WIESBADEN, GERMANY
APRIL 11, 2019

# BIOLOGICAL TOOLS IN WINEMAKING ADAPTED TO A CHANGING ENVIRONMENT

THE *XXIX*es *ENTRETIENS SCIENTIFIQUES LALLEMAND*

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PROCEEDINGS
OF

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**BIOLOGICAL TOOLS IN WINEMAKING ADAPTED TO A CHANGING ENVIRONMENT**

# 125th ANNIVERSARY OF THE GEISENHEIM YEAST BREEDING CENTER: LOOKING BACK


## Manfred Grossmann

Department of Microbiology and Biochemistry, Hochschule Geisenheim University, von-Lade-Strasse 1, 65366 Geisenheim, Germany


### The beginning

It all started in 1872 when Eduard von Lade was granted an allowance by Prussian King Wilhelm I to install the *Koenigliche Lehranstalt für Obst und Weinbau zu Geisenheim* with the goal of establishing applied research and study programs in the fields of horticulture and viticulture. Hermann Müller, the grapevine breeder known to this day for introducing the Müller-Thurgau grape, began his career at the Geisenheim Station in 1876 not only as a breeder but also a microbiologist (a lesser known fact about Müller).

Microbes and microbiology became increasingly important in the years that followed, especially after the revolutionary findings of Emil Christian Hansen (brewing) and Louis Pasteur (winemaking). Julius Wortmann continued to build on these developments when he became head of the Plant Physiology Experimental Station in Geisenheim in 1891. Through his research, he identified wine yeasts as the main microbes that determine quality during alcoholic fermentation. This prompted him to start the Geisenheim Yeast Culture and Breeding Station in 1894. From that point on, wine producers had access to pure, well-tested yeast strains they could use to inoculate their grape musts.

Today, the department sees 1894 as the starting point for wine microbiology R&D and instruction at Geisenheim.

### Brief timeline:

***1894: Julius Wortmann.*** The isolation of pure yeast cultures and their practical applications marked a milestone for improving quality in wine production.

***1924:*** The Yeast Station was integrated as part of the Plant Physiological Research Station of the Geisenheim Research Center headed by Karl Kroemer.

***1932:*** The Plant Physiological Research Station was renamed the Botanical Institute and was led by *Hugo Schanderl*. The main research focus at that time was to look at problematic film-forming yeasts and other spoiling yeasts and their interaction with pure yeast cultures. Hugo Schanderl wrote the first textbook on the microbiology of must and wine.

***1966: Helmut Hans Dittrich*** stepped in as head of the department. Research focused on the physio-metabolic activities of microorganisms in must. Priority areas included the fermentation processes and selection of yeasts with low formation of $SO_2$-binding substances as well as investigations into the origins of and potential strategies for avoiding the main aroma off-flavours, such as acetic notes, ester notes, sulfur off-flavours, sweet wine disease, and so on, which can be caused by microorganisms.

***1994: Manfred Grossmann*** took over as head of the Department of Microbiology and Biochemistry**.** The department's research focused on stress research, aroma development and biotechnological implementation of microbial processes in juice, wine and wine-associated products.

***2019: Jürgen Wendland*** is now head of the department.

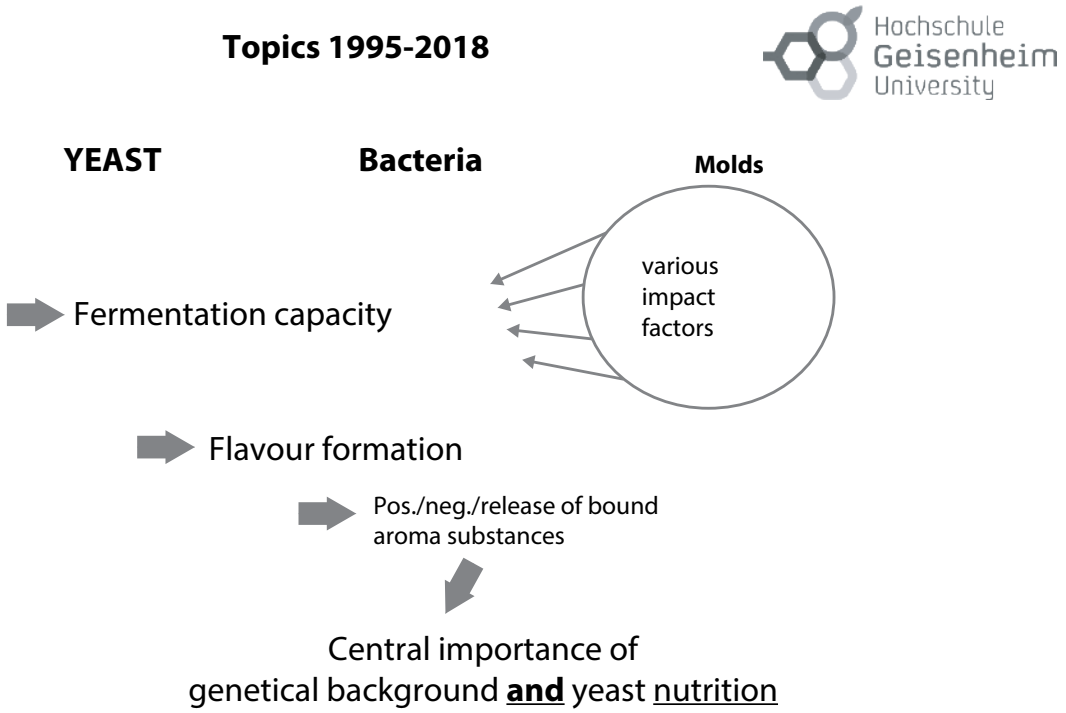


**Figure 1**: Department of Microbiology and Biochemistry: 1995–2018 R&D plan

## From 1995 to 2018

Figure 1 presents a brief summary of the main R&D topics over the last 23 years. The bulk of the department's work involved research on yeasts relevant to winemaking, followed by bacteria (mainly lactic acid bacteria) and, to a lesser extent, molds (mainly the impact of *Botrytis*-infected grapes on yeast activity and flavour formation).

Yeast nutrition was identified as a very important key for regular fermentations and the formation of pleasing fermentation flavours. Thanks to the findings of the Geisenheim Center, which were also backed by the results French and Austrian researchers, the European wine act allowed

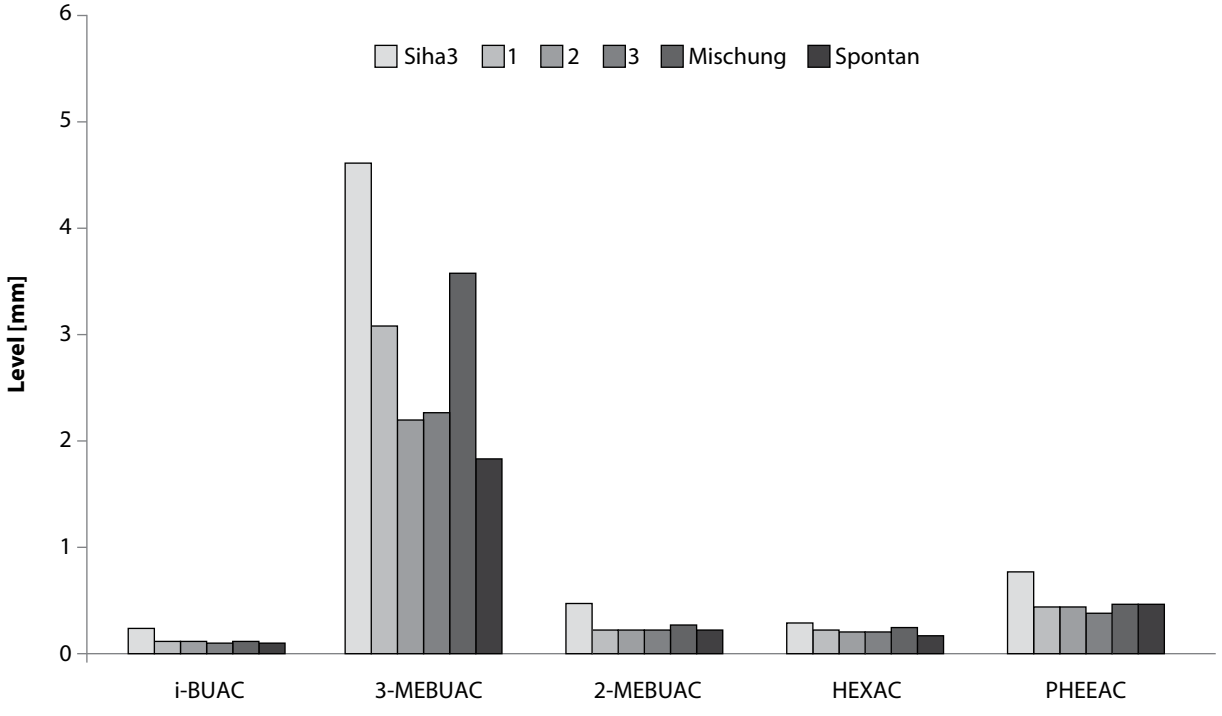


**Figure 2:** Comparison of ester contents of single strains versus mixed culture (Mischung)

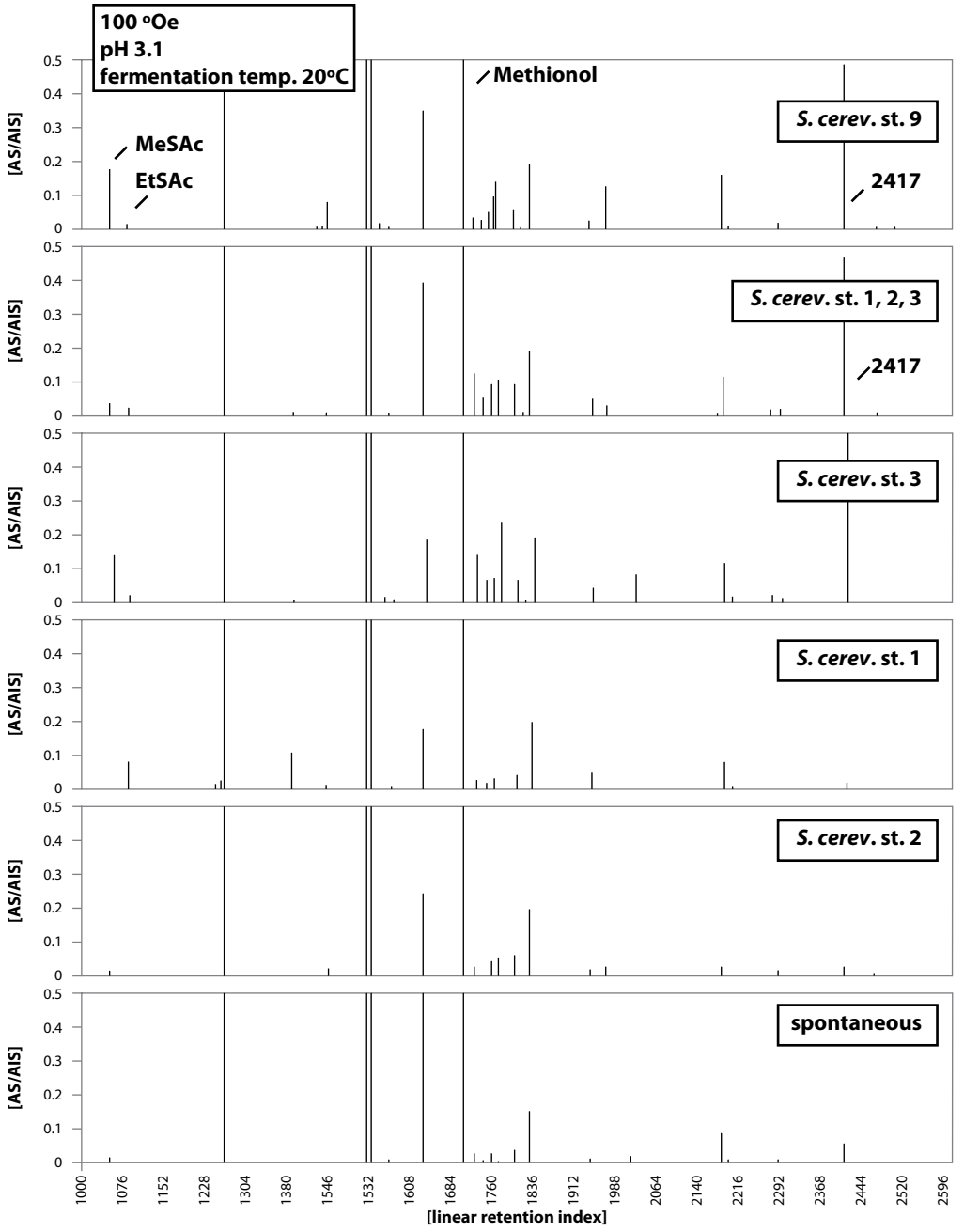


**Figure 3:** Formation of sulfur-containing esters by single strains vs. their use in mixed culture

the addition of 100 g/hL of fermentation salts (diammonium-phosphate (DAP) and ammonium-sulfate) in 2003. Prior to that, the limit had been set at only 30 g/hL. The increase in nitrogen concentration also helped considerably in the prevention of sulfur off-flavours, also known as reductive flavours.

The Geisenheim department also initiated the use of mixed yeast cultures on a commercial scale. The first step in the process was to closely examine mixtures of *Saccharomyces cerevisiae* strains. Although the activity of mixed cultures is closer to the conditions in spontaneous fermentations, it turned out that unwanted effects can occur if strains come together that do not act synergistically. Proper and intensive testing of their behaviour is absolutely necessary before they can be used at the commercial scale. Knowing the properties of each yeast strain used within such a mixture provides no clue as to what the outcome of such a mixed fermentation will be. Sought-after flavour compounds could wind up being less formed in a mixed culture than in single strains. Figure 2 gives an example comparing the flavour profiles of single strains versus their use as a mixed culture.

