pretorius, I.S., 2000. Tailoring wine yeast for the new millennium: novel approaches to the Ancient art of winemaking. *Yeast.* 16: 675–729.

Zarraonaindia, I., S.M. Owens, P. Weisenhorn, K. West, J. Hampton-Marcell, S.Lax, et al. 2015. The Soil Microbiome Influences Grapevine-Associated Microbiota. *mBio* 6(2):e02527-14.


A

143 organisms found

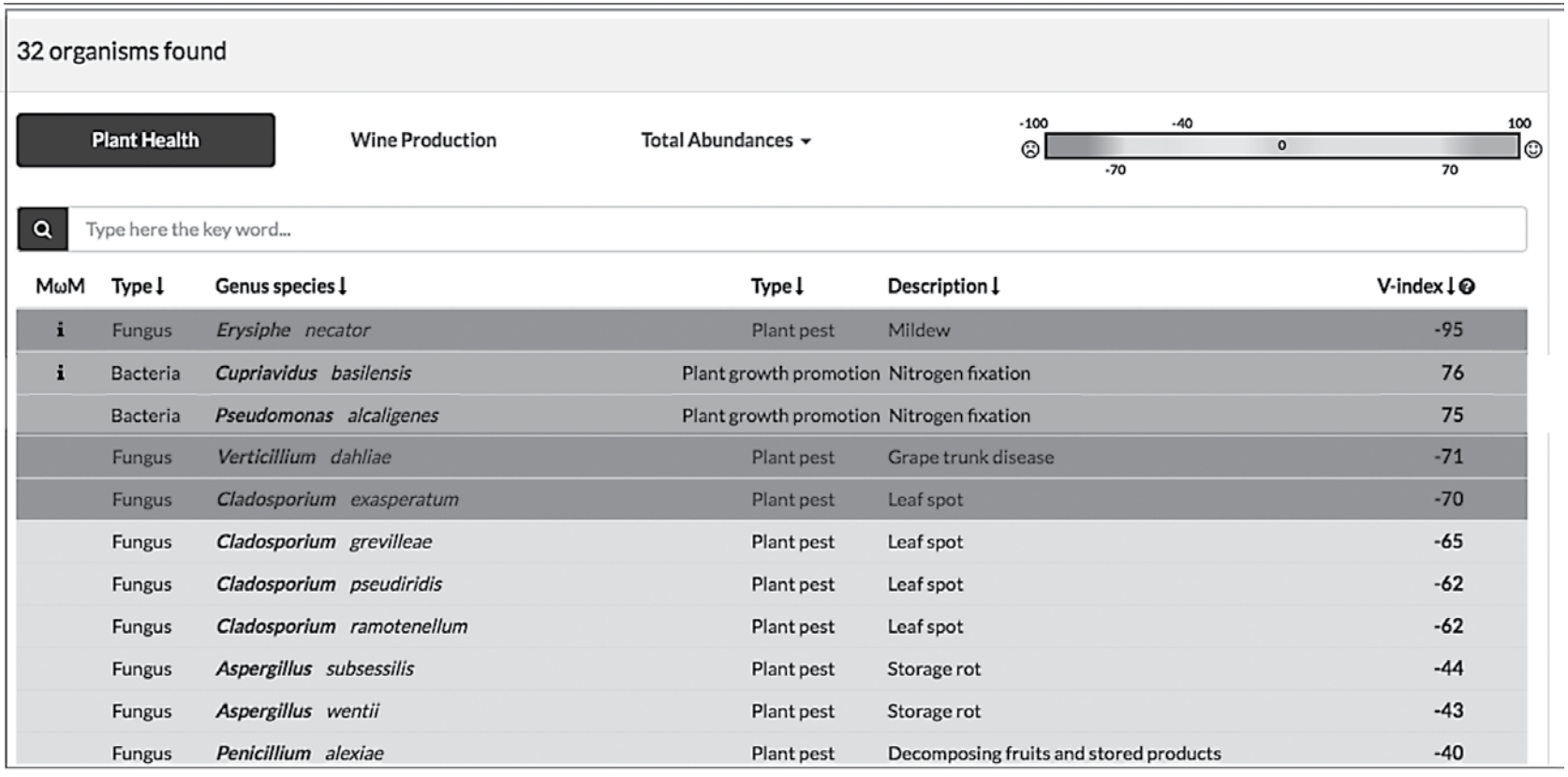
Plant Health | Wine Production | Total Abundances

Type here the key word...

| Type | Genus species | % |
|---|---|---|
| Fungus | *Pleosporales* sp. | 28.7 |
| Fungus | *Tremellales* sp. | 10.36 |
| Fungus | *Cryptococcus victoriae* | 10.21 |
| Fungus | *Cryptococcus terreus* | 5.87 |
| Fungus | *Fellomyces mexicanus* | 3.35 |
| Fungus | *Ascochyta fabae* | 2.94 |
| Fungus | *Auriculariales* sp. | 2.86 |
| Fungus | *Cryptococcus laurentii* | 2.45 |
| Fungus | *Mortierella* sp. | 2.02 |

**Figure 1.** Partial results of the microbiome analysis of a soil. A) Raw metagenomic data, sorted by relative abundance. B) Data processed with the WineSeq® algorithm for vine health. C) B) Data processed with the WineSeq® algorithm for wine production.

**B**



32 organisms found

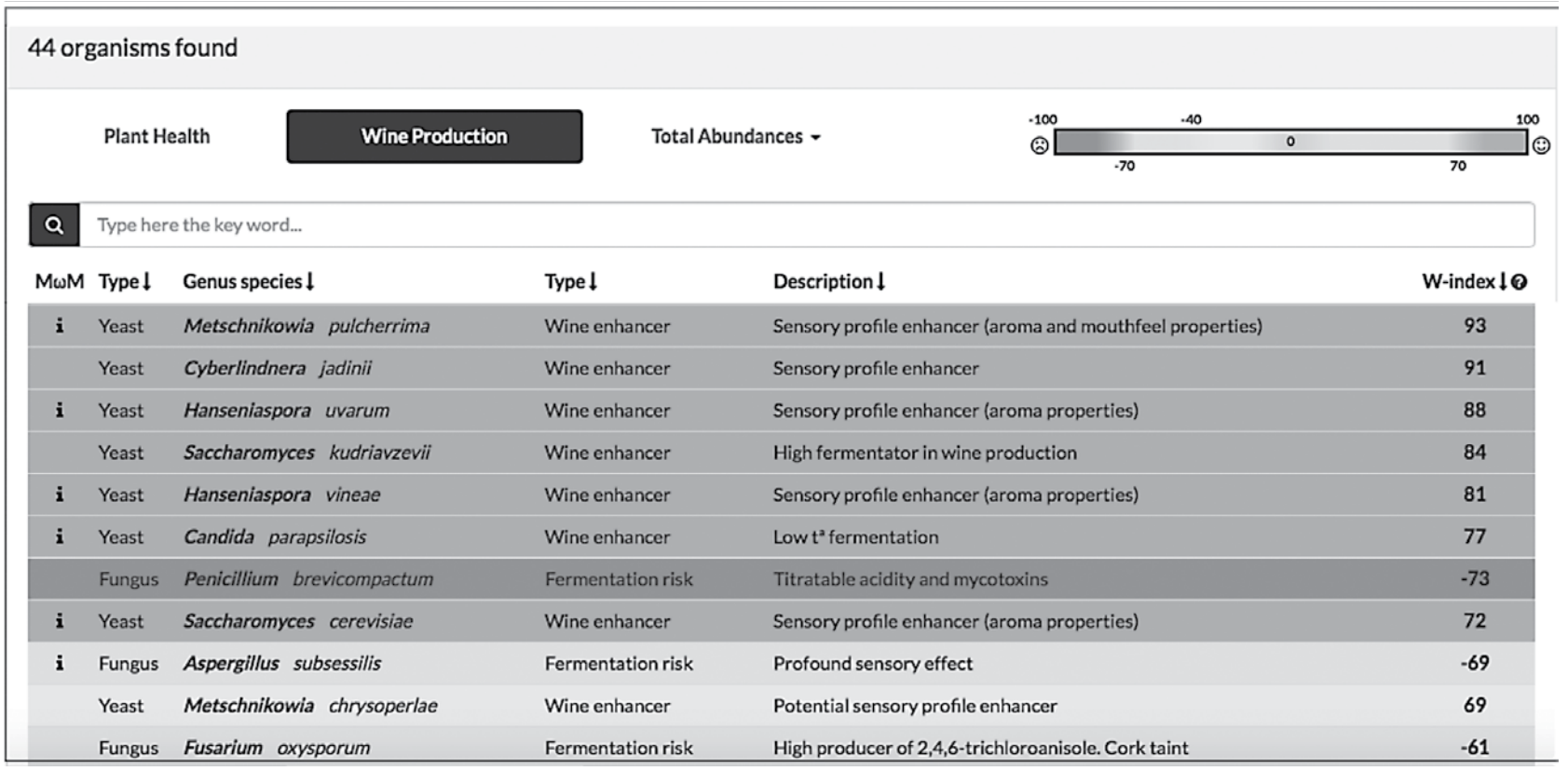
Plant Health | Wine Production | Total Abundances

-100 -40 0 100 -70 70

Type here the key word...

| MωM | Type | Genus species | Type | Description | V-index |
|---|---|---|---|---|---|
| i | Fungus | *Erysiphe necator* | Plant pest | Mildew | -95 |
| i | Bacteria | ***Cupriavidus*** *basilensis* | Plant growth promotion | Nitrogen fixation | 76 |
| | Bacteria | ***Pseudomonas*** *alcaligenes* | Plant growth promotion | Nitrogen fixation | 75 |
| | Fungus | ***Verticillium*** *dahliae* | Plant pest | Grape trunk disease | -71 |
| | Fungus | ***Cladosporium*** *exasperatum* | Plant pest | Leaf spot | -70 |
| | Fungus | ***Cladosporium*** *grevilleae* | Plant pest | Leaf spot | -65 |
| | Fungus | ***Cladosporium*** *pseudiridis* | Plant pest | Leaf spot | -62 |
| | Fungus | ***Cladosporium*** *ramotenellum* | Plant pest | Leaf spot | -62 |
| | Fungus | ***Aspergillus*** *subsessilis* | Plant pest | Storage rot | -44 |
| | Fungus | ***Aspergillus*** *wentii* | Plant pest | Storage rot | -43 |
| | Fungus | ***Penicillium*** *alexiae* | Plant pest | Decomposing fruits and stored products | -40 |