However, one important outcome of our mixed culture studies points to the possibility of even using strains that

might have an attractive fermentation flavour but also a tendency to produce reductive flavours under certain conditions. Normally such behaviour would preclude the use of this yeast as a single strain culture. However, such a strain can be combined with another strain able to metabolise sulfur compounds. Such mixtures make it possible to use partially problematic strains by including a "helper/rescue" strain. This supports the idea of using mixed yeast cultures, and can also be extended to mixtures of *Saccharomyces* with non-*Saccharomyces* yeast strains.

Figure 3 demonstrates the decrease in unwanted sulfur compounds by a helper strain.

The use of gene technology/genetic engineering is a hot topic among wine consumers. This is fueled by consumer apprehensions about genetic engineering in food production in general and concerns about a loss of tradition and culture in winemaking.

To learn more about the possible effects of genetically modified yeasts, the German Federal Ministry of Nutrition, Agriculture and Consumer Protection (BMELV) financed a comprehensive study covering all stages of the winemaking process, from vineyard to bottle. The results showed that for both genetically modified and commercial yeast strains, once they are used and brought into the environment, they inevitably become part of the respective microbiota. Release of yeasts always occurs when yeast lees are used as fertilisers in the vineyards. Another release occurs when tanks, barrels and winery equipment are cleaned and yeasts leave the winery with the waste water. Another finding: yeasts are not killed during sewage treatment!

In conclusion, it was shown that yeasts used in a winery are always released into the environment, regardless of whether they are spontaneous yeasts, commercial non-engineered or genetically engineered yeasts strains.

With the steady increase in commercially available yeast strains from various yeast suppliers, it is increasingly difficult for wine producers to decide which product is best for them. Decisions also have to factor in a variety of specific conditions, including grape variety, health of grapes, stress conditions during grape ripening, cellar equipment (e.g., cooling facilities), and, last but not least, consumer expectations regarding the varietal character or fruitiness/freshness of the wine.

To address this, the Geisenheim Yeast Finder (*Geisenheimer Hefefinder*) was developed with the help of software specialists from the Wiesbaden University of Applied Sciences. This open-access platform allows winemakers to see a ranking of the best-suited commercial wine yeast strains for them based on their answers to a 17-question survey. All data on the yeast properties is delivered by the yeast manufacturers.

Figure 4 shows an example of the computer display.

## 1995 to 2018: Continuity

"It's all about FLAVOUR!" Wine consumers decide whether or not they like a given wine in a matter of seconds—and the decision largely hinges on flavour and taste. That is why it is so important to know as much as possible about the flavour compounds produced either by the grapes themselves or by yeasts during fermentation. Lactic acid bacteria also play an important role in flavour.

Figure 5 presents the different flavour R&D clusters investigated by the department.

In addition to the flavour clusters, the department's other research areas are summarized in Figure 6.

**Dazu empfehlen wir Ihnen folgende Hefe**

| Rank % | Bezeichnung | Lieferant | |
|---|---|---|---|
| 100 | Uvaferm CGC 62 | Lallemand/Begerow | Datenblatt |
| 100 | Lalvin W | Lallemand/Begerow | Datenblatt |
| 100 | Uvaferm CS2 | Lallemand/Begerow | Datenblatt |
| 95.8 | Uvaferm 228 | Lallemand/Begerow | Datenblatt |
| 91.6 | Lalvin R2 | Lallemand/Begerow | Datenblatt |
| 91.6 | Enoferm T306 | Lallemand/Begerow | Datenblatt |
| 91.6 | SIHA Cryarome | Begerow | Datenblatt |
| 91.6 | Fermicru VB1 | DSM Food Specialties | Datenblatt |
| 91.6 | Uvaferm SLO | Lallemand/Begerow | Datenblatt |
| 91.6 | Uvaferm CEG | Lallemand/Begerow | Datenblatt |
| 91.6 | Lalvin V | Lallemand/Begerow | Datenblatt |
| 91.6 | Uvaferm PM | Lallemand/Begerow | Datenblatt |
| 91.6 | VIN 13 | Anchor Yeast, Südafrika / C. Schliessmann GmbH & Co. KG; Auwiesenstr. 5; 74523 Schwäbisch Hall | Datenblatt |
| 91.6 | Oenoferm Tipico | Erbslöh Geisenheim Getränketechnologie; D-65366 Geisenheim, Erbslöhstr. 1 | Datenblatt |
| 91.6 | Uvaferm CM | Lallemand/Begerow | Datenblatt |
| 91.6 | Oenoferm Freddo | Erbslöh Geisenheim Getränketechnologie; D-65366 Geisenheim, Erbslöhstr. 1 | Datenblatt |

**Figure 4:** Example of the parameters factored into a ranking of suitable yeast strains

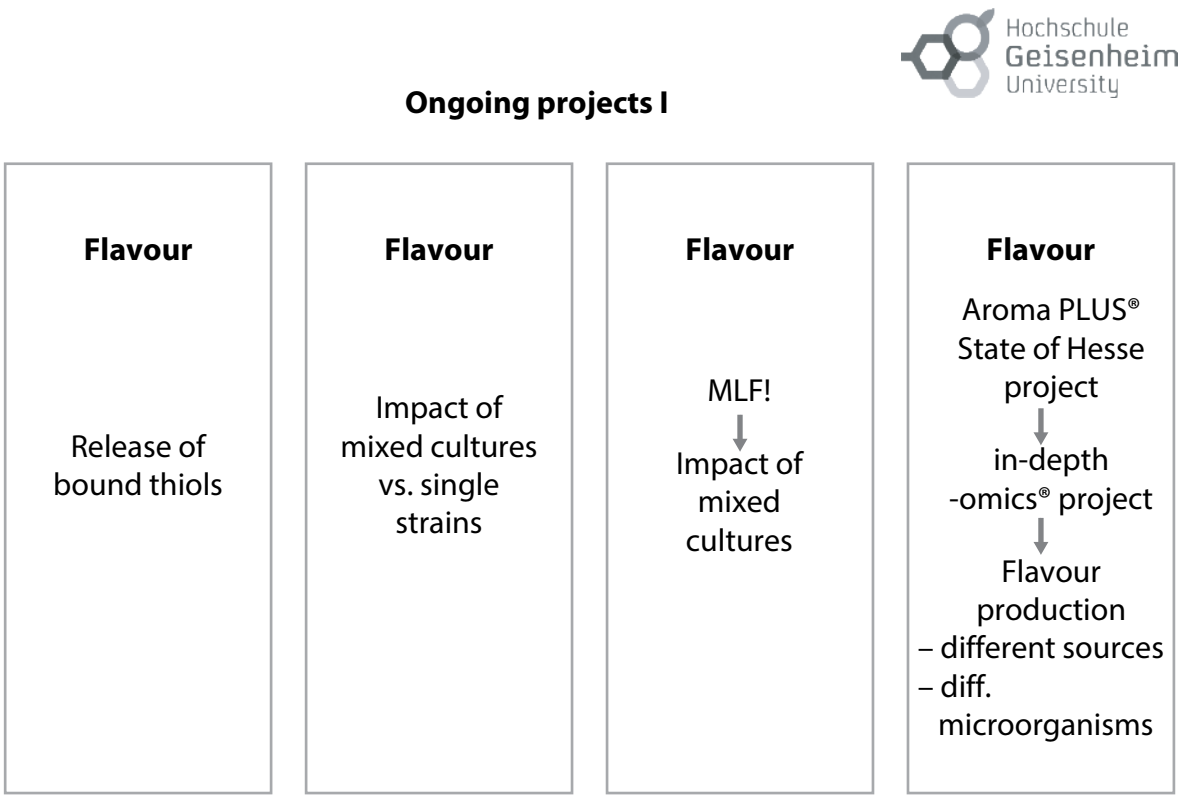


**Figure 5:** Flavour R&D clusters of the department

All in all, a lot of uncharted territory still remains, and the Geisenheim Department of Microbiology and Biochemistry will stay very busy seeking more insights into the microbially impacted steps of the winemaking process.

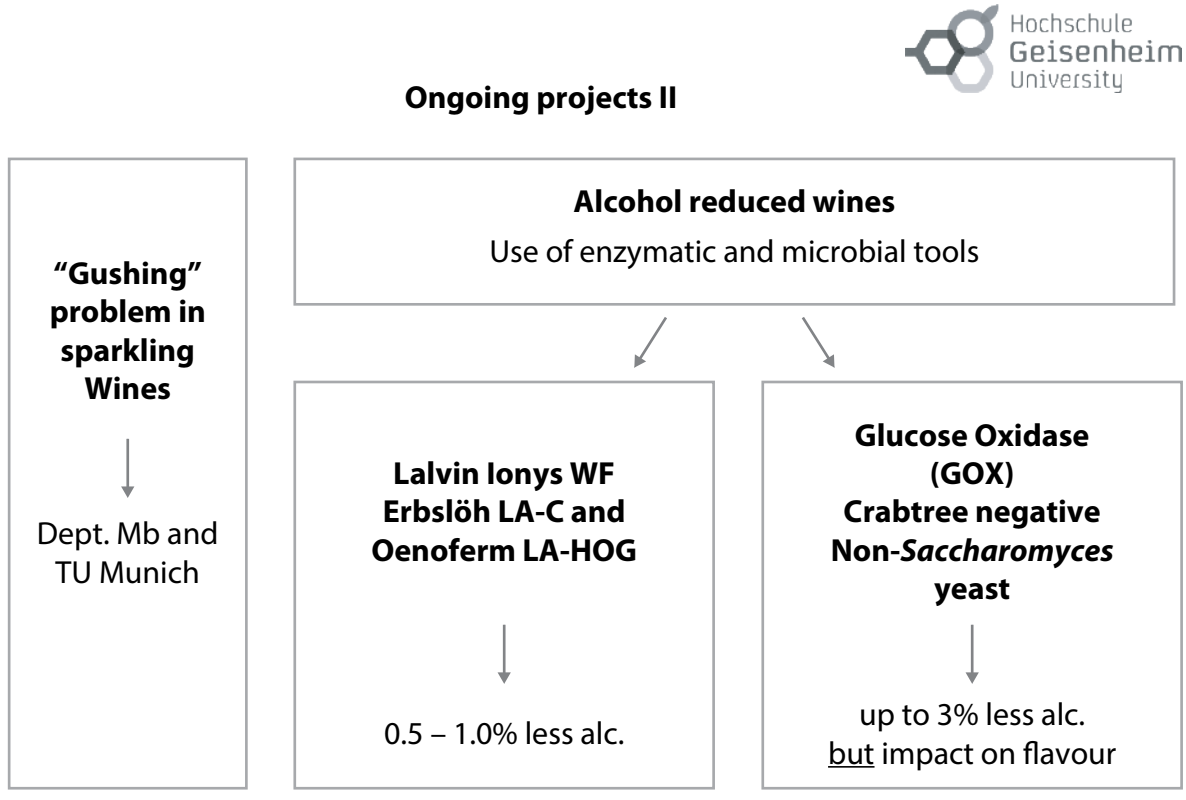


**Figure 6:** Working clusters: gushing and reduced alcohol wines

# 125th ANNIVERSARY OF THE GEISENHEIM YEAST BREEDING CENTER


## Jürgen Wendland

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**Abstract:**

Yeast has been used in fermentations for millennia. Yet, it was not until 1883, with the work of Emil Chr. Hansen at the Carlsberg Laboratory in Copenhagen, that pure culture yeast strains were introduced in the beverage industry. The use of pure cultures for fermentation then spread to the distillery and dairy industries and, with the work of Julius Wortmann, to winemaking. As a result of these developments, in 1894 the Geisenheim Yeast Breeding Center was founded and headed up by Dr. Wortmann. Since then a diverse set of yeast strains has been used in wine fermentations. More dedicated strains for specific types of fermentation are now available; however, more could still be done today to generate improved, targeted strains using modern non-GMO techniques. With the 125th anniversary of the Geisenheim Yeast Breeding Center, efforts are stepping up to study better yeast strains for alcoholic beverage fermentations. Here, we present a short historic overview of and the strategies and targets for improving yeast strains for both lager and wine yeasts.

Besides *Saccharomyces cerevisiae,* the biodiversity of yeast, which encompasses thousands of ascomycetous yeasts, holds a cornucopia of untapped resources that may be utilized in co-fermentations, e.g., with the aim of reducing the alcohol content in fermented beverages. The use of non-conventional yeasts may also generate more complex and flavour-rich products or, as is shown for yeasts belonging to the genus *Saccharomycopsis,* that may be utilized as biocontrol agents against fungal plant pathogens.


## 1. Introduction

Fermented beverages have been used in human civilizations for millennia. Still today, in some places fermented beverages often constitute the only source of a clean drinkable liquid (i.e., free of fecal bacteria) with nourishing value (Wendland, 2014). Fermentation is a way to preserve food and beverages, much in the same way that methods such as pickling, heating, drying or smoking and salting of food (Jans et al., 2016) are used. Pasteur's discovery of yeast's microbial contribution to the beer fermentation process did not immediately change the general process of repitching, i.e., the reuse of slurries from previous fermentations to start the next ones (Pasteur, 1876). The game changer was when Emil Chr. Hansen isolated pure cultures of *Saccharomyces* and it was demonstrated that a single strain could be used to ferment wort into an acceptable product (Hansen, 1883). This breakthrough opened the door to industrialized fermentation processes and led to a huge improvement in quality. From then on fermentation could be monitored either microscopically to detect bacterial contaminations or chemically by measuring the pH, given that a lower pH would also indicate souring of the beer by bacterial contamination (Sörensen, 1909; Meyers, 2010).

The breakthrough research conducted by Hansen (and later Sörensen) at the Carlsberg Laboratory was immediately adapted at other laboratories and in the industry at large. The use of pure cultures then started to replace previous processes largely based on spontaneous fermentations because they were often quite unpredictable and resulted in inferior beverages. In Germany, Julius Wortmann propa-

gated the use of pure cultures for wine fermentation. He published a book summarizing the application and properties of pure yeasts in winemaking (Wortmann, 1895). There he described analyses of different yeasts other than *Saccharomyces* that can be identified in spontaneously fermenting musts, including *Mucor*, *Torulaspora* and *Hanseniaspora*. His studies determined that (i) different yeast isolates fermented the same must in different ways, particularly with regard to final alcohol content, (ii) the amount of glycerol produced by various yeast strains differed significantly and (iii) the generation of aroma compounds was also dependent on the yeast strain.

The Geisenheim Yeast Breeding Center was founded in 1894 to promote the efforts of using pure yeast cultures. This made it possible to collect and characterize hundreds of yeast strains that were then made available to winemakers. A particular problem regarding yeast supplies for the wine industry emerged. Beer can be made all year long, making it more flexible and less dependent on harvesting of raw materials. In contrast, winemaking is fully dependent on the seasonal availability of grapes and must be undertaken immediately after harvest. Moreover, in winemaking the need for fresh yeast starter cultures is concentrated around harvest time, putting pressure on yeast suppliers to obtain sufficient amounts of fresh yeast in liquid culture. It was not until the 20th century that active dry yeast (Fleischmann's, during World War II) and instant dry yeast (Lesaffre in 1973) formulations were developed. This has helped provide yeast starters with prolonged shelf live.

The identification of yeasts for specific purposes has led to a large variety of yeasts that are commercially available for the various fermentation industries.

## 2. The awesome power of yeast genetics

The next huge advance in yeast research was brought about by one of Hansen's successors: Ojvind Winge. He and his coworkers determined the sexual cycle of *Saccharomyces* (Winge, 1935; Winge and Laustsen, 1937). This made it possible to combine the favourable traits of two strains into one novel yeast strain and helped unlock the awesome power of yeast genetics. *Saccharomyces cerevisiae* then became the model eukaryotic organism due to the availability of stable haploid and diploid cell lines, the ease of growing and mating yeasts (and performing tetrad analysis) and the ability to conduct facile genetic manipulation (e.g., using PCR-based gene targeting tools).

Mapping of the yeast genome sequence opened the door to the study of fermentation-related traits first at the gene level, then at the genome and transcriptome levels (Goffeau et al., 1996; Velculescu et al., 1997). Lager yeast genomics took another ten years to develop, thanks mainly to the availability of cheaper sequencing methods (Nakao et al., 2009; Walther et al., 2014). By pairing yeast genomics with targeted yeast breeding, it is now possible to combine molecular markers and traits of different yeast strains in targeted strain improvement efforts.

It is important to note that specialty yeasts are used in brewing different styles of beer. For example, ale and stout beers are brewed with ale yeasts that are *S. cerevisiae* strains. In contrast, lager beer is fermented by lager yeasts. Lager yeasts are triploid (group I) or tetraploid (group II) hybrids between *S. cerevisiae* and *S. eubayanus*. All lager yeasts share a common origin, i.e., they are descended from an original Ur-hybrid strain. Subsequent evolution and re-hybridization events led to a variety of strains with regional profiles (Walther et al., 2014; Wendland, 2014; Okuno et al., 2016).

While lager yeasts can be viewed as domesticated strains, wine yeasts bear more similarity with wild *S. cerevisiae* strains. But even in wine yeasts, hybrids have been detected, e.g., between *S. cerevisiae* and *S. kudriavzevii* (Peris et al., 2016).

## 3. Targets of yeast strain improvement

Molecular yeast breeding as a tool to improve pure culture yeast strains by marker-assisted breeding is becoming more popular given the ease with which large genomic datasets—even on yeast populations—can be obtained (Peter and Schacherer et al., 2016). However, methods for manipulating yeast strains are split along two rather dogmatic lines: the first, which is based on consumer preferences and embraced by the food and beverage industry, accepts only non-GMO yeast strains for the production of consumer goods, while the other promotes the use of modern genetic engineering tools to improve yeasts more rapidly (Jagtap et al., 2017; Pretorius, 2017).

In either school, several opportunities could be explored to generate improved yeast strains for use in different fermentation regimes.

The first of these is to look at (i) strain variability. Here it is important to analyze the stability of a pure culture yeast strain and potentially, via several rounds of selection, pick the best in class. This can be combined by assaying the within-progeny variability, which can be enhanced by intermittent sexual production. Synthetic biology tools may actually facilitate the analysis of hundreds of progeny (Ravasio et al., 2014). Second (ii), yeast breeding

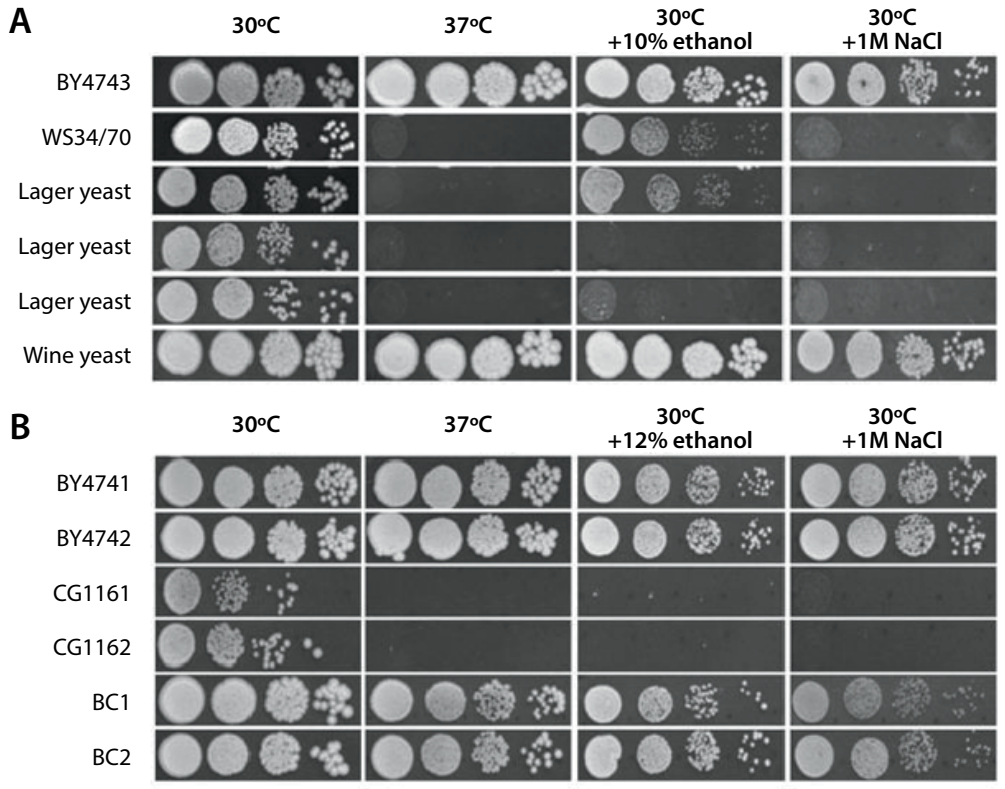


**Figure 1.** Lager yeasts are more sensitive to high-temperature and high-salt stress than *S. cerevisiae* strains. Yeast strains were grown overnight in YPD with 2% glucose, washed with water and adjusted to an OD600nm = 0.08, followed by 1:10 serial dilutions that were spotted on YPD plates supplemented with 10–12% ethanol or 1 M NaCl where indicated. Plates were incubated at either 30°C or 37°C (A, B). Representative pictures were taken after 5 days of growth. *S. cerevisiae* strains: BY laboratory yeast strains and a wine yeast strain. Lager yeast strains are commonly referred to as *S. carlsbergensis*. CG strains are lager yeast spore clones and BC strains are derived from crosses of BY and CG strains

can generate novel hybrids that show improved characteristics (hybrid vigor) compared to their parental strains (Shapira et al., 2014). This has been used, for example, to improve the stress resistance of lager yeast by breeding with *S. cerevisiae* (Figure 1 taken from Garcia Sanchez et al., 2012, with the publisher's permission). Additionally, meiotic recombination may eliminate aging phenotypes in yeast cells (Unal et al., 2011). The third (iii) involves generating strains that produce larger amounts of volatile aroma compounds, e.g., by upregulating the Ehrlich pathway (Hazelwood et al., 2008).

In Figure 1 we show that lager yeast strains do not grow at elevated temperatures and are sensitive to high-salt conditions. These are traits found in all lager yeasts in addition to phenotypic traits that underline a common evolutionary origin. Interestingly, by breeding lager yeast spore clones (CG1161 and CG1162) with haploid laboratory *S. cerevisiae* strains (BY4741 and BY4742), new hybrids were obtained that inherited the temperature and high-salt resistance of the *S. cerevisiae* parental strains, indicating that simple yeast breeding displays great improvements as early as the F1-generation.

## 4. Use of non-conventional yeasts to improve fermented beverages

Additionally, fermentation outcomes may be positively influenced to generate more complex aroma profiles using multiple yeast strains. These may generally include a *Saccharomyces* strain to promote rapid fermentation to dryness as well as non-conventional yeast strains (Varela et al., 2016; Canonico et al., 2017; Holt et al., 2018; Ravasio et al., 2018; Morales et al; 2019).

Several non-conventional yeast strains have been tested in co-fermentations. However, there is no clear picture emerging on how to use these strains. Co-fermentations could be generated by (i) using each yeast strain separately and at some point combining the different liquids and end-fermenting the mixture with *S. cerevisiae*; (ii) inoculating the non-conventional yeast strain(s) first and only later adding *S. cerevisiae* to finish the fermentation or (iii) co-inoculating all strains with equal amounts or in different ratios in which the cell numbers of the individual strains used may vary by several orders of magnitude (see for example Morales et al., 2019).

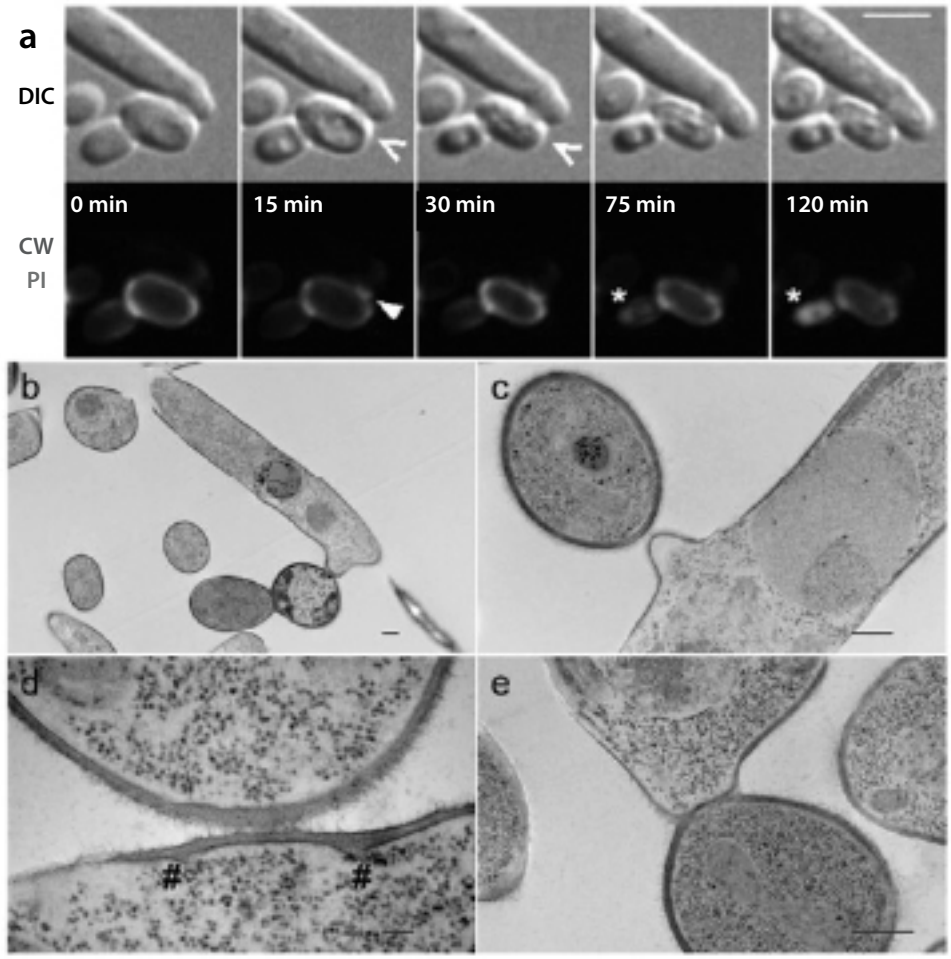


**Figure 2.** *S. schoenii* attacks and kills *C. auris.* (a) *S. schoenii* and *C. auris* NCPF8985#20 stained with Calcofour White (CW, cyan, bottom panel), a fluorescent dye that stains chitin-rich cell walls and septa, and propidium iodide (PI, red, bottom panel), a fluorescent dye that stains nucleic acids of cells with a compromised cell membrane, i.e., dead or dying cells. We captured images twice per minute for two hours and found that at 15 min., a penetration peg [Δ] from *S. schoenii* was visible by CW. The *C. auris* prey cell subsequently collapsed in size at between 15 min. and 30 min. (Λ). While the attacked *C. auris* cell was not stained by PI, its daughter cell accumulated PI at between 75 min. and 120 min. (*). (b–e) TEM images of *S. schoenii* and *C. auris* that had been co-cultured for 1 hr. Scale bar 500 nm in (b,c and e). Scale bar 100 nm in (d). (b) A dimorphic *S. schoenii* cell formed a penetration peg to contact, attack and kill an ovoid *C. auris* cell. (c) A *S. schoenii* cell with a penetration peg protruding towards a prey cell. (d) Early interactions between *S. schoenii* and *C. auris* visualize potential penetration peg start sites (#). fe) Partial disintegration of the *C. auris* cell wall.