**C**



44 organisms found

Plant Health | **Wine Production** | Total Abundances

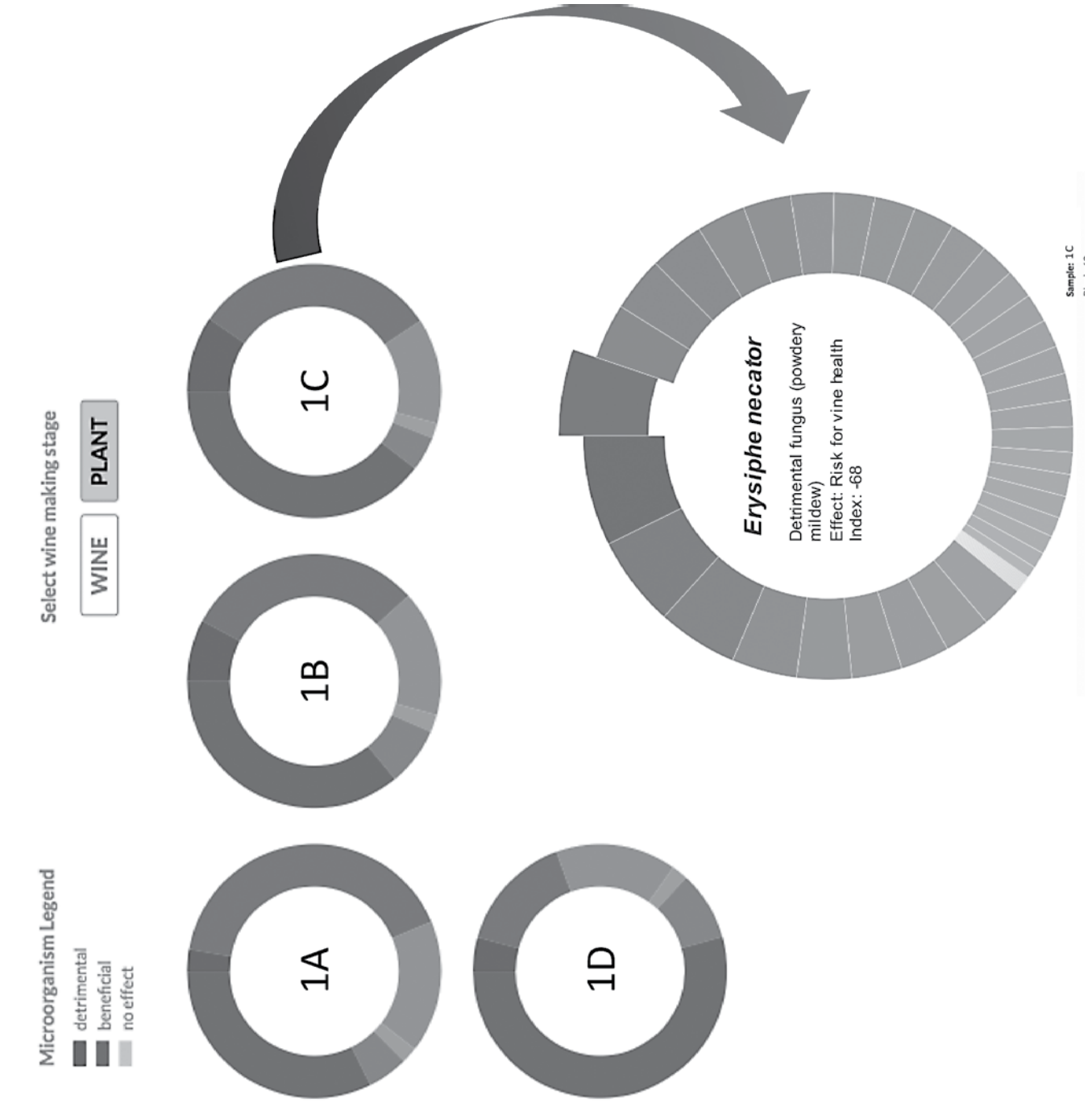
-100 -40 0 100 -70 70

Type here the key word...

| MωM | Type | Genus species | Type | Description | W-index |
|---|---|---|---|---|---|
| i | Yeast | *Metschnikowia pulcherrima* | Wine enhancer | Sensory profile enhancer (aroma and mouthfeel properties) | 93 |
| | Yeast | *Cyberlindnera jadinii* | Wine enhancer | Sensory profile enhancer | 91 |
| i | Yeast | *Hanseniaspora uvarum* | Wine enhancer | Sensory profile enhancer (aroma properties) | 88 |
| | Yeast | *Saccharomyces kudriavzevii* | Wine enhancer | High fermentator in wine production | 84 |
| i | Yeast | *Hanseniaspora vineae* | Wine enhancer | Sensory profile enhancer (aroma properties) | 81 |
| i | Yeast | *Candida parapsilosis* | Wine enhancer | Low $t^a$ fermentation | 77 |
| | Fungus | *Penicillium brevicompactum* | Fermentation risk | Titratable acidity and mycotoxins | -73 |
| i | Yeast | *Saccharomyces cerevisiae* | Wine enhancer | Sensory profile enhancer (aroma properties) | 72 |
| i | Fungus | *Aspergillus subsessilis* | Fermentation risk | Profound sensory effect | -69 |
| | Yeast | *Metschnikowia chrysoperlae* | Wine enhancer | Potential sensory profile enhancer | 69 |
| | Fungus | *Fusarium oxysporum* | Fermentation risk | High producer of 2,4,6-trichloroanisole. Cork taint | -61 |

A


**Figure 2.** Integrated view of WineSeq microbiome data from different vineyards. A) Geographical distribution of the studied vineyards. B) Visual comparison of the vine health/microbiological potential status of the studied vineyards. Green represents the proportion of beneficial microorganisms and red represents the proportion of detrimental microorganisms for vine health. An enlarged version of the diagram for sample "1C" is also shown, indicating that Erysiphe necato is the main microbiological risk in this vineyard for vine health.


B
Microorganism Legend
detrimental
beneficial
no effect
Select wine making stage
WINE
PLANT
1A
1B
1C
1D
Erysiphe necator
Detrimental fungus (powdery mildew)
Effect: Risk for vine health
Index: -68
Sample: 1C

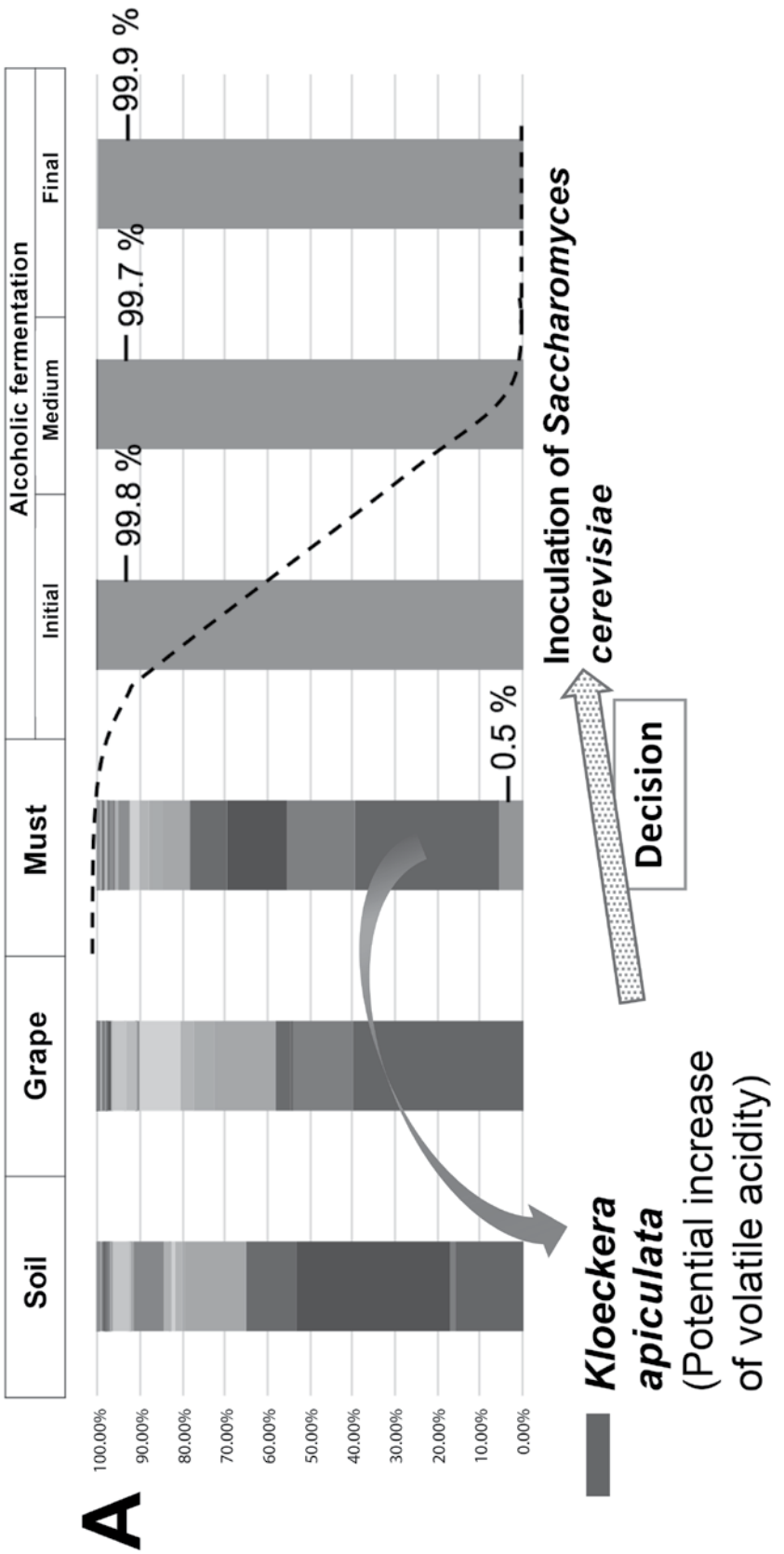
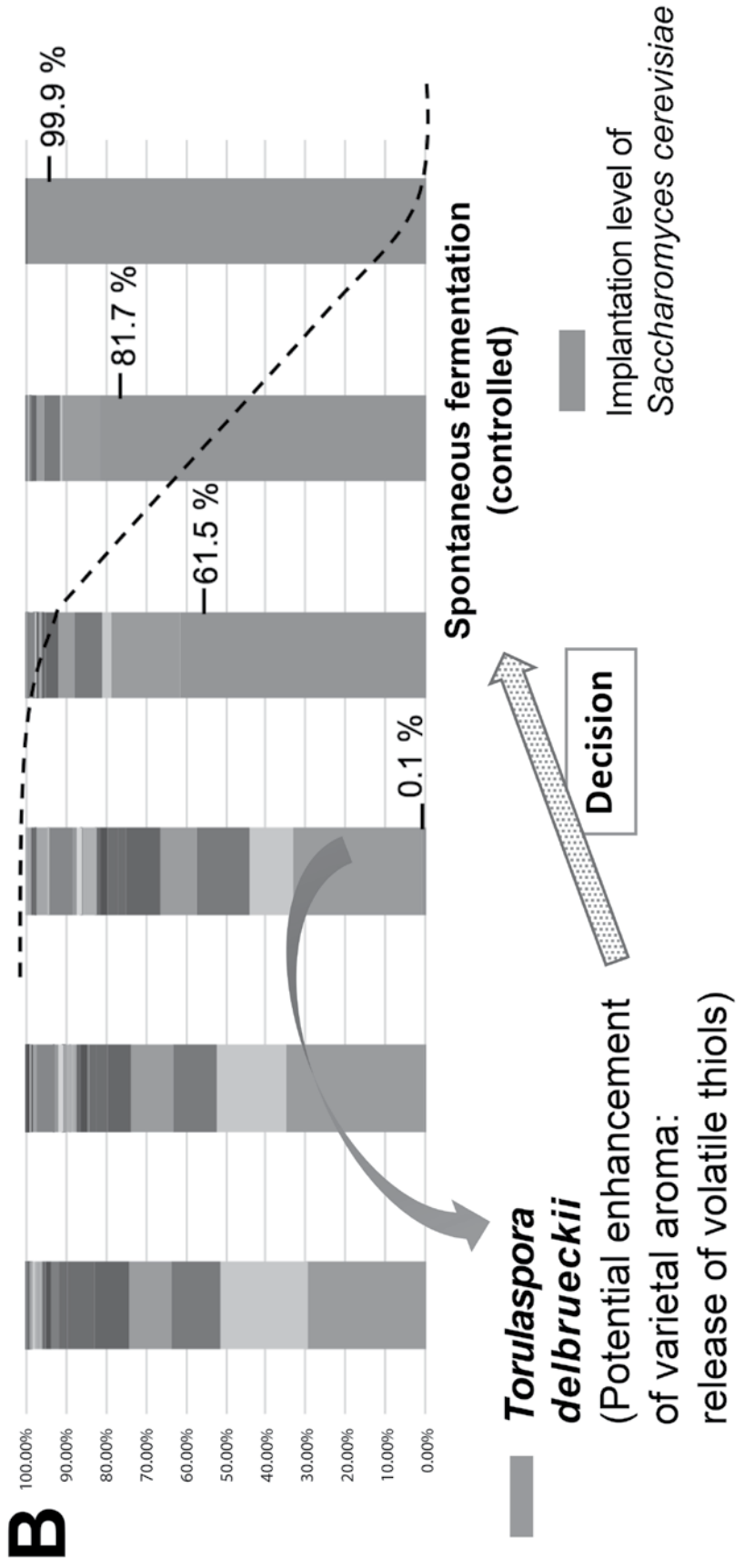