Among non-conventional yeasts, *Saccharomycopsis* species have been found to promote fermentations of a variety of foods and beverages, and one strain in particular, *S. fibuligera*, has been employed in alcoholic fermentations in Asia (Lee et al., 2018).

## 5. Biocontrol with *Saccharomycopsis* predator yeasts

Another rather unique feature of the *Saccharomycopsis* species is its biocontrol ability. When starved, *S. schoenii* cells can change their life style and exhibit necrotrophic mycoparasitism. By generating penetration pegs, these predator yeasts attack, penetrate and kill their fungal prey cells (Figure 2, reprinted from Junker et al., 2018). These yeasts and their predatory behaviour have been described only recently (Lachance and Pang; 1997). The host range is apparently rather wide, i.e., the predator yeasts are able to attack both filamentous and yeast-like ascomycetes. Recently we have shown that they are able to kill several members of human fungal pathogens belonging to the *Candida* clade including *Candida auris* (Junker et al., 2018). This makes them ideal biocontrol strains that could provide us with a new weapon against a variety of fungal infections, e.g., vineyard pathogens. Very recently, we examined the predation mechanisms using multi-omics technologies and have started to decipher the genetic background of predation (Junker et al., 2019).

After 125 years of research, the Geisenheim Yeast Breeding Center (which is part of the Department of Microbiology and Biochemistry) is now entering a new era. Three

major areas of research are slated for further development: First, Molecular Yeast Breeding of wine and beer yeasts to obtain superior yeast strains that combine favourable traits; second, the use of Non-Conventional Yeasts, i.e., non-*cerevisiae* yeasts in fermentations to introduce diversity and flavour complexity and, finally, the use of *Saccharomycopsis* yeasts in biocontrol applications as well as in fundamental research to investigate the molecular mechanisms required for predation. This promises to be an exciting journey and the basis of many international collaborations.

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# COMPARING EFFICIENCY STRATEGIES IN YEASTS WITH ECONOMICALLY SUCCESSFUL WINERIES

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## Abstract

In order for wine yeasts to be able to adapt and survive under harsh conditions, especially during alcoholic fermentation, they need cellular regulation systems that can respond quickly and efficiently to outside factors. Similarly, the success and survival of winemakers and wine retailers also hinge on their ability to rapidly adapt to changing economic conditions. Comparisons of both these systems (e.g., enzyme presence and activity in yeasts vs. workforce activity in a winery) reveal interesting similarities.

In living cells, enzymes are the "workers," and their presence and activity must be regulated so that they can help the cells survive, even under difficult outside conditions. By the same token, any winery that wants to be successful and able to weather difficult economic conditions also needs efficient, cost-effective workforce management.

When looking at different employment systems in wineries and comparing them to cellular enzymes, certain similarities clearly emerge. Dedicated, permanent employees can be found in core units of wine production and trade. The direct parallel to this in yeast cells is enzymes, because they are permanently required to produce energy or ensure cell wall integrity. And while some needs are continuous, others are periodic and only require specific workers (or enzymes) for specific activities at specific times (e.g., grape pickers at harvest time and enzymes needed for chaptalized grape musts).

This paper will draw parallels between enzyme regulation systems and different business employment systems in terms of numerical flexibility, functional flexibility or temporal flexibility.

## 1. Introduction

Henry Ford is often brought up in discussions about successful business strategies. Although he did not invent assembly line production of goods and automobiles as such, Ford initiated mass production by introducing sequential improvements to various aspects of car making and workline assemblies for high through-put production of automobiles. His significant impact was not restricted to car production, but also extended to the life, work and wages of his workers ("Fordism") in the 1920s.

By comparison, the evolution of life started millions of years ago with single-cell organisms, which are now viewed as "simple" when compared to the complexity of multicellular organisms. Nevertheless their metabolism was clearly efficient enough to allow their survival and development. *Saccharomyces cerevisiae* yeasts were among the earliest microorganisms at the start of evolution at the eukaryotic level and can therefore be used as model organisms when looking at the development of yeast physiology and regulation of metabolic activities. We will compare the efficiency strategies that have evolved in living organisms with manmade forms of work and business organization.

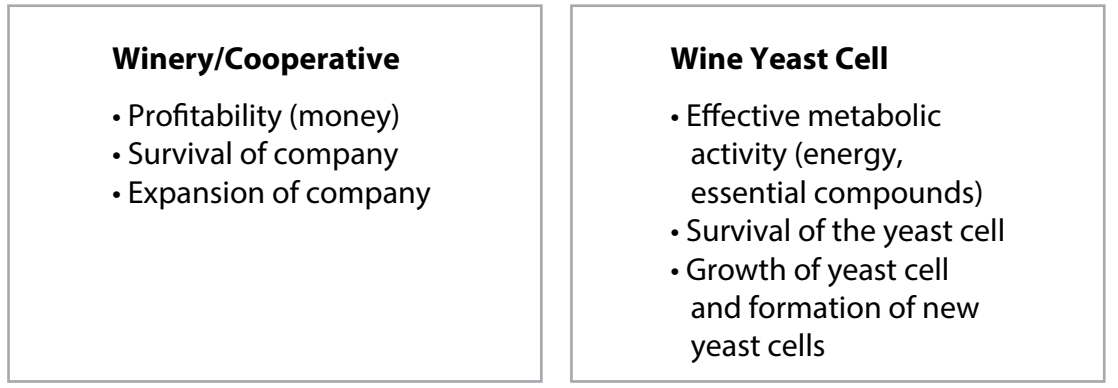


**Figure 1.** Similarities between the aims of wineries and yeast cells

## 2. Comparison of yeast regulation vs. commercial-scale production

### 2.1 General remarks

Biological systems and businesses have similar aims in terms of growth and expansion and the need to achieve them (Figure 1).

Successful wineries or cooperatives follow business plans with common components, such as SWOT analyses, financial planning, product descriptions, and production or marketing plans, to name a few. One question that is relevant to both systems is this: What is the purpose of regulation systems and what do they look like? This research seeks to determine whether there really are similarities between the two or whether they follow completely different strategies.

### 2.2. General workforce comparisons

Yeasts are organized so that metabolic reactions can be grouped into several units (e.g., carbohydrate metabolism, energy metabolism or lipid metabolism). In total, hundreds of biochemical reactions are running at the same time. For the organisms to evolve, there must be a way to guarantee that only those reactions that are absolutely necessary at a given time are running and, more importantly, that energy is not wasted. All of these reactions are managed by a huge number of different enzymes, which can be viewed as the workforce within a yeast cell. In a business context, employees/workers are the counterpart of enzymes.

Human workers demonstrate multi-tasking properties, whereas enzymes, as "biocatalysts," normally focus on just one substrate which is converted in one specific reaction into a product. At first glance, it may seem like "multitasking" has an advantage over "singletasking." However, the success of Henry Ford's automotive assembly line clearly illustrates the advantages of singletasking. A million-year-old biological strategy is thus mirrored in the manmade economy.

### 2.3 Quantity and quality of workforce

Figures 2A and 2B clearly indicate the overall similarities in terms of the different production steps and workforce (human or enzyme) needs. In both cases, productivity is determined by the quantity and quality of the workforce.

Wineries and yeast cells both operate in changing environments. Wineries have to contend with both high and low quality vintages, high and low demand for wine, and consumer preferences for new wine styles. Flexibility is therefore essential for wineries to adapt to changing conditions.

Yeasts are also confronted with changes in their environments. These changes occur while the yeasts are living in the grapes on the vine, but are even more pronounced and quicker to occur during alcoholic fermentation. During fermentation, the lack of oxygen, increased ethanol concentration and decrease in available nutrients are predominant factors.

Thus, the ability to adapt to changes is of the utmost importance in both systems.

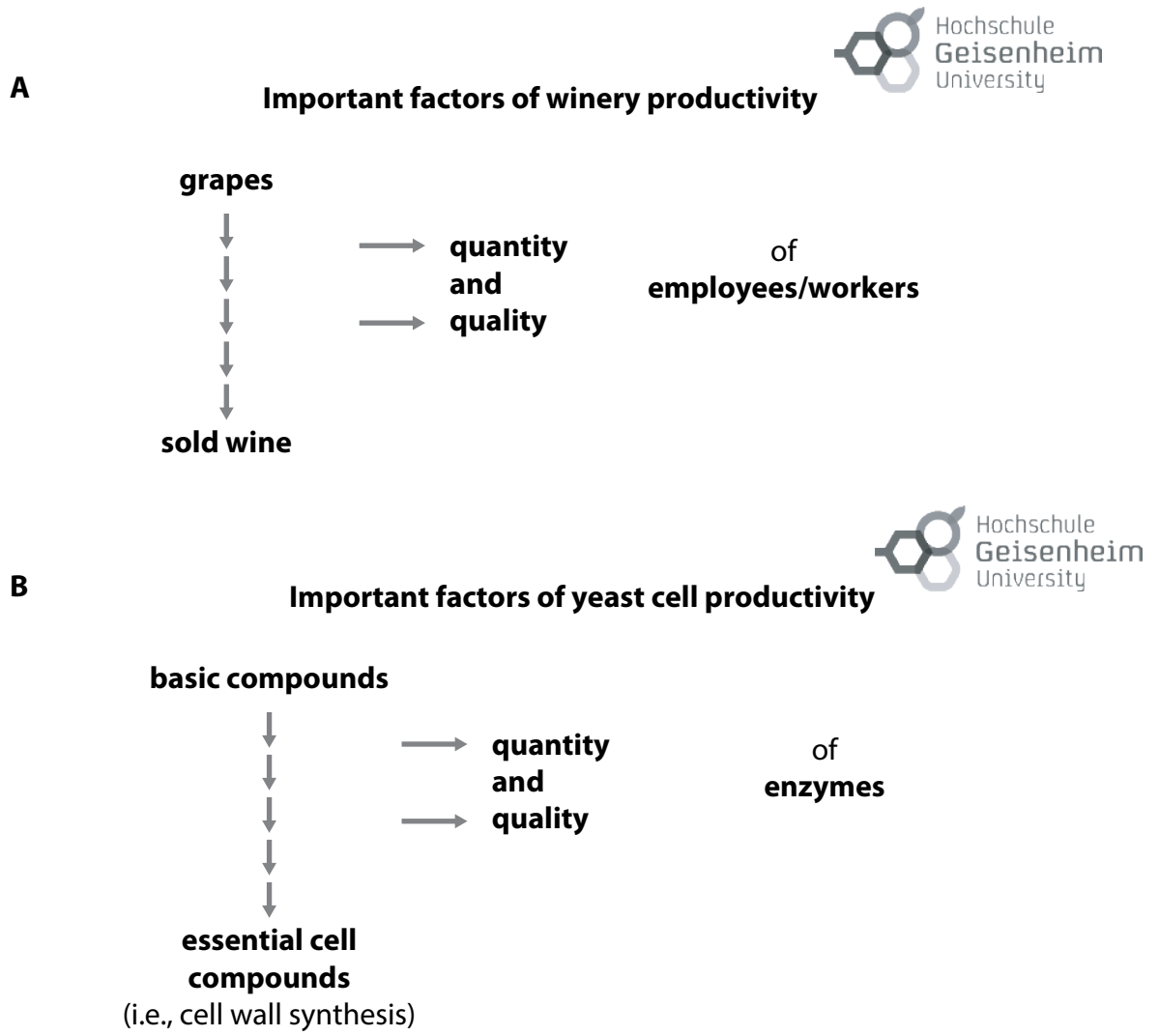


**Figure 2A and 2B.** Demonstration of quantity and quality of workforce

### 2.4 Comparisons of regulation strategies in businesses and in yeasts

As discussed in Section 2.2, workers at a winery and enzymes in yeasts display the following interesting similarities.

#### Working time

Since productivity gradually declines as workers get tired, the economical solution is to implement **shiftwork**. During harvest season it is often necessary to keep the presses running day and night. The only way to do this is to have employees work in shifts (day, evening, night). In yeasts, to avoid decreasing biochemical activity, enzymes are often replaced by new ones with the same specificity every 20 to 30 minutes.

#### Assembly-line work

The best way to achieve high productivity, whether in yeast cells or in wineries (e.g., bottling), is to assign specific "workers" to perform specific tasks.

#### Permanent workforce

No matter what the outside circumstances, it is always critical to have a dedicated workforce. In yeasts, examples of this include the enzymes needed for cell wall or membrane synthesis. In wineries this could be anyone from winemakers to salespeople and administrative staff. So, the similarities are clear.

#### Possibilities for workforce on demand

Biosynthesis of enzymes involves a lot of other enzymes as well as energy, which has to be generated by breaking down energy-rich compounds like glucose or fructose

(ATP). Given these prerequisites, only those enzymes that are truly needed should be synthesized. The same holds true for businesses, which assign only those workers who are truly needed for the job in order to keep costs in check.

In response to outside conditions, yeasts and wineries have to be flexible enough to adapt their workforce (enzymes/workers) to the tasks at hand.

- **Induction of enzyme synthesis vs. hiring of new personnel**

Synthesis of amino acids is a very important part of metabolic activity in yeast since they are the basic building blocks for all enzymes. If there is a shortage of amino acids in a given must, then synthesis of relevant amino acids is triggered via transcription of relevant DNA sequences and translation into respective enzymes. By the same token, when a special workforce is needed at a winery, the appropriate personnel are brought in.

- **Repression of enzyme synthesis vs. hiring freeze**

Once yeast cells recognize that the amount of required compounds is sufficient, translation of enzyme-determining DNA sequences is halted to avoid creating a surplus of enzymes that are no longer needed. Businesses stop hiring workers as soon as the existing number of workers can handle the workload.

- **Activation of "dormant" enzymes (pro-enzymes) vs. reinstatement of former employees**

Some enzymes are produced in an inactive form. They are only activated if a special activator molecule is present, thereby allowing the requested biochemical reaction to begin. Similar scenarios can play out in wineries, for example when former employees are reinstated to help out during harvest when more grape pickers are needed.