**Figure 3.** Dynamic analysis of the microbiome of two samples, from soil to the end of alcoholic fermentation. It shows the incidence of inoculation of selected *Saccharomyces cerevisiae* strains on the microbial population of the fermentation (A) versus the microbial evolution of a spontaneous fermentation (B). The percentage of implantation of *S. cerevisiae* is represented by light blue bars and the fermentative kinetics (sugar consumption) is represented by the dotted curve.


B
100.00%
90.00%
80.00%
70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
99.9 %
81.7 %
61.5 %
0.1 %
Spontaneous fermentation
(controlled)
Implantation level of
Saccharomyces cerevisiae
Decision
Torulaspora
delbrueckii
(Potential enhancement
of varietal aroma:
release of volatile thiols)

# THROUGH THE LOOKING GLASS: WHAT REALLY HAPPENS IN YOUR FERMENTATIONS

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## Introduction

In the winemaking process, yeast populations can be diverse and dynamic, both before and during primary fermentation. Identifying the diversity present in the yeast population and the changes that occur during fermentation provides a tool for winemakers to better understand what is occurring within the yeast population throughout the fermentation process.

ETS Laboratories utilizes a method of DNA fingerprinting known as multi-locus variable copy number tandem repeat analysis (MLVA) to discriminate between closely related strains of indigenous and commercial *Saccharomyces cerevisiae*. The MLVA method detects differences in the number of tandem repeat DNA sequences present in individual strains. The genomes of the target organisms contain many regions with tandem repeat DNA sequences. These regions are amplified using the polymerase chain reaction (PCR). The resulting length of the amplified piece of DNA is directly related to the number of tandem repeat sequences present at a particular location in an individual microbial strain.

A single location may contain enough variation to distinguish between several strains. Multiple locations provide the potential to distinguish between an unlimited numbers of strains. The ETS MLVA for *Saccharomyces cerevisiae* analyzes five unique locations, enabling winemakers to detect and identify both commercial and non-commercial strains of *Saccharomyces cerevisiae*. The five loci were selected by screening 23 published loci (Field and Wills, 1998; Legras et al., 2005) and determining the most informative loci for creating a multiplex assay. ETS utilizes a 6-plex polymerase chain reaction amplification process to amplify highly variable regions in the yeast genome. This involves amplifying five target sequences specific to *Saccharomyces cerevisiae* and one universal target that can distinguish between most species of yeast. Capillary electrophoresis is used to separate the amplified fragments by size, forming a unique DNA fingerprint for individual strains *of S. cerevisiae*.

Clients use this technology to monitor yeast populations in both inoculated and non-inoculated fermentations. Analyzing fermentations at the beginning, middle, and end points provides a view of the changes occurring in the non-*Saccharomyces* and *Saccharomyces* populations throughout the fermentation. This analysis can be used to monitor native fermentations as well as to characterize the efficiency of commercial strains inoculated into musts. The ability to monitor the yeast population during fermentation ensures that process decisions affecting wine production are made based on actual data from the winery's fermentations. Decisions regarding the selection of strains can be based upon observations on their ability to perform in a clients' specific wine style. The analysis can also be used as a quality control tool to verify that desired strains are dominating the individual fermentations, resulting in more consistent fermentations

A multi-year study was conducted on non-inoculated fermentations using grapes from six vineyards at six wineries participating in the trial. Grapes from three vineyards were fermented in three different wineries. Grapes from three additional vineyards were fermented in the other three wineries. Grape samples from the vineyards were analyzed by MLVA to determine the yeast species/strains present in the vineyards. Fermentation samples were submitted from the wineries for MLVA analysis at the beginning, middle, and end of fermentation in order to monitor changes in yeast population structure within the individual fermentations.

The results from the study will be presented in a manner that addresses the following questions asked by winemakers in regards to yeast populations in their non-inoculated, aka native/indigenous, fermentations.

- Is it possible to have a fermentation driven by indigenous *Saccharomyces cerevisiae* yeast?
- Do indigenous *Saccharomyces cerevisiae* strains from the vineyard persist through the fermentation?
- Are vineyard yeast strains, including *Saccharomyces cerevisiae* and non-*Saccharomyces*, the same from vintage to vintage?
- What yeast strains dominate non-inoculated fermentations in wineries that have previously used or currently use commercial (ADY) *Saccharomyces cerevisiae* yeast?
- Do yeast from the vineyard or "resident/house" yeast strains in the winery drive non-inoculated fermentations?

## Materials and Methods

Isolation of yeast from the vineyard

Clusters were collected in the vineyard and directly placed in new, one-gallon zip-lock bags to avoid contamination with winery yeast strains. Fruit was shipped to ETS Laboratories where the fruit was crushed directly in the shipping bag. The juice was aseptically transferred to sterile 1 L flasks with a fermentation trap and fermented at room temperature (~68˚F). Starting sugar was measured and initial samples were pulled for analysis. Additional samples were collected at approximately 6% ethanol and after fermentation stopped.

Isolation of yeast from juice and wine samples

Yeast cell counts were determined in juice and wine samples using a Beckman-Coulter Vi-Cell XR and samples were dilution plated to approximately 30–50 colonies per plate. Plates were incubated at 30˚C for 2–3 days. Sixteen colonies were randomly selected and analyzed to determine the yeast population structure in the sample.

VNTR Analysis

DNA was extracted directly from selected yeast colonies using a proprietary method in a multi-well format. Sample DNA was added to a multiplex reaction containing primer sets for the five published loci (Table 1) and a primer set for the internal transcribed spacer of the yeast 5.8S ribosomal sequence. Post PCR, the samples were cleaned using column-based technology and run on a Beckman Coulter CEQ8000 genetic analyzer.

**Table 1.** Primer used for ETS VNTR analysis

| ETS MLVA Locus | Published Locus |
|---|---|
| ETS SC-1 | C5 |
| ETS SC-2 | Sc8132x |
| ETS SC-3 | C11 |
| ETS SC-4 | C12b |
| ETS SC-5 | YOR267C |

Data generated from the CEQ8000 was exported and further analyzed, using proprietary software, to compare MLVA profiles from yeast selected for analysis to profiles from a library of 140 commercial yeast strains. Both commercial strains and unidentified yeast are reported as a percent of the population. *Saccharomyces cerevisiae* strains not present in our commercial library are classified as putative native/indigenous yeast strains. This process enables discrimination between most commercial *S. cerevisiae* strains as well as native strains of *S. cerevisiae*, enabling characterization of the yeast population at a specific time point in the fermentation process.

## Results

### Is it possible to have a fermentation driven by indigenous *Saccharomyces cerevisiae* yeast?

Analysis of the 18 fermentations in 2015 indicates at least four of the fermentations did not contain any yeast strains present in our library of commercial *Saccharomyces cerevisiae* strains (Table 2). These include the fermentations from Winery F, where no *S. cerevisiae* strains similar to commercial strains were recovered. However, the fermentations from Winery A were dominated by a single strain of yeast that had the same MLVA profile as the Lallemand strain Enoferm Syrah. This commercial yeast strain was used in this facility during the 2015 vintage. The fermentations from the other wineries were a mix of putative native strains only or a combination of putative native strains and commercial strains. The fermentations from Winery C contained only putative native strains at the mid fermenta-

tion point, but finished with commercial strains present. This type of shift in yeast population profiles has been observed in many non-inoculated fermentations, suggesting that commercial yeast strains are more competitive as the ethanol level increases.

**Do indigenous *Saccharomyces cerevisiae* strains from the vineyard persist through the fermentation?** Observations from both vintages indicate that *Saccharomyces cerevisiae* strains from the vineyard can be recovered from mid- and end-stage winery fermentations. Analysis of the winery fermentations indicates that yeast strains observed in the vineyard were observed in 6 of 18 fermentations in 2014 and 8 of 18 fermentations in 2015. In 2014, the percent of yeast strains observed in the vineyard that were present at the end of fermentation ranged from 0 to 75%; in 2015, that number ranged from 0 to 25%. Although vineyard yeasts can be found in the fermentations, it is unusual for them to dominate the fermentation. Examples of yeast strains observed in the vineyard persisting in the fermentations can be seen in figures 1 and 2.