- **Transient inactivation of enzymes vs. V-time schedules**

In yeast cells, biosynthesis of amino acids is thoroughly regulated, as different amino acids are needed in very different amounts at different stages of cell growth and the cell cycle. To achieve fast regulation of enzymatic activities, the relevant amino acid can act as a repressor molecule as soon as its concentration is sufficient. Once the concentration decreases, the repressor molecule is released and enzymatic activity is restored once again. This type of feedback inhibition enables a very specific and fast adaption to the situation of the 20 different amino acids needed for protein synthesis.

In business, a voluntary reduced worktime (V-time) schedule is an arrangement between the company and employees giving employees the flexibility to work a non-standard schedule (between full- and part-time) based on the needs of the company and the employee. Wineries often use this kind of flexible arrangement to offset seasonal shifts in work demand.

- **Active breakdown of existing enzymes vs. dismissal of employees**

When enzymes are either no longer needed or at the end of their life cycle, they are actively broken down into their basic compounds (amino acids) by proteases and peptidases. As such, these compounds become available for the biosynthesis of new enzymes, thereby avoiding the energy cost of synthesizing new amino acids.

When a company's financial situation deteriorates over an extended period of time, employee layoffs becomes unavoidable. In both systems, workforce inactivation is a strategic tool used to adapt to difficult situations.

## 3. Conclusion

In business, companies need economical strategies to grow and/or survive difficult situations. How these strategies have developed into various employment systems can be traced back to the Industrial Revolution. Development and survival are just as critical for all living organisms, right down to the "simplest" form of eukaryotic cells, yeasts. The fact that they have been in existence for millions of years and have a metabolism with various catabolic and anabolic pathways and hundreds of enzymatically catalysed reactions clearly demonstrates the efficiency of the regulation methods at play. The quantity and quality of workers in a winery and enzymes in a yeast cell are managed in very similar manners. It might be worthwhile to delve deeper into (micro)biological regulation systems and compare these with economic systems. The findings could prove interesting and surprising. In our next study, we will look at "border control," by comparing import/export systems in wineries with uptake/secretion systems in yeasts.

# ENHANCING PHENOLIC MATURITY OF SYRAH WITH THE APPLICATION OF A NEW FOLIAR SPRAY

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Short version of the title: Enhancing Phenolic Maturity of Syrah

Climate change is inducing earlier grape ripening, especially in warm vintages. This phenomenon is resulting in unbalanced wines with too high an alcohol concentration and low titratable acidity along with a high pH level, without the desired level of phenolic maturity. Final wine quality notably depends on the phenolic composition of grapes and the extractability of these compounds. This research was designed to test a new foliar spray, called LalVigne® MATURE for its capacity to create a balance between sugar development and phenolic maturity. It is a formulation of 100% natural, inactivated wine yeast derivatives. This foliar spray was tested on Syrah vines in two vintages (2012, 2013) in a cool climate wine region (Eger, Hungary). It was acting as an elicitor, stimulating the synthesis of several secondary metabolites. Changes in anthocyanin extractability and texture characteristics of the grape berries were monitored during ripening. Experimental wines were made at three separate harvest times in each vintage. Standard analytical parameters for grapes and wines as well as resveratrol were evaluated. Grapes from treated vines had thicker skins than controls on all sampling dates in both vintages. The phenolic potential (especially anthocyanin concentration and its extractability) of the foliar spray–treated grapes was greatly improved. Our experiment showed that phenolic ripening can be enhanced using the foliar spray, and its application is useful in different vintages.

## INTRODUCTION

Nowadays wine consumers prefer well structured wines with deep colour, fruit scents, soft tannins and pleasant mouthfeel (Bruwer *et al.,* 2011). These kinds of wines can be made from well ripened grapes with an optimal level of phenolic and technological (sugar) maturity, but not from overripened grapes. Nevertheless, the changing climate is notably modifying the ripening process. In cool climate wine regions such as the Eger wine district in

Hungary we can count on more frequent extreme weather events, including uneven precipitation, heat waves and droughts (Schultz 2000). In dry and hot vintages, the ripening process is faster, and the balance between phenolic and technological (sugar) maturity may not be maintained (Hannah *et al.,* 2013). This results in an increase in the sugar concentration, and in parallel, a rapid decrease in the titratable acidity resulting in unbalanced and overly alcoholic wines. At the same time, the lack of optimal phenolic maturity results in wines with green and astringent tannins (Jones *et al.,* 2005). On the other hand, in a rainy, cool vintage, ripening is slowed, and late ripening varieties (such as Cabernet Sauvignon, Cabernet Franc, Syrah) cannot reach optimal maturity (Jackson & Lombard 1993).

Several technological applications can be used in order to reduce these negative effects. Cluster thinning (Guidoni *et al.,* 2002; Prajitna *et al.,* 2007), girdling (Singh Brar *et al.,* 2008; Koshita *et al.,* 2011) and early defoliation (Poni *et al.,* 2006; Poni *et al.,* 2009; Kemp *et al.,* 2011; Gatti *et al.,* 2012; Lee & Skinkis 2013) are reported to have a beneficial effect on phenolic maturity, especially on anthocyanin and flavonoid synthesis. The resveratrol content of the grape varies considerably and depends on many viticultural factors, including climate, terroir, grape variety, fungal infections and yield (Jeandet *et al.,* 1995; Bavaresco 2003; Bavaresco *et al.,* 2007; Prajitna *et al.,* 2007). There are also some papers that look at increasing resveratrol concentration in grapes using elicitors (Vezzulli *et al.,* 2007; Santamaria *et al.,* 2011).

Beyond the abovementioned techniques a new foliar spray for enhancing phenolic maturity was recently developed, and its effects were examined. In addition, Syrah is a new cultivar to the Eger wine region, which has only limited cultivation experience with this varietal.

The aim of this study is 1) to describe the effects of the application of this new foliar spray on grape phenolic maturity and 2) to describe some aspects of the responses of a "new" variety (Syrah, *Vitis vinifera* L.) in a cool climate wine region (Eger, Hungary).

## MATERIALS AND METHODS

### Description of the experimental site and the experimental design

The experiment took place in the Eger wine region (in Northeast Hungary) in a commercial vineyard (lat. 47°55'31.84" N; long. 20°24'42.32" W, elevation: 430 m asl). The vineyard's shallow soil is based on limestone. This site met the criteria for an investigation of a new foliar spray designed to enhance phenolic maturity, because in warm vintages the sugar accumulation is very fast at the Nagy-Eged-hill, leading to overly alcoholic, unbalanced wines. Besides, the desired level of phenolic maturity cannot be achieved in most of the vintages. The trial was performed over two consecutive vintages in 2012 and 2013.

Ten-year-old Syrah (clone ENTAV-INRA® 877) vines grafted onto Teleki 5C at a spacing of 2.4 m x 0.8 m with south-north row orientation were investigated. Vines were trained to a unilateral cordon at a height of 0.6 m, and were pruned to four spurs, each bearing two nodes. A trial site of 6 rows was selected for each treatment (with 3 control [unsprayed, C] rows and 3 treated [sprayed, LM] rows). Each row was divided into 3 blocks. One block contained 25–29 vines. At the same harvest time, 3 blocks/treatments were harvested, which resulted in 3 replicates/treatments. The LalVigne® MATURE leaf spray is a formulation of 100% natural, inactivated wine yeast (*Saccharomyces cerevisiae*) derivatives (specifically designed to be used with the patent foliar application technology WO/2014/024039, Lallemand Inc., Canada). It is non-pathogenic, non-hazardous, food grade and non-GMO. The product is already registered in many countries and in the process of being authorized in others. Two applications of 1 kg/ha were done. The first was at the beginning of veraison, the second 12 days later. The powder was diluted in water without using an adjuvant. The whole canopy was sprayed with a motorized backpack sprayer.

There were three harvest dates (September 6, 13, and 27 in 2012 and September 12 and 19 and October 3 in 2013) in each vintage for both the control and treated vines. The second harvest (the Gróf Buttler winery's commercial harvest date) was established as the reference, and the first trial harvest date was set one week earlier and the third harvest two weeks later than the reference. One vine block represented one wine repetition per treatment at each harvest date. Veraison commenced in the first week of August in 2012, and one week later in 2013.

### Climate data

Climate data was monitored by an automatic weather station (Boreas Ltd. Érd, Hungary), approximately 300 m from the trial site.

### Berry sampling

Three 20 kg sets of grapes, with each set including 25–29 vines, were carefully hand-harvested for both treatments at each harvest date and transported immediately to the

experimental winery. Three 1 kg samples for each treatment were collected at random from several clusters before vinification. The berries were selected randomly from the upper, middle, and lower parts of the bunches. All berry samples were prepared and analyzed within 2 hours of harvest.

For the texture analysis, 50 berries were randomly removed from the clusters with pedicels and visually examined before texture analysis. One berry represents one repetition by this measurement. Damaged berries were rejected.

150 berries were separately selected for phenolic measurement (Glories method), and these berries were subdivided into two equal groups for the pH 1 and pH 3.4 solutions. The measurement was performed in triplicate. 25 berries were used for each repetition.

Three additional sets of 100 grape samples were selected for weight determination and grape composition analysis.

## Grape analysis

The analytical methods recommended by the OIV (2014) were used to determine the titratable acidity and pH of the grapes. The sugar content (expressed as °Brix) of the grape juices was determined at 20°C using a hand-held refractometer (Atago MASTER-α, Japan).

### *Assessment of grape phenolic maturity*

The phenolic potential of grapes was calculated according to the method described by Saint-Cricq *et al.* (1998). This involved grinding the grapes with a blender and macerating for 4 hours with buffer solutions at two pH values (1.0 and 3.4). The original method proposed a pH 3.2 buffer, but this was adjusted to 3.4, as it is more relevant to the grapes from this region. The indices of phenolic maturity were calculated according to Glories & Augustin (1993): potential anthocyanins (A1), extractable anthocyanins (A3.4), cell maturity index (EA%) and seed maturity index (SM%). All the measurements were performed in triplicate.

The following equations were used:

$$EA\ (\%) = [(A1 - A3.4) / A1] \times 100$$

$$SM\ (\%) = [(A280 - ((A3.4 / 1000) \times 40)) / A280] \times 100$$

### *Measurements of berry physical properties*

A TA.XTplus Texture Analyzer (Stable Micro System, Surrey, UK) with an HDP/90 platform and 30 kg load cell was used to monitor grape physical properties. The Exponent 6.1.4.0 software was used for data evaluation. All operative conditions were applied according to Letaief *et al.* (2008b) and Zsófi *et al.* (2014). Briefly, a P/35 probe was used to determine berry hardness (BH). Berries of approximately the same size, with their pedicel attached, were gently removed from the bunch and laid on the analyzer plate. After this, they were compressed to 25% of their diameter. The P/2N needle was applied to conduct a puncture test. A second set of berries with their pedicel attached were removed from the bunch, were laid on the analyzer plate and then punctured in the lateral face (Letaief *et al.,* 2008a). The skin break force ($F_{sk}$), skin break energy ($W_{sk}$) and Young's modulus of berry skin ($E_{sk}$) were calculated from the puncture test data using Exponent 6.1.4.0 software. Berry skin thickness ($Sp_{sk}$) was measured using a P/2 probe with 2 mm diameter. For this measurement, approximately 0.25 $cm^2$ of skin was removed from the lateral face of the berry. The skin was carefully and gently cleaned of pulp and then placed on the platform. The test was conducted as described by other authors previously (Letaief *et al.,* 2008a; Letaief *et al.,* 2008b; Río Segade *et al.,* 2008). The skin thickness is given by the distance (travel) between the point corresponding to the probe's contact with the berry skin and the platform base during the compression test. For seed hardness tests, one seed was removed from the berry and placed on the platform on its lateral side. The seeds were crushed by the P/35 probe. The seed break force ($F_s$), seed break energy ($W_s$) and Young's modulus of the seed ($E_s$) were also calculated by Exponent 6.1.4.0.

## Wine analysis

The analytical methods recommended by the OIV (2014) were used to determine the ethanol content, titratable acidity and pH of the wines.

Total phenolics of the wines were analyzed by the Folin-Ciocalteu method (Singleton & Rossi 1965) and the results expressed as gallic acid equivalents (GAE mg/L). The quantity of leucoanthocyanins (flavan-3,4-diols) was determined as described by Flanzy *et al.* (1969). The bisulfite bleaching method was used to determine the anthocyanin content of grape extracts and wines (Ribéreau-Gayon & Stonestreet 1965), while the total catechins (flavan-3-ols) were measured using the vanillin assay according to Amerine & Ough (1980). The colour intensity ($A_{420}+A_{520}+A_{620}$) and hue ($A_{420}/A_{520}$) of the wines were determined using the method described by Glories (1984). Phenolic components were measured by spectrophotometer (UVmini-1240 CE UV-VIS, Shimadzu, Japan). The gelatin and HCl indices (Ribéreau-Gayon *et al.,*

2006) were also calculated. All the measurements were performed in triplicate.

### *Qualitative and quantitative determination of resveratrol components in wines by HPLC*

The analysis of resveratrol compounds was carried out according to Kállay & Török (1997). The wine samples were filtered first on filter paper, then on a membrane of 0.45 µm. The eluent for the isocratic HPLC analysis consisted of a 5:5:90 mixture (v/v%) of acetonitrile: methanol: redistilled water. All the measurements were performed in triplicate, and the wine samples were directly injected after filtration, without dilution, in a quantity of 20 µl. Operating conditions and chromatograph settings are as follows: an HP Series 1050 HPLC-apparatus with a normal phase LiChrospher® 100 CN (250x4mm, 5 µm) column (Merck, Germany) was used during the measurements. The detector was an HP Series 1050. The flow was set to 2 ml/min at 30°C with detection wavelength at 306 nm. The methanol and acetonitrile used for the experiment are of HPLC grade, and other chemicals were of analytical purity. *Trans*-resveratrol (99%) standard was purchased from Sigma-Aldrich (Germany). *Trans*-piceid standard was received from the San Michele all'Adige Research and Innovation Centre. *Cis*-isomers are produced by UV irradiation of the *trans*-isomers (Sato *et al.,* 1997). The detection limit was 0.1 mg/L.

### *Microvinification process*

Three 20 kg sets of grapes were crushed, destemmed and sulfited (1 ml of 5% aqueous $SO_2$ solution for every 1 L of mashed grape) in the experimental winery at each harvest date. Macerations were conducted in 30 L plastic containers, and all grape repetitions were separately fermented. Three experimental wine replicates were made at each harvest time for each treatment respectively. After grape processing, the containers were transported immediately to the cellar to ensure constant ambient temperature (13°C) from the beginning to the end of maceration. After 24 hours of cold maceration, selected active dry yeasts (20 g of dry yeast/100 kg of processed grapes) (Uvaferm VN, Lallemand Inc.) and yeast nutrients (30 g/100 kg of processed grapes) (Uvavital, Lallemand Inc.) were added. The maceration lasted for 23 days. The cap was punched down twice a day throughout the skin contact period. The wines were also inoculated with 10 mg/L lactic acid bacteria (Uvaferm Alpha, Lallemand Inc.) at the end of alcoholic fermentation. After 23 days the wines were pressed at 1.5 bar in a 30 L membrane press. Free-run and press wines were mixed. After malolactic fermentation had occurred, the wines were racked and transported to the laboratory for analysis. All the wines were stored at 13°C for several days until the moment of the analysis, and no sulfur was added prior to analysis.

### Sensory analysis

All the wines were tasted by a group of 17 expert oenologists. Blind tests were carried out by comparing in pairs (control [C] vs. treated [LM]) the wines obtained from the three different harvest dates in both vintages. The wines were subject to sensory analysis by the 100-point OIV (1994) method. In all cases, the objective was for the experts to name which wine they preferred and why.

### Statistical analysis

Statistical analysis was conducted by IBM SPSS 20 (IBM Corp., Armonk, NY, USA) software. Values were compared by multivariate ANOVA test with three factors: the effects of vintage (2012 and 2013), treatment (C [control] and LM [LalVigne® MATURE]) and harvest dates. This was followed by a between-subjects effect test. Homogeneity of variances was checked using Levene's test. In case of significant effect of harvest dates, Tukey's or Games-Howell post hoc test was used for mean separation, depending on whether or not the homogeneity of variances was held.

## RESULTS

### Climate characteristics for 2012 and 2013

Fig. 1 shows the climate characteristics of the two vintages. The weather of 2012 can be considered as dry (total rainfall was 439.2 mm compared to the 50-year average of 589.6 mm) and warm (average year temperature was 12.5°C compared to the 50-year average of 10.7°C). On the other hand, 2013 can be regarded as a cooler vintage (total rainfall: 663 mm, average year temperature: 12.2°C), although the weather was somewhat cooler with more rain during the flowering and ripening stage than in 2012.

### Yield, grape juice sugar concentration, acidity, pH, berry weight, cell and seed maturity indices

The average yield per vine was 0.63 kg (control) and 0.65 kg (treated) in 2012, and 0.99 kg (control) and 0.92 kg (treated) in 2013. An average of seven bunches were grown per vine in both years.

Table 1 (see page 31) shows the standard grape juice parameters. The grapes reached a greater level of technological maturity in 2012 (maximum sugar concentration: 24.3 °Brix) compared to 2013 (maximum sugar concentration: 21.2 °Brix). Indeed, the berry sugar concentration in 2012 exceeded 2013 by 15–25%. There were also notable differences in the case of titratable acidity, with the 2013 values being significant higher. The lowest concentration was 8.6 g/L. The weight loss of the berries during ripening is due to dehydration. There was some rain between the second and the third harvest dates in 2012, however, which resulted in heavier berries. Clearly, the vintage had a very strong effect on all the parameters as can be seen in Table 1.

The Glories indices, which provide a prediction on phenolic compounds in the resulting wines (Kontoudakis *et al.,* 2010), are given in Table 2 (see page 32). In general, the lower the EA% and SM% values, the riper the berry. In most cases the regular range for A1, EA% and SM% varies from 500 to 2,000 mg/L, 70% to 20% and 60% to 0%, respectively (Ribéreau-Gayon *et al.,* 2006). The A1 and A3.4 values indicate a good anthocyanin concentration especially in 2012. Interestingly, the EA% values showed an increase in some cases during ripening, implying that the extractability of the anthocyanins decreased. None of the factors affected the seed maturity index (SM%).

## Grape texture properties

Table 3 (see page 33) shows the texture parameters of the berries. The berries became softer (BH) during ripening. The significant increase observable in 2012 is due to the rainfall during the second and third harvest periods. Changes in skin break force ($F_{sk}$) showed a very similar pattern to $W_{sk}$ related to the treatments and the harvest time. The impact of the leaf spray caused a significant increase in skin thickness ($Sp_{sk}$). The values were above 0.2 mm in the case of treated grapes at all harvest dates and in both years. There was no correlation between skin thickness ($Sp_{sk}$) and skin break force ($F_{sk}$) values. The seed texture parameters remained unchanged despite the treatment between the harvest dates. However, the vintage had a very strong effect on these parameters.

## Wine composition

Table 4 (see page 34) summarizes the main wine parameters. The wines had a wide range of alcohol concentration (between 11.28% v/v and 15.55% v/v). The foliar spray did not influence this parameter, however. We found significant differences between the titratable acidity and pH in the first phase of ripening, but the differences were no longer significant by the second and third harvest dates.

The total polyphenol values were independent of the foliar spray treatment. In 2012 we measured significantly higher (above 2,000 mg/L) values than in 2013 (concentration between 1,025 and 1,304 mg/L). The leucoanthocyanin and anthocyanin concentrations were found to be significantly higher in the treated wines in three instances: in 2012 at the second and third harvest dates, and in 2013 at the second harvest date (although only for anthocyanins). The weather conditions in 2012 favoured anthocyanin synthesis up to 796 mg/L. By contrast, in 2013, the unfavourable vintage resulted in significantly lower anthocyanin concentration (Table 4). The impact of the foliar spray and harvest date on catechin levels is unclear. The colour intensity ($A_{420}+A_{520}+A_{620}$) correlated well with the increasing concentration of anthocyanins. The values of colour hue ($A_{420}/A_{520}$) represent a bluish tone, but this is typical for young red wines (Boulton 2001).

The gelatin index increased significantly in 2012 between the first and the third harvest dates in the foliar spray–treated grapes. In 2013 the differences between harvest dates were smaller, and the values were also much lower than in 2012 and less than the optimal value due to the unfavourable weather conditions (Ribéreau-Gayon *et al.,* 2006). During tastings, the wines were characterized by green, unripe tannins. HCl indices show a marked variation from 4.34 to 12.99. The foliar spray treatment increased this parameter, but the difference was significant only at the second harvest date in 2012 and at the third harvest date in 2013.

Table 5 (see page 35) shows the changes in resveratrol concentration in the wines. The majority of resveratrol was found in the wines as the isomeric forms of piceid (resveratrol glycoside). In 2012 and 2013, *cis*- and *trans*-resveratrol were not detected in the control wines at the first harvest date. *Trans*-resveratrol was also absent in 2013 in the treated wines at the second harvest date. Treated wines contained this compound from the first harvest date. Under the effect of the foliar spray, total resveratrol concentration increased especially in the first phase of ripening. The differences in total resveratrol concentration were not significant in three cases: at the second harvest dates in both years, and at the third harvest date in 2012.

## Sensory analysis

All the tasters were able to differentiate between the control and treated wines. Wines made from foliar treated grape were preferred and received higher scores than

controls (data not shown). Vintage had a very strong effect on the sensory quality. In 2013 the average points were much lower for all the wines, but the positive impact of the foliar spray remained noticeable.

## DISCUSSION

The foliar spray treatment had a significant effect on the titratable acidity and pH of the grapes, with the treated berries containing less acid. This is probably due to the higher berry respiration as an effect of faster ripening (Sweetman *et al.,* 2009). There was a positive effect of the leaf spray treatment on both total (A1) and potential (A3.4) anthocyanins, favouring their accumulation in both years and at nearly all harvest dates. Several phenomena may generally trigger the higher anthocyanin concentration of the wines. These include a beneficial change in the berry skin/flesh ratio (Kennedy *et al.,* 2002; Ojeda *et al.,* 2002), increased extractability (Río Segade *et al.,* 2011) and intensive anthocyanin synthesis (Downey *et al.,* 2004; Yamane *et al.,* 2006; Koshita *et al.,* 2011). In addition, during anthocyanin extraction in winemaking, it is also necessary to take into account the changes in grape skin cell-wall composition and structure, because this can modify the extractability process (Hanlin *et al.,* 2010). The foliar spray–treated grapes reached a greater level of phenolic maturity in both years, as can be seen in the results for the first and third harvests (values of EA [%] are lower, see Table 3). The absolute (A1) and extractable pigment (A3.4) concentration were also higher due to the foliar spray in both years, except one instance in 2012. At the third harvest date, the treated grape had a lower A1 value. Vintage had a significant influence on all the Glories parameters except SM%. As can be seen from the data in Table 2, SM% values did not match the optimal criteria (Ribéreau-Gayon *et al.,* 2006) for ripeness in several cases. Values higher than 60% mean that the seeds were not sufficiently ripe, and thus a long fermentation maceration would not be recommended. Neither the vintage nor the foliar spray treatment affected the SM% values significantly.

The foliar spray resulted in a significant increase in berry skin thickness ($S_{psk}$) at all sampling dates. The harvest date and the vintage did not influence the skin thickness significantly. The skin hardness ($F_{sk}$) values were significantly lower for treated grapes in three cases (first harvest date in 2012, second and third harvest dates in 2013). Our results show that the concentration of anthocyanins was higher in the thicker skins and also in the case of lower skin hardness ($F_{sk}$). This is the opposite of other findings, where thinner (Río Segade *et al.,* 2011) and harder skins (Rolle *et al.,* 2008, 2009) contained more anthocyanins. However, thicker and softer skins may also contain more anthocyanins due to the increased flavonoid synthesis and higher berry skin/flesh ratio. The enhanced pigment accumulation due to the foliar spray is also supported by Duo *et al.* (2014) and Lissarrague *et al.* (2014). Berry texture parameters were strongly modified by vintage effect, as seen before (Letaief *et al.,* 2008a; Río Segade *et al.,* 2008). Young's modulus of berry skin ($E_{sk}$), berry hardness (BH) and seed texture properties were the parameters most affected, as can be seen in Table 3. It seems cooler weather results in harder skin and softer seed. In 2012 the seeds were harder than in 2013. In 2013 the $F_s$ values remained under 36 N, and the values of work needed for the break ($W_s$) were under 6 mJ, indicating softer seeds. There was no difference in seed texture parameters ($F_s$, $E_s$, $W_s$) between the control and treated berries. Further, the harvest date had no effect on these parameters.

Torchio *et al.* (2010) reported decreasing Young's modulus of the berry skin ($E_{sk}$) as ripening progresses. This was observed only in the 2013 season and can most probably be explained by the combined effects of changes in the cell-wall structure, ripening processes and the water content of the berry. With respect to other berry physical properties, only the BH values, which reflect berry softness, decreased with ripening as expected. The only increase in BH values (Table 3) can be seen between the second and third harvest dates in 2012, due to a rainy period at that time.

The increased values of HCl and gelatin indices for the wines from foliar spray–treated grapes in 2012, and to some extent in 2013, indicate a more polymerized and balanced tannin structure compared to control wines. Sensory analysis supported these facts. All the tasters were able to differentiate between the control and treated wines. The wines made from foliar sprayed grapes had more intense flavour, better mouthfeel, higher varietal character and a longer finish. In all cases, the tasters preferred wines made from treated grapes. This capacity to achieve a higher phenolic maturity is a potential benefit of the foliar spray treatment. Interestingly, there was a lower concentration of monomeric catechins in wines from the foliar spray–treated grapes in 2012. This observation may be explained by the higher polymerized phenolic compound concentration. HCl indices of the wines were between 4 and 12. A wine suitable for aging has a value of 10–25 (Ribéreau-Gayon *et al.,* 2006). Only two wines met this criterion. Both wines were made from foliar spray–treated grapes in 2012 at the second and third harvest dates.

Resveratrol synthesis was also positively affected by the foliar spray, especially in the first phase of ripening. The differences disappeared by the second harvest in both vintages, however. Significantly higher concentration was found for the first treated wines in both vintages and for the third treated wine in 2013. The causes may be the same as in the case of higher anthocyanin concentration since resveratrol can also be found in the berry skins. Vintage strongly affected the amount of total resveratrol. It seems the lower average temperature during the ripening phase (Figure 1) is delaying stilbene synthesis. The cooler vintage in 2103 also reduced the impact of the foliar spray, resulting in lower resveratrol concentration at the first harvest date. *Trans*-piceid was the most abundant stilbene compound. This is in accordance with other findings (Bavaresco *et al.,* 2007).

The observed changes (the treated berries had higher anthocyanin content along with thicker skins) could be explained with vine-pathogen interaction. Vine recognizes the yeasts in the foliar spray, which is activating some defense mechanisms (Langcake & Pryce 1976; Hahn 1996; Garcia-Brugger *et al.,* 2006; Santamaria *et al.,* 2011). In this way secondary metabolism is enhanced in the berries (Zhao *et al.,* 2005).