**Table 2.** Number of *Saccharomyces cerevisiae* strains observed at fermentation mid- and end-point for the trial. *S. cerevisiae* strains whose MLVA profiles did not match any of the 140 strains in our library of commercial S. cerevisiae strains were categorized as putative native strains.

| | Total Strains | Similar to Commercial | Putative Native |
|---|---|---|---|
| Winery A | | | |
| Vineyard 1 mid ferment | 2 | 1 | 1 |
| Vineyard 1 end ferment | 1 | 1 | 0 |
| Vineyard 2 mid ferment | 1 | 1 | 0 |
| Vineyard 2 end ferment | 1 | 1 | 0 |
| Vineyard 3 mid ferment | 1 | 1 | 0 |
| Vineyard 3 end ferment | 1 | 1 | 0 |
| Winery B | | | |
| Vineyard 1 mid ferment | 4 | 3 | 1 |
| Vineyard 1 end ferment | 6 | 1 | 5 |
| Vineyard 2 mid ferment | 6 | 1 | 5 |
| Vineyard 2 end ferment | 9 | 1 | 8 |
| Vineyard 3 mid ferment | 9 | 0 | 9 |
| Vineyard 3 end ferment | 8 | 2 | 6 |
| Winery C | | | |
| Vineyard 1 mid ferment | 6 | 0 | 6 |
| Vineyard 1 end ferment | 7 | 2 | 5 |
| Vineyard 2 mid ferment | 11 | 0 | 11 |
| Vineyard 2 end ferment | 9 | 2 | 8 |
| Vineyard 3 mid ferment | 11 | 0 | 11 |
| Vineyard 3 end ferment | 10 | 2 | 8 |

| | Total Strains | Similar to Commercial | Putative Native |
|---|---|---|---|
| Winery D | | | |
| Vineyard 4 mid ferment | 12 | 3 | 9 |
| Vineyard 4 end ferment | 9 | 2 | 7 |
| Vineyard 5 mid ferment | 10 | 0 | 10 |
| Vineyard 5 end ferment | 11 | 0 | 11 |
| Vineyard 6 mid ferment | | | |
| Vineyard 6 end ferment | 10 | 1 | 9 |
| Winery E | | | |
| Vineyard 4 mid ferment | 6 | 1 | 5 |
| Vineyard 4 end ferment | 7 | 1 | 6 |
| Vineyard 5 mid ferment | 6 | 1 | 5 |
| Vineyard 5 end ferment | 5 | 1 | 4 |
| Vineyard 6 mid ferment | 1 | 0 | 1 |
| Vineyard 6 end ferment | 3 | 0 | 3 |
| Winery F | | | |
| Vineyard 4 mid ferment | 9 | 0 | 9 |
| Vineyard 4 end ferment | 7 | 0 | 7 |
| Vineyard 5 mid ferment | 10 | 0 | 10 |
| Vineyard 5 end ferment | 8 | 0 | 8 |
| Vineyard 6 mid ferment | 7 | 0 | 7 |
| Vineyard 6 end ferment | | | |

**Are vineyard yeast strains, including *Saccharomyces cerevisiae* and non-*Saccharomyces*, the same from vintage to vintage?** In the 2014 vintage, grapes were submitted from five vineyards. Three of the grape cluster samples contained *S. cerevisiae* with a total of 29 putative native strains observed. In the 2015 vintage, grapes were submitted from all six vineyards and *S. cerevisiae* was found in all six vineyard samples, with a total of 31 putative native strains observed. Comparative analysis was done on the 60 strains observed over the two vintages. A single strain was observed in both vintages from Vineyard 6. The grape cluster fermentations from Vineyard 6 had the largest number of *Saccharomyces cerevisiae* strains as compared to the other vineyards, with 19 strains observed in 2014 and 10 strains in 2015.

Differences were also observed in the non-*Saccharomyces* yeast strains between the 2014 and 2015 vintages (Table 3). Generally speaking, *Hanseniaspora* spp are the most prevalent non-*Saccharomyces* yeast observed on the grapes. However, in 2014, the most prevalent yeast in Vineyard 2 was a *Picha* spp. and in 2015 it was a *Kazachstania* spp. Although this is a small data set, it appears there was less diversity in the non-*Saccharomyces* species present on the grapes in 2015 as compared to 2014.

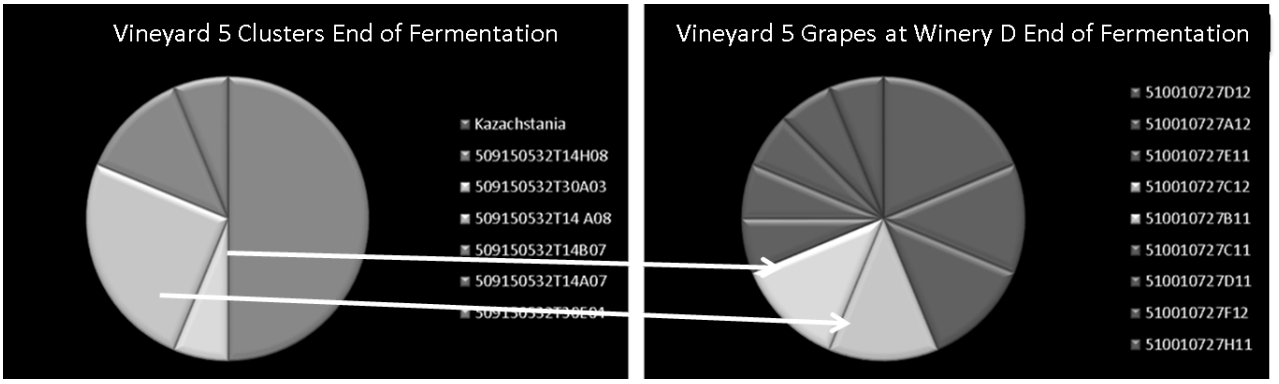



**Figure 1.** *Saccharomyces cerevisiae* strains observed in the Vineyard 5 cluster fermentation and recovered at the end of fermentation with those grapes at Winery D.

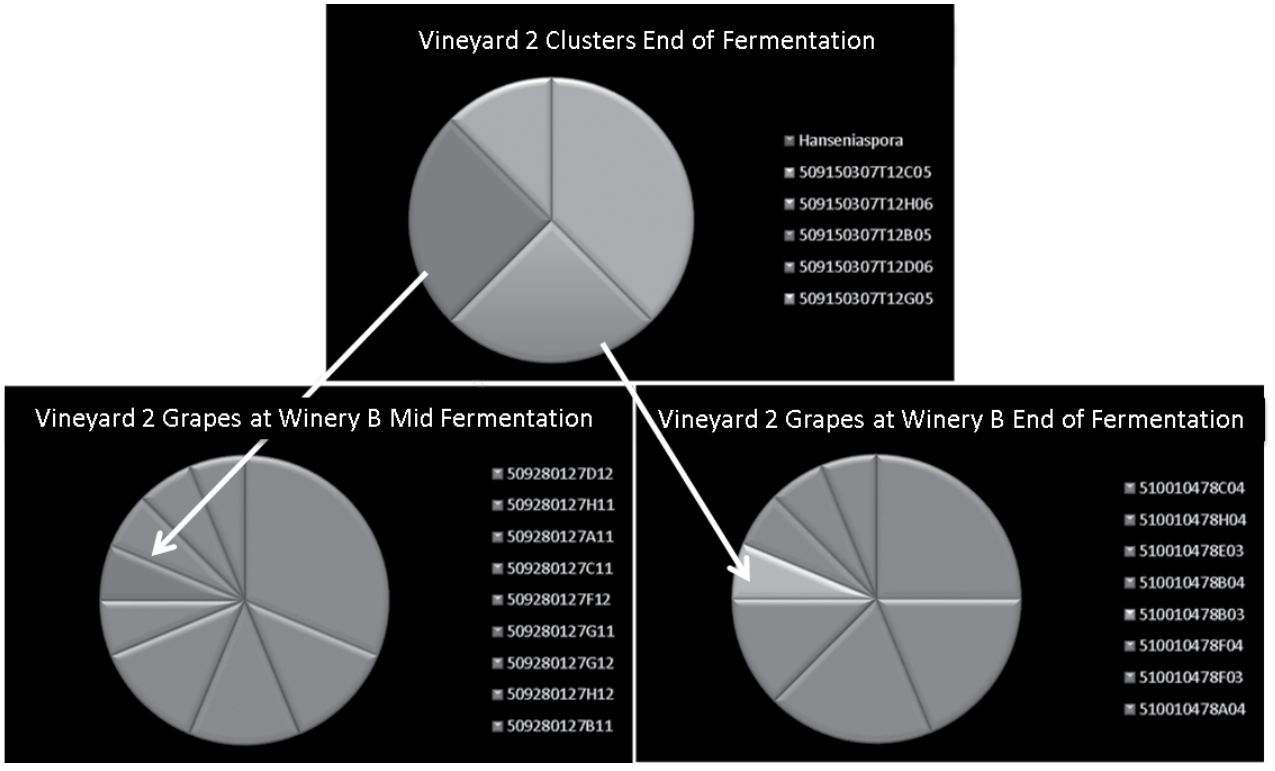
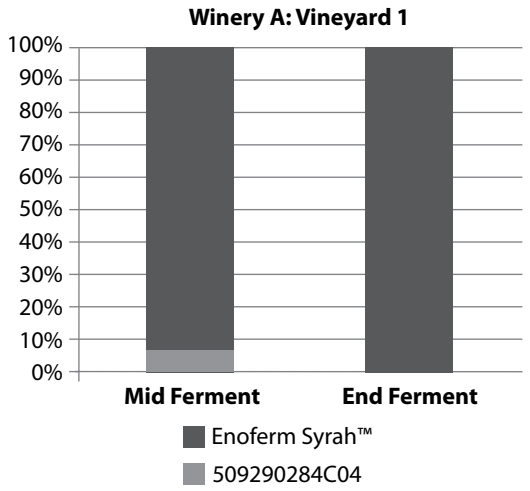
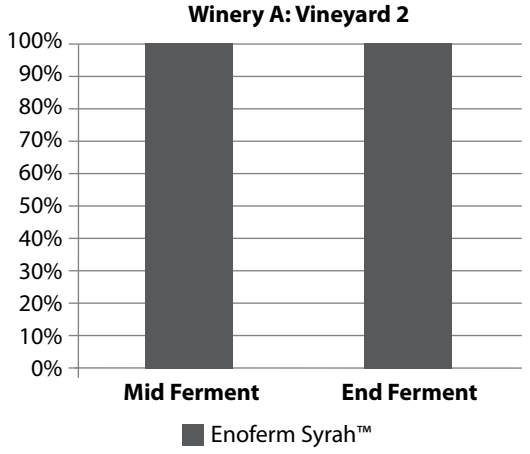



**Figure 2.** *Saccharomyces cerevisiae* strains observed in the Vineyard 2 cluster fermentation and recovered at both the middle and end of fermentation with those grapes at Winery B.

**Table 3.** Vineyard non-*Saccharomyces* yeast strains observed in the 2014 and 2015 vintages

| | *Hanseniaspora* spp. | *Metschnikowia* spp. | *Pichia* spp. | *Kluyveromyces* spp. | *Kazachstania* spp. |
|---|---|---|---|---|---|
| Vineyard 1 | | | | | |
| 2014 | 88% | 12% | | | |
| 2015 | 100% | | | | |
| Vineyard 2 | | | | | |
| 2014 | 6% | | 94% | | |
| 2015 | | | | | 100% |
| Vineyard 3 | | | | | |
| 2014 | | | | | |
| 2015 | 12% | | | | 88% |
| Vineyard 4 | | | | | |
| 2014 | 88% | 6% | | 6% | |
| 2015 | 100% | | | | |
| Vineyard 5 | | | | | |
| 2014 | 88% | 6% | 6% | | |
| 2015 | 100% | | | | |
| Vineyard 6 | | | | | |
| 2014 | 100% | | | | |
| 2015 | 100% | | | | |



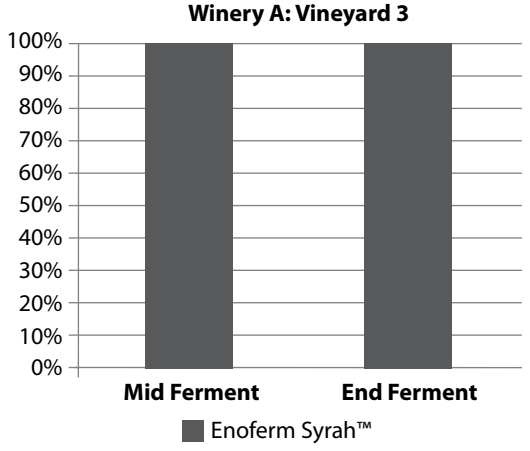




**Figure 3.** Fermentations done at Winery A using grapes from Vineyards 1, 2, and 3. The non-inoculated fermentations were dominated by the commercial yeast strains Lalvin Enoferm Syrah™ at the middle and end points.

**What yeast strains dominate non-inoculated fermentations in wineries that have previously used or currently use commercial (ADY) *Saccharomyces cerevisiae* yeast?**

All of the wineries that participated in the study have used commercial yeast strains in past vintages and most facilities used them in the 2014 and 2015 vintages. Commercial *Saccharomyces cerevisiae* strains are selected for many traits, but competiveness is a major factor when identifying potential strains for commercial application. The results in Table 2 indicate that commercial yeast strains are often present in non-inoculated fermentations as evident in Wineries A, B, C, D and E. An extreme example of this occurred in the fermentations conducted at Winery A. All three of the non-inoculated fermentations were dominated by the commercial Lallemand strain Enoferm Syrah (Figure 3, previous page). This commercial strain was used in the winery during the 2015 vintage. Observations on numerous non-inoculated fermentations indicate that commercial yeast strains tend to show up later in the fermentation as alcohol levels increase.

**Do yeast from the vineyard or "resident/house" yeast strains in the winery drive non-inoculated fermentations?**

In order to determine the origin of the yeast driving these non-inoculated fermentations, comparative analyses were conducted. The analyses looked at similarities between fermentations using grapes from the same vineyard at different wineries (Same Vineyard Different Winery) and between fermentations using grapes from different vineyards at the same winery (Same Winery Different Vineyard). The expectation would be that vineyard yeast dominance would result in similarities between the fermentations using the same grapes, but at different wineries. Likewise, resident yeast dominance would result in similarities between fermentations using different grapes, but at the same winery.

In the 2014 vintage, more similarity was observed between yeast populations in fermentations at the same winery, but using grapes from different vineyards (Figure 4). Less similarity was observed between fermentations using the same grapes, but at different wineries. The results from 2014 suggest that resident yeast at a winery was more prevalent in the fermentations at that winery than yeast originating from the vineyard. The majority of the similarity observed in fermentations at different wineries, but using grapes from the same vineyard, was due to the presence of similar commercial strains at both facilities.

Likewise, the results from the 2015 vintage suggest that resident yeast was more prevalent in fermentations at a facility than vineyard yeast. More similarity was observed between yeast populations in fermentations at the same winery, but using grapes from different vineyards (Figure 5). Less similarity was observed between fermentations using the same grapes, but at different wineries. Once again, the majority of the similarities in yeast populations observed between fermentations at different wineries, but using grapes from the same vineyard, were due to the presence of similar commercial yeast strains in the fermentations.

## Conclusions

**Is it possible to have a fermentation driven by indigenous *Saccharomyces cerevisiae* yeast?**

The results from seven years of analyzing client samples indicate that it is possible to have non-inoculated fermentations driven by indigenous strains of *Saccharomyces cerevisiae*. The past or current use of commercial yeast strains in a facility and ineffective winery sanitation will decrease the likelihood of indigenous yeast strains dominating the non-inoculated fermentations at a particular facility. The ability to utilize commercial yeast strains in a facility and have non-inoculated fermentations driven by indigenous strains in that facility requires a fastidious winery sanitation program.

**Do indigenous *Saccharomyces cerevisiae* strains from the vineyard persist through the fermentation?**

*Saccharomyces cerevisiae* strains identified from vineyard samples can be recovered from winery fermentations. In the trial study on non-inoculated fermentations, vineyard yeast recovered in the winery fermentations ranged from 0 to 80% in the 2014 vintage and 0 to 25% in the 2015 vintage. In the 2014 vintage, the winery fermentations with the highest percentage of vineyard yeast recovered were both from the same vineyard. However, in 2015 no vineyard yeast was recovered in winery fermentations from that vineyard.

**Are vineyard yeast strains, including *Saccharomyces cerevisiae* and non-*Saccharomyces*, the same from vintage to vintage?**

A total of 59 strains of *Saccharomyces cerevisiae* were recovered from the cluster fermentations in the first two years of the trial. Of these, only one strain was observed in both vintages, indicating significant population diversity between the two vintages. Observations on non-*Saccharomyces* yeast populations indicate differences were also observed between the two vintages. This data is only based on two years of analysis, but it suggests the vineyard yeast population is dynamic vintage to vintage.

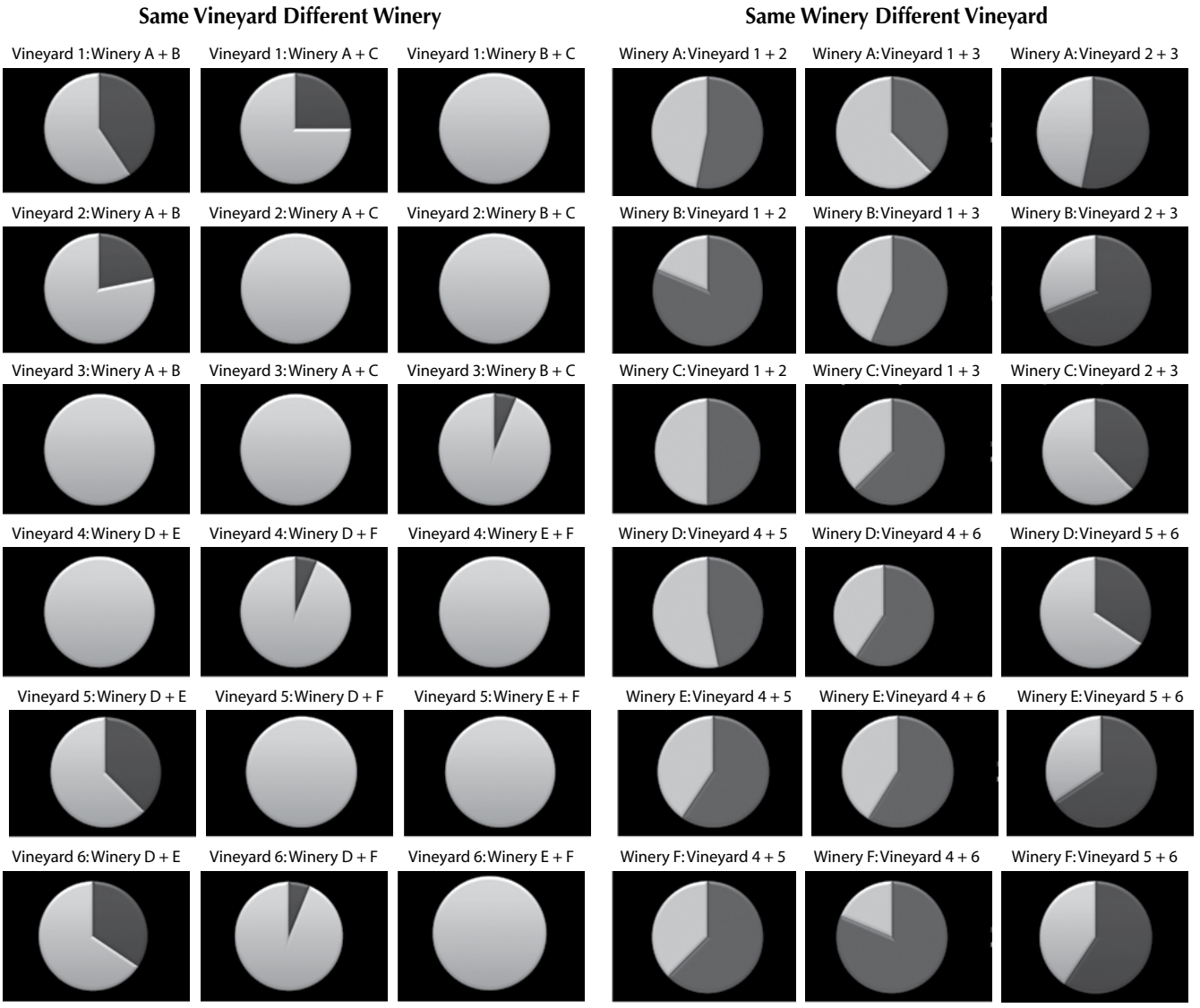



**Figure 4.** Comparison of yeast populations recovered from fermentations in 2014. Yeast populations were compared between fermentations using grapes from the same vineyard, but done at different wineries, and between fermentations at the same winery, but using grapes from different vineyards. Dark grey indicates similarity between yeast populations present in the two fermentations and light grey indicates no similarity.

**What yeast strains dominate non-inoculated fermentations in wineries that have previously used or currently use commercial (ADY) *Saccharomyces cerevisiae* yeast?**

Commercial (ADY) yeast strains have been used in all of the wineries participating in the trial. In the 2015 vintage, commercial *Saccharomyces cerevisiae* strains were recovered from fermentations in five of the six participating wineries. In these non-inoculated fermentations containing commercial strains, the percentage of commercial strains in the individual fermentations ranged from 10% to 100%. However, the majority of non-inoculated fermentations had a higher number of putative indigenous strains than commercial strains of *S. cerevisiae*. Overall, good winery sanitation and awareness of the potential for cross contamination should minimize the appearance of commercial strains in non-inoculated fermentations.