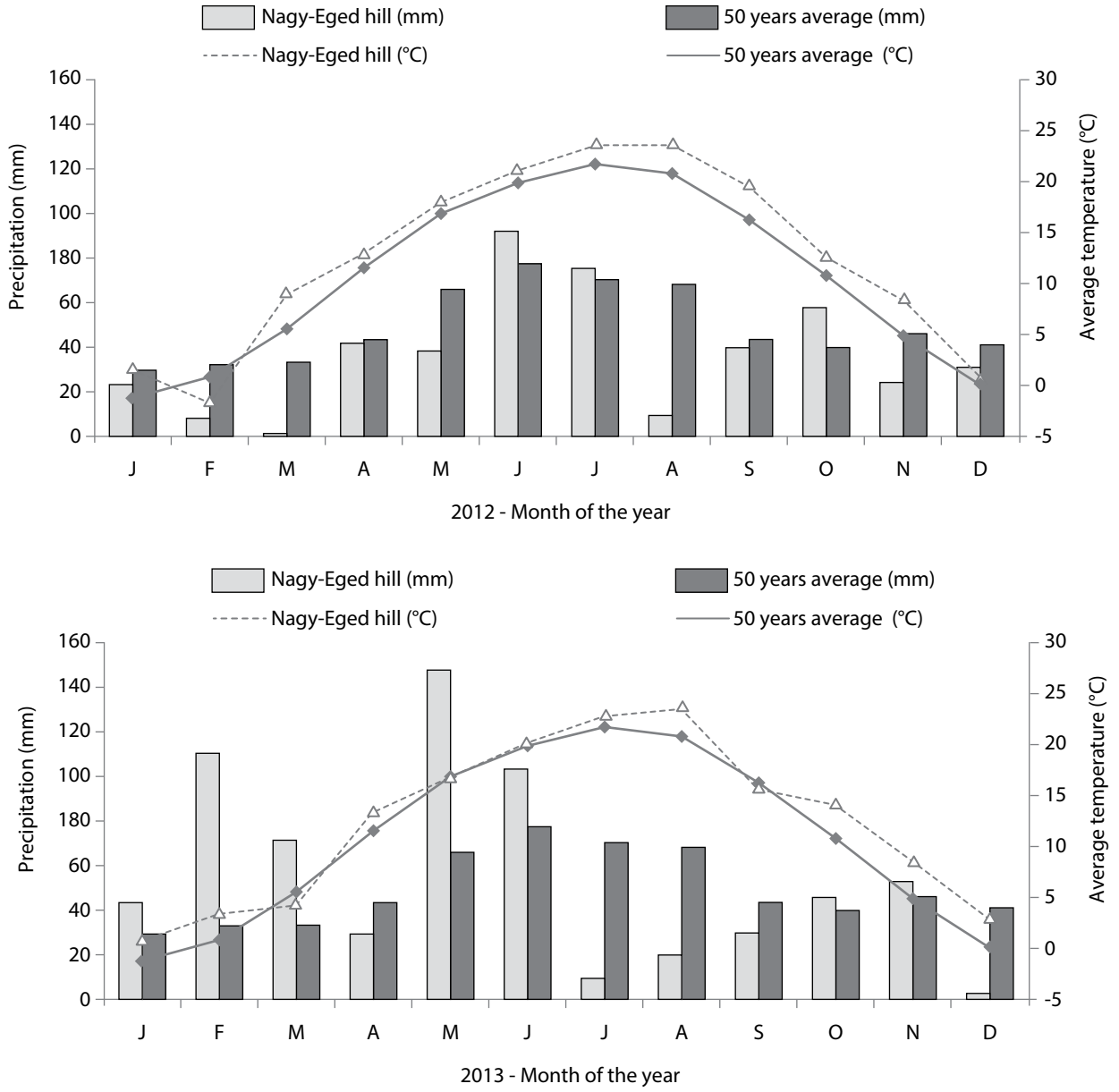


**FIGURE 1** Average air temperature (lines) and monthly sum of precipitation (bars) for 2012 and 2013 at the experimental site (data from automatic weather stations)

Overall, it seems that the impact of the foliar spray is stronger in the earlier phases of the grape ripening process. As the ripening went forward the differences decreased between the treatments, while remaining noticeable until the end of the ripening.

## CONCLUSION

We examined the impacts of yeast derivatives applications (LalVigne® MATURE, Lallemand Inc.) on Syrah grape phenolic maturity as well as wine phenolic composition and concentration. The results from two vintages indicate that its application leads to more optimal harvest conditions. In addition, a higher level of phenolic maturity was achieved in both warm (2012) and cool (2013) vintages. The application of this foliar spray results in wines that are more balanced, showing more flavours and complexity than those made from unsprayed vines. Preliminary evidence was also obtained to suggest that LalVigne® MATURE may also help in cooler and less optimal vintages by enhancing the ripening process, leading to wines with greater oenological potential. Moreover, thicker grape skins and accumulation of resveratrol in early phases could also play an important role in plant protection.

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**Table 1:** Standard grape composition parameters.

| Parameter | Vintage | Harvest date | | | | | |
|---|---|---|---|---|---|---|---|
| | | 2012.09.06 / 2013.09.12 | | 2012.09.13 / 2013.09.19 | | 2012.09.27 / 2013.10.03 | |
| | | Treatment | | | | | |
| | | C | LM | C | LM | C | LM |
| °Brix | 2012 | 22.9 ± 0.3aα | 23.6 ± 0.1bα | 23.7 ± 0.1aβ | 24.0 ± 0.2bβ | 24.3 ± 0.1aγ | 24.3 ± 0.1aβ |
| | 2013 | 18.5 ± 0.2aα | 18.2 ± 0.1aα | 19.0 ± 0.31aα | 20.4 ± 0.2bβ | 21.2 ± 0.3aβ | 21.0 ± 0.2aγ |
| | | * | * | * | * | * | * |
| Titratable acidity (g/L) | 2012 | 7.6 ± 0.1aα | 6.3 ± 0.0bα | 5.1 ± 0.1aβ | 5.3 ± 0.1bβ | 5.5 ± 0.1aγ | 5.9 ± 0.0bγ |
| | 2013 | 10.8 ± 0.1aα | 9.4 ± 0.1bα | 10.2 ± 0.1aβ | 8.9 ± 0.1bβ | 8.6 ± 0.1aγ | 9.2 ± 0.1bγ |
| | | * | * | * | * | * | * |
| pH | 2012 | 3.14 ± 0.02aα | 3.23 ± 0.00bα | 3.32 ± 0.01aβ | 3.34 ± 0.01bβ | 3.25 ± 0.01aγ | 3.34 ± 0.01bβ |
| | 2013 | 2.90 ± 0.01aα | 2.89 ± 0.00aα | 2.93 ± 0.01aβ | 3.02 ± 0.02bβ | 2.94 ± 0.01aβ | 2.91 ± 0.01bα |
| | | * | * | * | * | * | * |
| Weight of 100 berries (g) | 2012 | 127.83 ± 1.39aα | 134.68 ± 2.16bα | 125.23 ± 3.10aα | 121.54 ± 1.24aβ | 134.60 ± 2.51aβ | 136.92 ± 3.09aα |
| | 2013 | 173.45 ± 3.43aα | 178.98 ± 4.61aα | 171.41 ± 6.89aα | 175.60 ± 6.06aα | 147.11 ± 5.47aβ | 147.46 ± 5.79aβ |
| | | * | * | * | * | * | * |

Values marked with different Roman letters mean significant differences between the treatments within the same year and same harvest date. Different Greek letters mean significant differences between harvest dates within the same year and same treatment. * means significant differences between the years within the same treatments and harvest dates. For separation, Tukey's and Games-Howell's post hoc test was used at p=0.05. Each value represents the average ± standard error of 3 replicates. C=control, LM=foliar sprayed.

**Table 2:** Measures of phenolic maturity in grapes.

| Parameter | Vintage | Harvest date | | | | | |
|---|---|---|---|---|---|---|---|
| | | 2012.09.06 / 2013.09.12 | | 2012.09.13 / 2013.09.19 | | 2012.09.27 / 2013.10.03 | |
| | | Treatment | | | | | |
| | | C | LM | C | LM | C | LM |
| A1 (mg/L) | 2012 | 1754 ± 41aα | 1781 ± 82aα | 1781 ± 48aα | 1888 ± 34bα | 1834 ± 124aα | 1736 ± 112aα |
| | 2013 | 1084 ± 61aα | 1273 ± 68bα | 1038 ± 58aα | 1386 ± 49bαβ | 1356 ± 57aβ | 1433 ± 46aβ |
| | | * | * | * | * | * | * |
| A3.4 (mg/L) | 2012 | 828 ± 79aα | 958 ± 26bα | 801 ± 84aα | 839 ± 26aβ | 725 ± 49aβ | 792 ± 16bγ |
| | 2013 | 559 ± 37aα | 702 ± 40bα | 593 ± 22aα | 734 ± 47bα | 602 ± 28aα | 761 ± 29bβ |
| | | * | * | * | * | * | |
| EA (%) | 2012 | 52.9 ± 3.4aα | 46.1 ± 3.8bα | 54.9 ± 5.8aα | 55.5 ± 1.7aβ | 60.4 ± 3.2aβ | 54.2 ± 2.9aβ |
| | 2013 | 48.2 ± 6.4aα | 44.7 ± 5.6aα | 42.6 ± 5.1aα | 46.9 ± 4.8aα | 55.6 ± 1.5aβ | 46.9 ± 1.4bα |
| | | | | | * | | * |
| SM (%) | 2012 | 58.3 ± 2.7aα | 55.8 ± 2.5aα | 55.8 ± 9.1aα | 65.4 ± 1.0aα | 66.5 ± 5.8aα | 56.2 ± 8.7aα |
| | 2013 | 69.5 ± 3.5aα | 65.5 ± 3.8aα | 57.5 ± 10.6aα | 67.3 ± 2.0aα | 49.0 ± 14.2aα | 56.1 ± 14.0aα |
| | | * | * | | | | |

Values marked with different Roman letters mean significant differences between the treatments within the same year and same harvest date. Different Greek letters mean significant differences between harvest dates within the same year and same treatment. * means significant differences between the years within the same treatments and harvest dates. For separation, Tukey's and Games-Howell's post hoc test was used at p=0.05. Each value represents the average ± standard error of 3 replicates. C=control, LM=foliar sprayed.

**Table 3:** Berry physical properties.

| Parameter | Vintage | Harvest date | | | | | |
|---|---|---|---|---|---|---|---|
| | | 2012.09.06 / 2013.09.12 | | 2012.09.13 / 2013.09.19 | | 2012.09.27 / 2013.10.03 | |
| | | Treatment | | | | | |
| | | C | LM | C | LM | C | LM |
| BH (N) | 2012 | 3.271 ± 0.578aαβ | 3.552 ± 0.672bαβ | 3.114 ± 0.667aα | 3.252 ± 0.684aα | 3.450 ± 0.737aβ | 3.822 ± 0.947bβ |
| | 2013 | 3.940 ± 0.899aα | 4.011 ± 0.873aα | 3.751 ± 0.745aα | 3.183 ± 0.617bβ | 3.266 ± 0.768aβ | 3.134 ± 0.692aβ |
| | | * | * | * | | * | * |
| $F_{sk}$ (N) | 2012 | 0.472 ± 0.066aα | 0.433 ± 0.063bα | 0.409 ± 0.073aβ | 0.422 ± 0.087aα | 0.442 ± 0.077aαβ | 0.453 ± 0.102aα |
| | 2013 | 0.450 ± 0.106aα | 0.434 ± 0.097aα | 0.469 ± 0.098aα | 0.414 ± 0.105bα | 0.458 ± 0.094aα | 0.415 ± 0.089bα |
| | | | | * | | | |
| $E_{sk}$ (N/mm) | 2012 | 0.437 ± 0.111aα | 0.451 ± 0.107aα | 0.455 ± 0.091aαβ | 0.450 ± 0.128aα | 0.489 ± 0.076aβ | 0.520 ± 0.148aβ |
| | 2013 | 0.559 ± 0.103aα | 0.525 ± 0.085aα | 0.476 ± 0.077aαβ | 0.499 ± 0.077aα | 0.332 ± 0.042aβ | 0.371 ± 0.061bβ |
| | | * | * | | * | * | * |
| $W_{sk}$ (mJ) | 2012 | 0.270 ± 0.102aα | 0.260 ± 0.075aα | 0.232 ± 0.075aβ | 0.252 ± 0.104aα | 0.244 ± 0.071aβ | 0.247 ± 0.096aα |
| | 2013 | 0.226 ± 0.081aα | 0.233 ± 0.088aα | 0.283 ± 0.100aβ | 0.224 ± 0.101bα | 0.342 ± 0.102aγ | 0.271 ± 0.082bβ |
| | | * | | * | | * | |
| $Sp_{sk}$ (mm) | 2012 | 0.185 ± 0.038aα | 0.227 ± 0.042bα | 0.197 ± 0.028aα | 0.220 ± 0.037bα | 0.197 ± 0.038aα | 0.228 ± 0.030bα |
| | 2013 | 0.190 ± 0.033aα | 0.210 ± 0.028bα | 0.191 ± 0.030aα | 0.219 ± 0,030bα | 0.190 ± 0.030aα | 0.223 ± 0.035bα |
| $F_s$ (N) | 2012 | 38.50 ± 8.26aα | 38.88 ± 9.64aα | 38.52 ± 9.17aα | 37.61 ± 8.12aα | 37.68 ± 8.11aα | 39.91 ± 10.51aα |
| | 2013 | 30.77 ± 7.13aα | 33.85 ± 5.78aα | 35.60 ± 6.02aβ | 34.61 ± 6.42aα | 33.35 ± 6.14aαβ | 33.14 ± 8.11aα |
| | | * | * | | * | * | * |
| $E_s$ (N/mm) | 2012 | 69.66 ± 14.51aα | 73.46 ± 11.82aα | 68.31 ± 12.29aα | 68.58 ± 14.79aα | 73.94 ± 15.33aα | 73.12 ± 15.33aα |
| | 2013 | 77.67 ± 13.75aα | 78.64 ± 12.91aα | 82.55 ± 15.22aα | 87.36 ± 13.18aβ | 82.86 ± 14.24aα | 80.37 ± 16.54aα |
| | | * | * | * | * | * | * |
| $W_s$ (mJ) | 2012 | 9.73 ± 2.90aα | 9.77 ± 3.42aα | 9.92 ± 3.65aα | 9.56 ± 3.15aα | 9.48 ± 3.13aα | 10.25 ± 3.65aα |
| | 2013 | 5.77 ± 2.24aα | 6.85 ± 1.88aα | 7.13 ± 2.13aβ | 6.59 ± 2.32aα | 6.37 ± 1.78aαβ | 6.50 ± 2.27aα |
| | | * | * | * | * | * | * |

Values marked with different Roman letters mean significant differences between the treatments within the same year and same harvest date. Different Greek letters mean significant differences between harvest dates within the same year and same treatment. * means significant differences between the years within the same treatments and harvest dates. For separation, Tukey's and Games-Howell's post hoc test was used at p=0.05. Each value represents the average ± standard error of 50 replicates. C=control, LM=foliar sprayed.