**Do yeast from the vineyard or "resident/house" yeast strains in the winery drive non-inoculated fermentations?**

Many winemakers believe that the use of commercial strains in a winery will result in the development of resident strains of these highly competitive yeasts. One of the primary goals of this research was to determine if "resident" or "vineyard" yeast strains were the dominant yeast present in non-inoculated fermentations. The results from two years of analysis indicate that although vineyard strains can be recovered from non-inoculated winery fermentations, the fermentations appear to be driven by yeast strains resident in the winery. The resident strains appear to be a mix of commercial strains used in the winery as well as non-commercial strains.

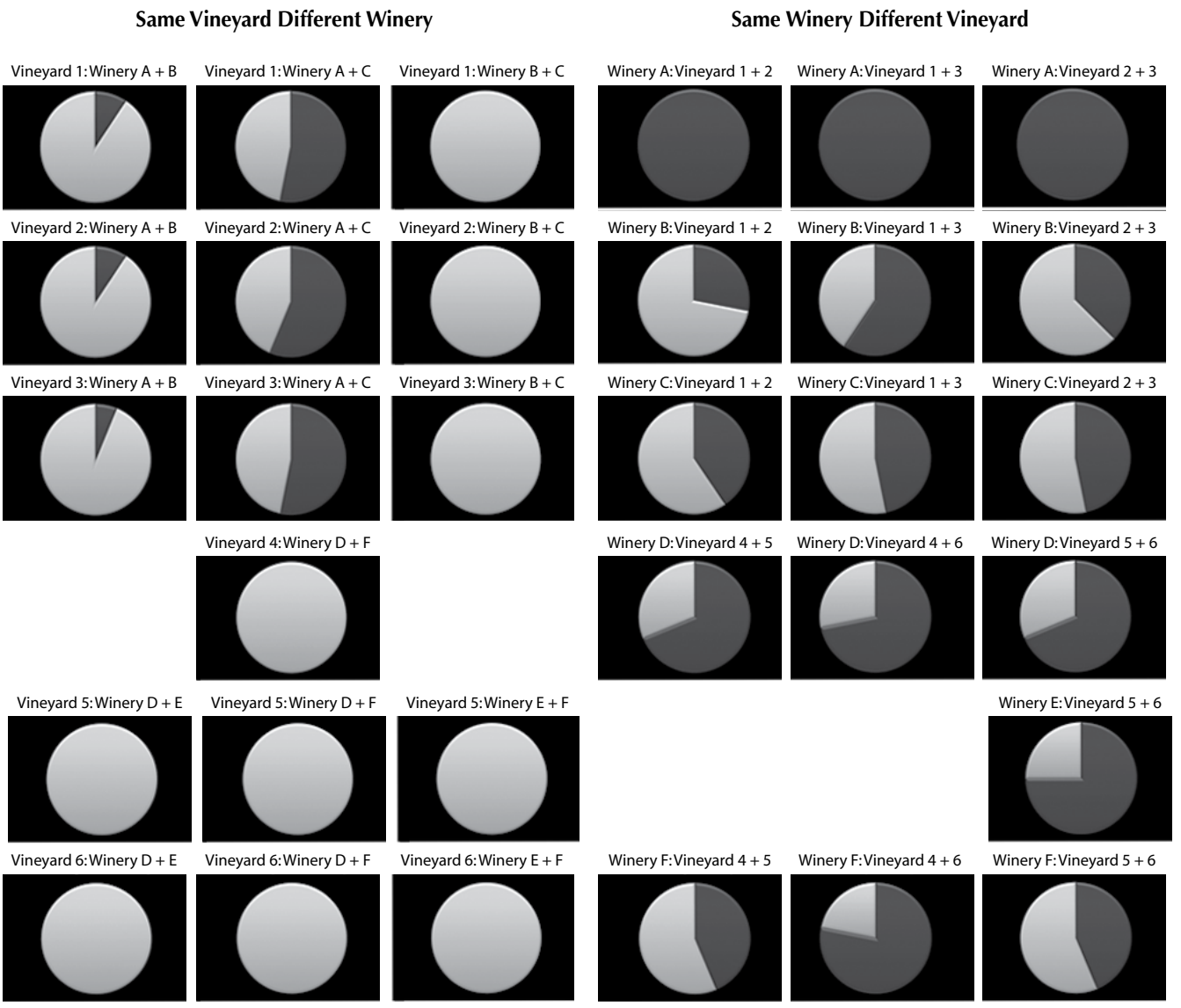



**Figure 5.** Comparison of yeast populations from fermentations in 2015 using grapes from the same vineyard, but done at different wineries. Similarly, yeast populations were compared between fermentations at the same winery, but using grapes from different vineyards. Dark grey indicates similarity between yeast populations present in the two fermentations and light grey indicates no similarity. No data was available from Winery E at the end point for the fermentation using grapes from Vineyard 4.

## Literature cited

Field, D. and C. Wills, 1998. Abundant microsatellite polymorphism in *Saccharomyces cerevisiae* and the different distributions of microsatellites in eight prokaryotes and *S. cerevisiae* result from strong mutational pressure and a variety of selective forces. *Proc. Natl. Acad. Sci. U.S.A.* 95:1647–1652

Legras, J-L., O. Ruh, D. Merdinoglu, and F. Karst. 2005. Selection of hypervariable microsatellite loci for the characterization of *Saccharomyces cerevisiae* strains. *Int. J. Food Microbiol.* 102:73–83

# MICROORGANISMS IN SERVICE OF TERROIR WINES

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Burgundy's winemaking region spans 28,000 ha and produces 1.5 million hL of wine. The region has 740 identified terroirs or "climates," including 640 deemed "Premier Cru." This winemaking structure gradually took shape over the centuries and was made official with the creation of "appellation d'origine controlée" certification in 1936. Advances in viticulture and oenology have steadily progressed with the development of knowledge and new equipment, as today's successful new approaches becomes tomorrow's traditions. But at the same time, microbiological phenomena have not evolved, and fermentations remain associated with potential alterations.

Wine microbiology is our topic of discussion, with the notion of typicity as the underlying theme. If the terroir conditions the quality of the grape, why wouldn't it also ensure the quality of the wine? We need to frame this question differently: Why would the vine naturally foster flora capable of fermenting large quantities of sugar or malic acid in an acidic environment? That said, biodiversity is nonetheless important for the vine's sustainable development, with soil maintenance and pest management, for example.

The notion of quality is subjective. A phenolic wine was and may still be considered a typical wine. A lactic or acetic wine may be considered a natural wine. But a balanced and fruity wine clearly has a more legitimate claim to a terroir. In a single-varietal region like Burgundy, this notion of terroir is all the more important.

## The microorganisms of the grape and alcoholic fermentation

The winemaker's goal is to produce a ripe grape that is in good health (except in instances of noble rot). This raises the question of the role that grape microorganisms play in the development of wine. To find answers, organically grown grapes were harvested, then processed in experimental fermenting rooms using disinfected equipment. The musts (sulphite-free and with a sulphite content of 5 g/hL) were placed in small stainless steel vats. After over a week of incubation at 20°C, development of various moulds was visible on the surface (Photos 1). It took more than ten days of incubation (Figure 1) to observe active alcoholic fermentation. This experiment clearly shows that the grape contains very few wine yeasts in its microbial flora, whereas the presence of mould is common.

To trigger alcoholic fermentation, the first solution is to allow *Saccharomyces cerevisiae* yeasts that have colonized the cellar and cellar equipment to develop in the must. But, the stricter the hygiene requirements, the less attractive this solution will be. The second solution is to seed the must with selected yeast. The challenge is selecting the yeast best suited to the oenological objective at hand. Yeasts must be selected individually, since a major yeast strain presents little or no persistence from one vintage to another.

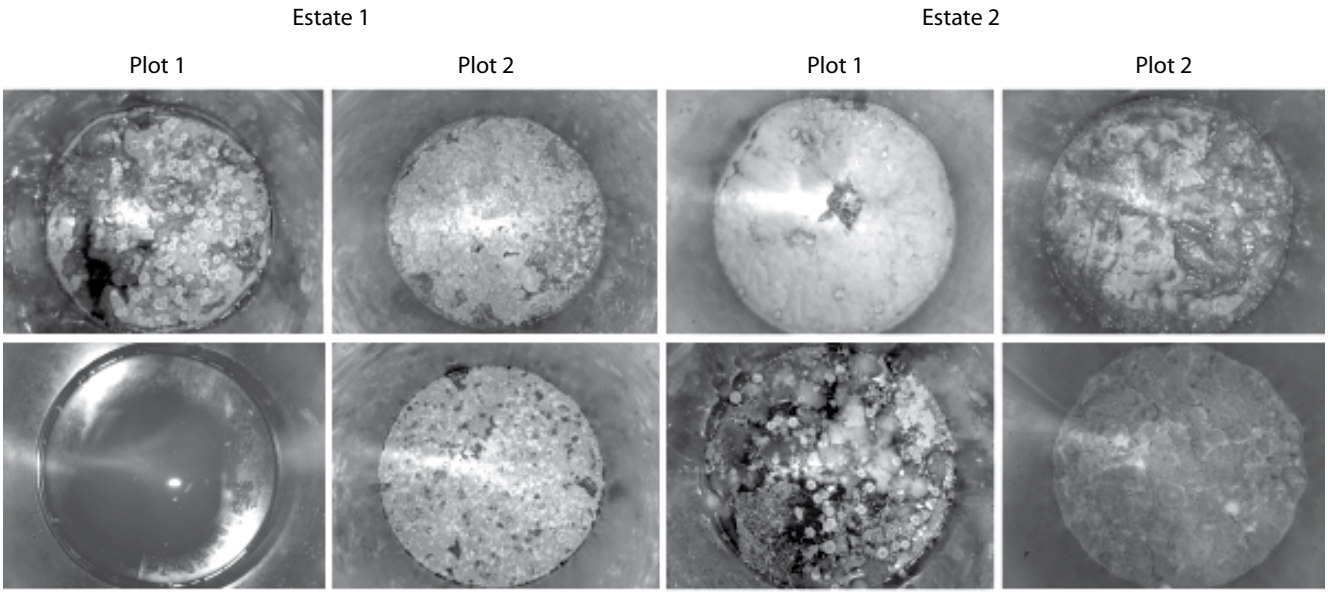
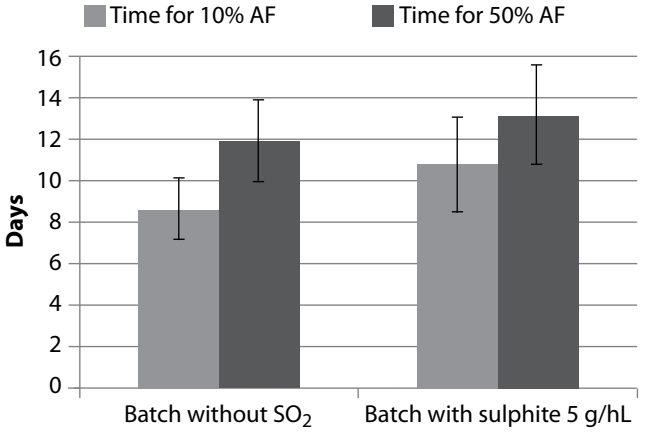



**Image 1**: Photographs of surfaces of aseptically treated grape musts after nine days of incubation at 20°C




**Figuree 1**: Average time required to obtain active fermentation in grape musts processed with disinfected equipment

## Cold pre-fermentation maceration and the fruit expression in Pinot Noir

*Kloeckera apiculata* (also known as *Hanseniaspora uvarum*) is a yeast better represented on the grape than *Saccharomyces cerevisiae*. This yeast presents an oxidative metabolism with a strong ability to produce acetic acid.

Contaminating a previously pasteurized Pinot Noir must with *Kloeckera apiculata* prompted rapid development, despite the cool temperature (15°C). In six days, the population grew from hundreds of cells to almost 100 million per mL (Table 1).

To prevent the development of *Kloeckera apiculata*, the classic solution is to pitch early with *Saccharomyces cerevisiae* to develop alcoholic fermentation. *Kloeckera apiculata* is inhibited by an alcohol content above 5 to 7% v/v. Early pitching with *Metschnikowia* is also an innovative solution for ensuring biological control in the must. This yeast, a common yeast on the grape, is non-fermenting, does not produce acetic acid, and has the potential to produce aromas. IFV and Lallemand have developed an isolated strain of *Metschnikowia fructicola* from Burgundy to control cold pre-fermentation maceration in red wines: Gaïa.

The Pinot Noir must contaminated with *Kloeckera apiculata* contained 0.30 g/L of acetic acid at the end of cold pre-fermentation maceration (Table 2), as well as the clearly discernible smell of ethyl acetate. Early pitching with *Metschnikowia fructicola* inhibits the metabolism of *Kloeckera apiculata*. The wine's acetic acid content at

**Table 1**: Growth of *Kloeckera apiculata* in a Pinot Noir must at 15°C (Sugars 230 g/L, pH 3.20, no $SO_2$)

| ***Yeasts in cells/mL*** | **T0** | **T 1 day** | **T 6 days** |
|---|---|---|---|
| **Control** (uncontaminated batch) | < 10 | < 10 | < 10 |
| ***Kloeckera apiculata*** (Contaminated batch) | 320 | 22,000 | 70,000,000 |

**Table 2**: *Kloeckera apiculata* activity in a Pinot Noir must with or without a biological control with *Metschnikowia* (pitching with *S. cerevisiae* at the end of cold pre-fermentation maceration)

| ***Acetic acid in g/L*** | **T 7 days (end of PFM)** | **T 21 days (end of AF)** |
|---|---|---|
| **Control** (uncontaminated batch) | 0.02 | 0.35 |
| ***Kloeckera apiculata*** (Contaminated batch) | 0.31 | 0.67 |
| ***Kloeckera* + Gaïa$_{Mf98.3}$** (Contaminated batch + biological control) | 0.10 | 0.36 |

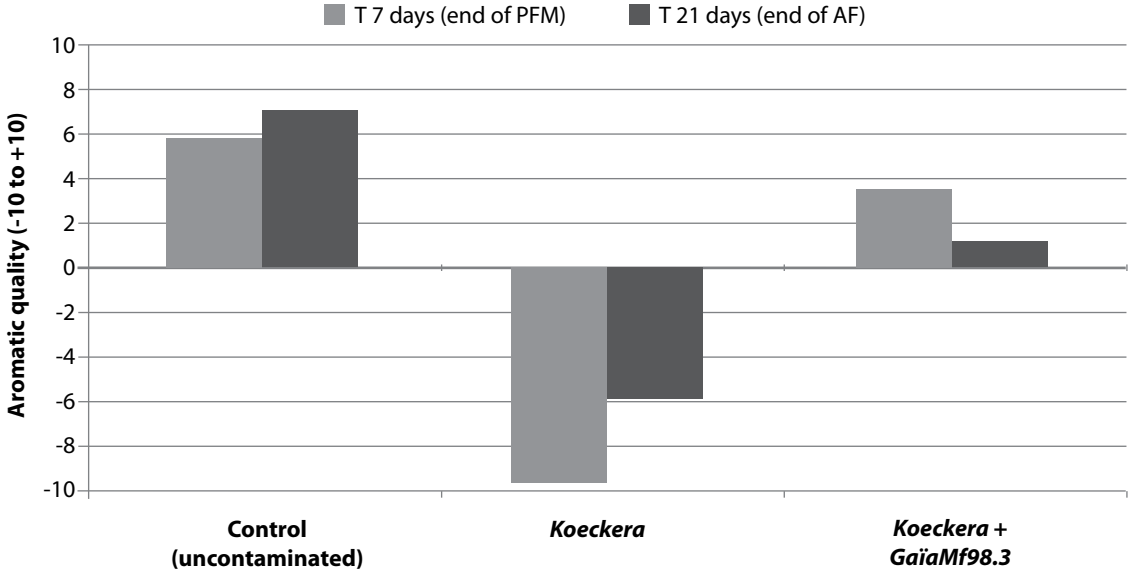



**Figure 2**: Qualitative influence of *Kloeckera apiculata* in a Pinot Noir with or without biological control with *Metschnikowia fructicola*

the end of alcoholic fermentation was thus equivalent to that of the uncontaminated control batch, or 0.35 g/L. The batch where *Kloeckera apiculata* contamination went unchecked had an acetic acid content of 0.67 g/L at the end of alcoholic fermentation. This batch showed clear signs of deterioration when tasted at the end of cold pre-fermentation maceration and at the end of AF (Figure 2). Biological control of *Kloeckera apiculata* using *Metschnikowia fructicola* provides a level of quality close to that of the uncontaminated batch.

## Control of alcoholic fermentation and the expression of fruit in Chardonnay

Yeast flora and the nutritional potential of the must impact the alcoholic fermentation process and the aromatic quality of the wine.

**Choice of yeast** – IFV and Lallemand have developed a yeast for northern Chardonnay wines: IOC TwICE™. Four levels of tests were conducted on an original collection of some 500 yeasts of Burgundy Chardonnay to narrow down the number of yeasts of interest. The selection criteria included high alcohol forming power, moderate and regular fermentation kinetics, positive interaction with the lactic bacterium Acidophil+ (selected for Chardonnay), measured acetic acid production, and fruity aromatic complexity. The selected yeast was tested in various Burgundy estates in the form of active dry yeast. On average, the final alcohol content was 13%v/v with less than 2 g/L residual sugar. Alcoholic fermentation was completed in one month, and malolactic fermentation in less than two months (inoculation with Acidophil+). The final volatile acidity content was 0.33 g/L $H_2SO_4$ on average. Test batches were tasted in a specific room by some 15 judges using the FIZZ software. The olfactory and gustatory qualities of the wines vinified in vats are markedly better with the TwICE yeast than the reference yeast (Figure 2a). The fruity and floral aromas are well expressed (Figure 2b).

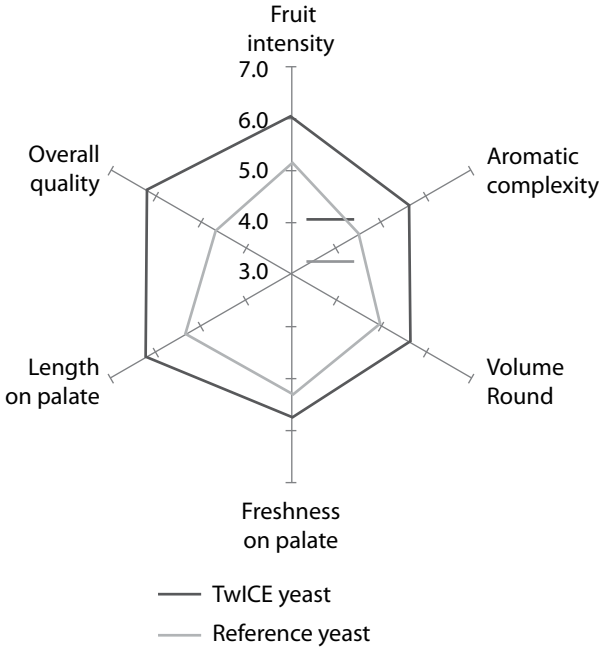



**Figure 3a**: Average sensory profiles for five tests of Chardonnay in vats (mark/10)

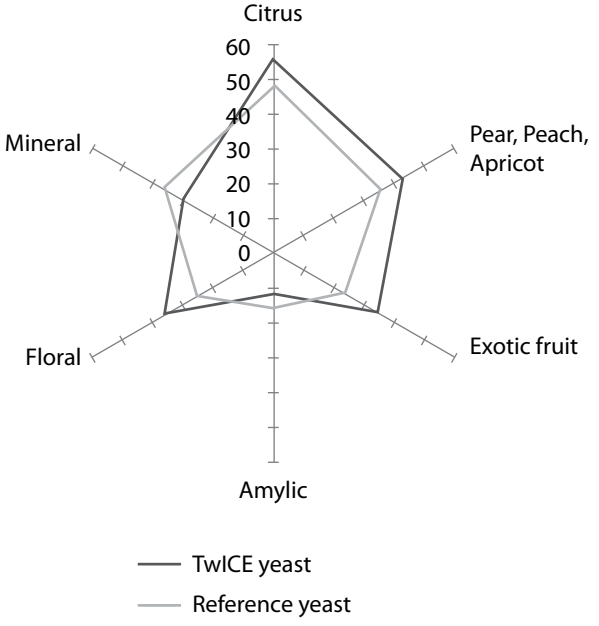



**Figure 3b**: Average aromatic descriptions for five tests of Chardonnay in vats (in % of the jury)

**Nitrogen nutrition of yeast** – Yeast nutrition is important for ensuring good alcoholic fermentation and to obtain good aromatic quality. The decision of whether or not to add a nutrient should take into account the amount of yeast-available nitrogen in the must. According to JM Sablayrolles (RFO 159, 1996), the most effective time to add ammonia nitrogen is mid-fermentation. Today, the oenological market offers many complex nutrients that can be used at different stages of alcoholic fermentation. An experiment was conducted with two Chardonnays presenting low YAN and high probable alcohol content (Table 3). Alcoholic fermentation was triggered with the "IOC TwICE™" active dry yeast, and the temperature was regulated at 19°C. Four batches were made: a control batch (without nutrient) and three batches where the nutrient (Activit™) was added at T0 (just prior to pitching), at 30%, and at 60% of alcoholic fermentation, respectively. The results were identical for the two control musts. In the T0 batch, the nutrient help initiate fermentation, but the end was protracted, similar to that of the control batch. The 30% and 60% batches initiated fermentation and ensured good consumption of sugars (Figure 4). Repitching was needed for the control batches and the batch where the nutrients were added at T0, with sugar consumption times doubled or tripled (Table 4). The tests confirmed that the nitrogen must be added at roughly mid-fermentation to be truly effective, especially if only a single dose is added. Aeration at the beginning of alcoholic fermentation is also important for vinification in vats.

With good control over the alcoholic fermentation process, it may be possible to trigger early malolactic fermentation. When trying to reduce doses of sulphite, good control over aging will foster the aromatic expression of Chardonnay and limit the risk of premature oxidation.

## Control of malolactic fermentation for quality aging

The time between alcoholic fermentation and malolactic fermentation is a critical period. The wine cannot be stabilized and is exposed to various risks of alteration. Triggering malolactic fermentation early is a compelling option to protect white Chardonnay wines against the risk of premature oxidation, and red wines against the microbiological risks tied specifically to *Brettanomyces*. In the particular case of Burgundy, malolactic fermentation must not be triggered too early to give the Chardonnay time to complete its alcoholic fermentation and allow for colour stabilization and tannin ripening in the Pinot Noir. A bacterial seeding in November at cool cellar temperatures (14/16°C) should make it possible to complete malolactic fermentation before Christmas, thereby leaving plenty of time to stabilize the wine prior to bottling.

**Investigation of indigenous lactic bacteria flora in Burgundy** – Pinot Noir and Chardonnay estate wines were sampled both during and at the end of malolactic fermentation. The Institute of Vine and Wine Sciences (ISVV) at the University of Bordeaux conducted a genetic identification test. Fifteen lactic bacteria clones were isolated for

**Table 3**: Analytical characteristics of test Chardonnays

| | **2015 Burgundy** | **2015 Beaune** |
|---|---|---|
| **Health status** | Oidium on grapes | Good health status |
| **Sulphiting** (pressing) | 40 mg/L | 30 mg/L |
| **Probable alcohol content** | 14.4% v/v | 13.7% v/v |
| **Available nitrogen** | 150 mg/L | 130 mg/L |
| **Turbidity** (enzymatic settling) | 20 NTU | 11 NTU |

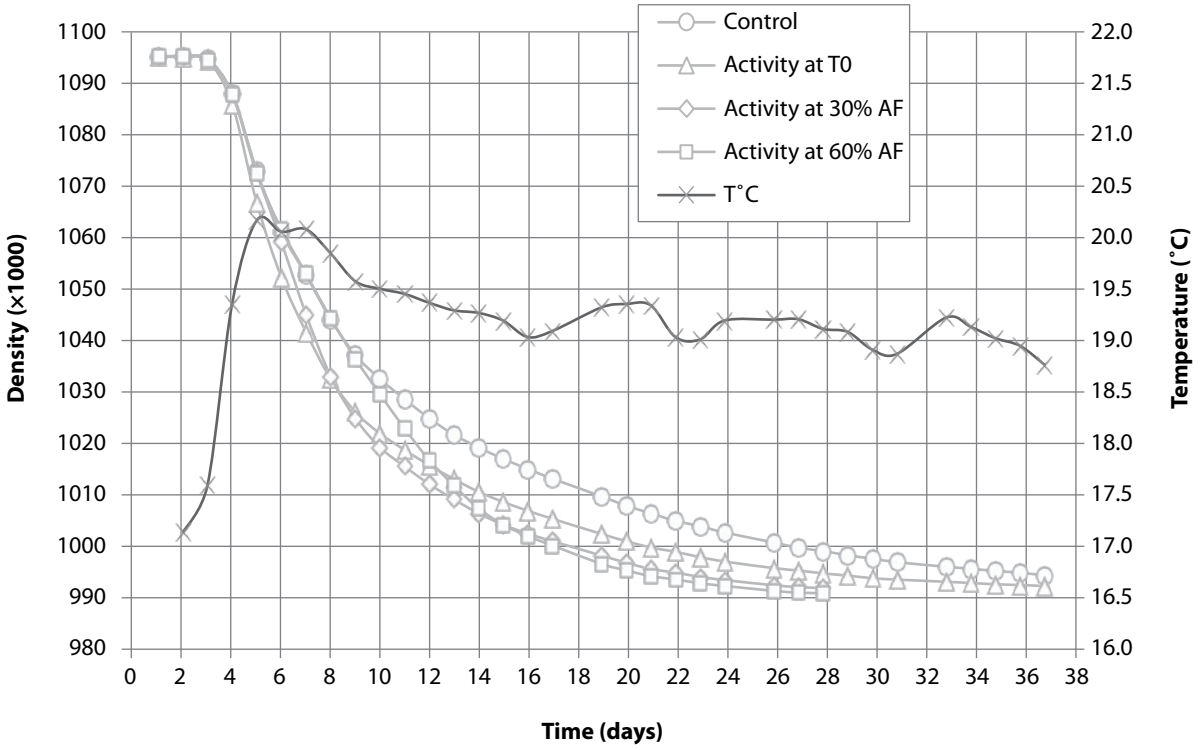



**Figure 4**: Changes in AF based on nitrogen nutrition (average values for two batches of Chardonnay)

**Table 4**: Fermentation balance

| ***Time in days*** | **2015 Burgundy** | | | | **2015 Beaune** | | | |
|---|---|---|---|---|---|---|---|---|
| | **Control** | **AF activator** | | | **Control** | **AF activator** | | |
| | | **T0** | **30% AF** | **60% AF** | | **T0** | **30% AF** | **60% AF** |
| **Repitching** | 45 | | No | | 51 | No | | |
| **Sugar < 2 g/L** | > 120 | > 120 | 38 | 32 | 68 | 51 | 38 | 34 |
| **Residual sugar (g/L)** | 3.3 | 2.3 | 1.6 | 0.7 | 1.8 | 1.8 | 1.5 | 0.8 |

**Table 5**: Investigation of the indigenous lactic bacteria flora in Bourgogne

| **CHARDONNAY** | **B1** | **B2** | **B3** | **B4** | **B5** | **B6** | **B7** | **B8** | **B9** |
|---|---|---|---|---|---|---|---|---|---|
| Stage of MLF when sample was taken | About 100% | | | | | | | | |
| *O. oeni* in 15 isolated clones | 0 | 6 | 15 | 15 | 14 | 6 | 15 | 0 | 1 |
| *P. damnosus* in 15 isolated clones | 15 | 9 | 0 | 0 | 0 | 9 | 0 | 15 | 14 |

| **PINOT NOIR** | **R1** | **R2** | **R3** | **R4** | **R5** | **R6** | | | |
|---|---|---|---|---|---|---|---|---|---|
| Stage of MLF when sample was taken | 70% +/- 30% | | | | | | | | |
| *O. oeni* in 15 isolated clones | 15 | 15 | 15 | 15 | 15 | 15 | | | |
| *Brettanomyces* (100 to 1,000 cells/mL) | No | Yes | Yes | Yes | Yes | No | | | |

**Table 6**: Volatile phenol detection threshold (value in µg/L)

| | **Chardonnay** | | **Pinot Noir** | | **Average threshold** (geometric average) |
|---|---|---|---|---|---|
| | **Unoaked** | **Oaked** | **Unoaked** | **Oaked** | |
| **Detection threshold** | **128** | **241** | **173** | **231** | **187** |

each of the batches. Five of the nine Chardonnay batches contained *Pediococcus damnosus*, which represented between 60 and 100% of the clones isolated (Table 5). All the clones isolated in the Pinot Noir batches were *Oenococcus oeni*. But four out of six batches also contained a population of *Brettanomyces*. These findings highlight the risks inherent to the indigenous lactic bacteria population.

**Determining the threshold for detecting volatile phenols in Burgundy** – The BET (best estimate threshold) method consists of conducting a series of triangle tests (first of three forced-choice tests) while considering a range of growing concentrations of volatile phenols (selected ratio: 2/3 ethylphenol and 1/3 ethylguaiacol). The results show that the threshold for detecting volatile phenols in Chardonnay or Pinot Noir is less than 200 µg/L for an unoaked wine, and just over 200 µg/L for an oaked wine (Table 6). The average threshold for detecting volatile phenols in a Chardonnay and Pinot Noir (oaked or unoaked) is 187 µg/L. These results show that volatile phenols have an impact in concentrations far lower than those currently permitted (approximately 400µg/L). Additional testing (non-reported) showed that the presence of volatile phenols must always be considered as negative for the quality of the wine, even at concentrations close to the detection threshold. Controlling the *Brettanomyces* population is therefore a major oenological challenge.

**Controlling malolactic fermentation to produce wines with aging potential** – Bacterial inoculation reduces the amount of time available for indigenous flora to multiply. Early malolactic fermentation in a Chardonnay prevents the possibility of an oily wine. For red wines, the main goal is to prevent the proliferation of *Brettanomyces* by enabling microbiological stabilization that is adapted to the situation. Sensory analysis demonstrates the qualitative interest of bacterial seeding by comparing late malolactic fermentation with a development of *Brettanomyces* (Figure 5).

Bacterial inoculation is a way of preventing biogenic amines from appearing in finished wines. The indigenous bacterial flora is generally able to degrade certain amino acids, particularly histidine into histamine. Selected bacteria biomasses do not have this aptitude, or at the very least have lost this unstable characteristic over the course of the isolation, selection, and production stages. Even though the impact of biogenic amines on the sensory or health quality of a wine may not be clear, their absence remains a sign of quality.

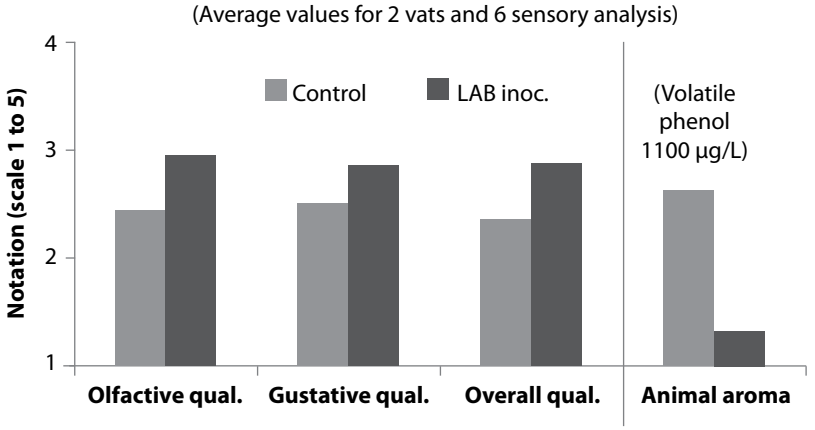



**Figure 5**: Control over malolactic fermentation to prevent volatile phenols

## Conclusions

The terroir (including human intervention) determines the quality of the grape. But this truth does not apply to the microbial flora of the vine, which presents aptitudes ranging from the desirable to the undesirable in winemaking. Biodiversity in nature is highly functional. Oenology isn't nature's problem; it is a human one.

The microorganisms in the must, whether a source of interest or alteration, must be controlled to transform a quality grape into a quality wine. This is one of the major challenges of oenology today.

Selecting yeasts and lactic bacteria and developing specific biomasses makes it possible to achieve this objective, provided the methods are accepted by professionals and put to good oenological use.

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# BIODIVERSITY MEETS TERROIR

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