**Table 4:** Wine composition parameters.

| Parameter | Vintage | Harvest date | | | | | |
|---|---|---|---|---|---|---|---|
| | | 2012.09.06 / 2013.09.12 | | 2012.09.13 / 2013.09.19 | | 2012.09.27 / 2013.10.03 | |
| | | Treatment | | | | | |
| | | C | LM | C | LM | C | LM |
| Alcohol (% v/v) | 2012 | 14.58 ± 0.09aα | 14.43 ± 0.20aα | 15.08 ± 0.26aαβ | 15.15 ± 0.21aβ | 15.35 ± 0.33aβ | 15.55 ± 0.31aβ |
| | 2013 | 11.28 ± 0.18aα | 12.11 ± 0.62aαβ | 11.87 ± 0.06aβ | 11.62 ± 0.23aα | 13.80 ± 0.50aγ | 13.12 ± 0.26aβ |
| | | * | * | * | * | * | * |
| Titratable acidity (g/L) | 2012 | 7.03 ± 0.06aα | 6.00 ± 0.20bα | 5.03 ± 0.06aβ | 5.47 ± 0.31aα | 5.87 ± 0.21aγ | 5.63 ± 0.06aα |
| | 2013 | 8.33 ± 0.15aα | 7.60 ± 0.10bα | 7.63 ± 0.12aβ | 7.00 ± 0.17bβ | 6.67 ± 0.15aγ | 6.97 ± 0.21aβ |
| | | * | * | * | * | * | * |
| pH | 2012 | 3.33 ± 0.01aα | 3.65 ± 0.05bα | 3.72 ± 0.04aβ | 3.81 ± 0.07aβ | 3.86 ± 0.04aγ | 3.69 ± 0.02bα |
| | 2013 | 3.02 ± 0.03aα | 3.16 ± 0.01bα | 3.15 ± 0.02aβ | 3.07 ± 0.01bβ | 3.11 ± 0.01aβ | 3.12 ± 0.02aγ |
| | | * | * | * | * | * | * |
| Total polyphenols (mg/L) | 2012 | 2562 ± 64aα | 2708 ± 83aα | 2944 ± 59aβ | 2928 ± 68aβ | 2782 ± 50aγ | 2850 ± 69bαβ |
| | 2013 | 1045 ± 47aα | 1035 ± 78aα | 1025 ± 91aα | 1117 ± 61aαβ | 1304 ± 165aα | 1260 ± 113aβ |
| | | * | * | * | * | * | * |
| Leucoanthocyanins (mg/L) | 2012 | 1641 ± 42aα | 1582 ± 105aα | 1543 ± 39aαβ | 1767 ± 111bα | 1449 ± 43aβ | 1770 ± 50bα |
| | 2013 | 1137 ± 103aα | 1248 ± 89aα | 1152 ± 41aα | 1386 ± 168aαβ | 1526 ± 102aβ | 1626 ± 141aβ |
| | | * | * | * | * | | |
| Catechins (mg/L) | 2012 | 1517 ± 73aα | 1184 ± 37bα | 1747 ± 65aβ | 1538 ± 109bβ | 1371 ± 48aα | 1421 ± 52aβ |
| | 2013 | 962 ± 85aα | 916 ± 64aα | 820 ± 33aα | 997 ± 62bα | 1048 ± 156aα | 1072 ± 87aα |
| | | * | * | * | * | * | * |
| Anthocyanins (mg/L) | 2012 | 740 ± 19aα | 793 ± 31aα | 736 ± 23aα | 796 ± 13bα | 688 ± 47aα | 762 ± 43aα |
| | 2013 | 340 ± 56aα | 406 ± 10aα | 408 ± 9aα | 463 ± 21bαβ | 526 ± 39aβ | 576 ± 51aβ |
| | | * | * | * | * | * | * |
| Colour intensity ($A_{420}+A_{520}+A_{620}$) | 2012 | 23.43 ± 0.86aα | 23.61 ± 0.64aαβ | 22.18 ± 0.48aα | 24.04 ± 0.07bα | 22.82 ± 0.14aα | 24.47 ± 0.07bβ |
| | 2013 | 14.68 ± 2.33aα | 20.49 ± 0.92bα | 17.70 ± 0.18aα | 20.16 ± 1.67aα | 23.34 ± 0.88aβ | 25.56 ± 1.75aβ |
| | | * | * | * | * | | |
| Colour hue ($A_{420}/A_{520}$) | 2012 | 0.60 ± 0.02aα | 0.64 ± 0.02aα | 0.63 ± 0.02aαβ | 0.64 ± 0.01aα | 0.65 ± 0.01aβ | 0.63 ± 0.00aα |
| | 2013 | 0.39 ± 0.01aα | 0.37 ± 0.01bα | 0.35 ± 0.00aβ | 0.34 ± 0.00bβ | 0.34 ± 0.00aβ | 0.34 ± 0.00aβ |
| | | * | * | * | * | * | * |
| HCl index | 2012 | 4.83 ± 0.15aα | 5.06 ± 3.16aα | 6.53 ± 0.35aβ | 12.99 ± 0.03bβ | 9.50 ± 0.36aγ | 11.16 ± 1.24aβ |
| | 2013 | 5.01 ± 0.53aα | 6.14 ± 0.54aα | 4.97 ± 0.73aα | 4.34 ± 0.61aβ | 4.43 ± 0.68aα | 6.27 ± 0.14bα |
| | | * | * | * | * | * | * |
| Gelatin index | 2012 | 46.91 ± 1.19aα | 51.58 ± 0.51bα | 52.32 ± 1.65aβ | 52.50 ± 0.21aα | 52.59 ± 0.91aβ | 56.58 ± 0.36bβ |
| | 2013 | 26.40 ± 2.52aαβ | 23.17 ± 1.85aαβ | 23.13 ± 0.93aα | 23.23 ± 0.35aα | 18.20 ± 0.30aβ | 18.90 ± 0.30bβ |
| | | * | * | * | * | * | * |

Values marked with different Roman letters mean significant differences between the treatments within the same year and same harvest date. Different Greek letters mean significant differences between harvest dates within the same year and same treatment. * means significant differences between the years within the same treatments and harvest dates. For separation, Tukey's and Games-Howell's post hoc test was used at p=0.05. Each value represents the average ± standard error of 3 replicates. C=control, LM=foliar sprayed.

**Table 5:** Resveratrol analysis of wines.

| Parameter | Vintage | Harvest date | | | | | |
|---|---|---|---|---|---|---|---|
| | | 2012.09.06 / 2013.09.12 | | 2012.09.13 / 2013.09.19 | | 2012.09.27 / 2013.10.03 | |
| | | Treatment | | | | | |
| | | C | LM | C | LM | C | LM |
| *Trans*-resveratrol (mg/L) | 2012 | n.d. | 0.10 ± 0.01α | 0.83 ± 0.25aα | 0.41 ± 0.01bβ | 0.30 ± 0.10aα | 0.23 ± 0.08aαβ |
| | 2013 | n.d. | 0.16 ± 0.14αβ | 0.10 ± 0.12α | n.d. | 0.63 ± 0.10aβ | 0.50 ± 0.11aβ |
| | | | | * | * | * | * |
| *Cis*-resveratrol (mg/L) | 2012 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| | 2013 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| *Trans*-piceid (mg/L) | 2012 | 1.07 ± 0.06aα | 1.39 ± 0.04bα | 0.57 ± 0.06aβ | 1.45 ± 0.05bα | 0.50 ± 0.05aβ | 0.55 ± 0.05aβ |
| | 2013 | 0.37 ± 0.28aα | 0.46 ± 0.16aα | 0.41 ± 0.07aα | 0.12 ± 0.11bβ | 0.47 ± 0.32aα | 0.74 ± 0.05aα |
| | | * | * | * | * | | * |
| *Cis*-piceid (mg/L) | 2012 | n.d. | 0.93 ± 0.15αβ | 1.20 ± 0.20aα | 0.90 ± 0.00aα | 0.87 ± 0.06aα | 0.61 ± 0.07bβ |
| | 2013 | 0.41 ± 0.09aα | 0.60 ± 0.34aα | 0.25 ± 0.02aα | 0.87 ± 0.19bαβ | 1.05 ± 0.31aβ | 1.63 ± 0.30aβ |
| | | * | | * | * | | * |
| Σ (mg/L) | 2012 | 1.07 ± 0.06aα | 2.42 ± 0.18bα | 2.60 ± 0.00aβ | 2.76 ± 0.06aα | 1.67 ± 0.20aγ | 1.39 ± 0.17aβ |
| | 2013 | 0.78 ± 0.32aα | 1.23 ± 0.26bα | 0.73 ± 0.11aα | 0.99 ± 0.10aα | 2.14 ± 0.69aβ | 2.87 ± 0.23bβ |
| | | | * | * | * | * | * |

Values marked with different Roman letters mean significant differences between the treatments within the same year and same harvest date. Different Greek letters mean significant differences between harvest dates within the same year and same treatment. * means significant differences between the years within the same treatments and harvest dates. For separation, Tukey's and Games-Howell's post hoc test was used at p=0.05. Each value represents the average ± standard error of 3 replicates. n.d. = not detectable, C=control, LM=foliar sprayed.

# *LACHANCEA THERMOTOLERANS*, THE ACIDIFYING YEAST

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## Abstract

*Lachancea thermotolerans* (LT) is an ellipsoidal budding yeast indistinguishable from *S. cerevisiae* by optical microscopy. LT is a ubiquitous yeast that has been found in many food and plant substrates including grapes. LT has interesting oenological properties: medium fermentative power 4–9% vol. of ethanol, low production of volatile acidity, and enhanced production of some floral and fruity esters such as 2-phenylethyl acetate or ethyl lactate. Nevertheless, the main metabolic feature is acidification by lactic acid production. With some exceptional strains, concentrations higher than 16 g/L have been observed after fermentation; however, it is not easy to find strains with productions higher than 4 g/L. The strong lactic acid production allows for changes of wine pH with reductions that can sometimes exceed 0.5 units. Some aspects affecting lactic acid production remain unclear and require further study. Lactic acid is a molecule that is stable under oenological conditions but also promotes higher microbiological stability by pH control and while at the same time providing more effective levels of molecular $SO_2$. LT must be used in sequential or mixed fermentations with *S. cerevisiae* or, alternatively, with high fermentative power non-*Saccharomyces* such as *Schizosaccharomyces pombe*, to obtain dry wines. Other fermentative applications of LT are also compelling for the fermentation of ciders and beers to deliver a better balance of freshness. Moreover it can be an interesting option to produce sweet wines in Mediterranean climates to yield a better balance between sweetness and acidity.

## Introduction

*Lachancea thermotolerans,* previously known as *Kluyveromyces thermotolerans* (Lachance & Kurtzman, 2011), is a budding yeast (Fig 1) with a medium fermentative power, and it is easy to find strains able to reach 7–9% vol. The first oenological applications of *L. thermotolerans* were described by Comitini et al (2011) and Gobbi et al (2013), and a recent review was done by Morata et al (2018). LT can be found in spontaneous fermentations when the alcohol content is in the range of 3–10% according to the principle of succession and is similar to other medium fermentative non-*Saccharomyces* such as *Torulaspora delbrueckii* or *Metschnikowia pulcherrima*. LT ferments glucose and fructose and is also involved in some variable fermentation of maltose, trehalose and raffinose. YAN requirements are higher than for *S. cerevisiae*, which is usually around 200 mg/L. Several extracellular enzymatic activities have been described, including esterases, pectinases and glucanases.

The production of acetic acid is very low, frequently lower than 0.4 g/L and, depending on the strain, it can also be used to control the final levels of volatile acidity. When used in sequential or mixed fermentations with *S. cerevisiae,* the final levels of volatile acidity are quite moderate. Although some LT strains can be very sensitive to sulfites, it is also possible to select strains with a suitable resistance. Some are able to ferment musts with concentrations of 60 mg/L, normally with a slight reduction in the final alcoholic content reached.

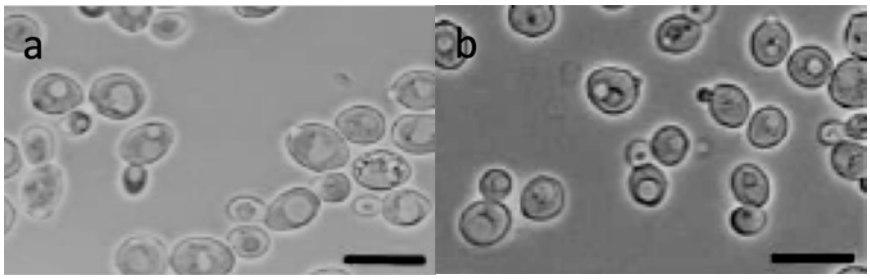


**Fig 1**. Optical microscopy of *S. cerevisiae* (a) and *L. thermotolerans* (b) during fermentation. Scale 10 µm.

In terms of fermentative power, most references describe a maximum value of 9% vol. However, recently we have been working as part of a global project led by Lallemand FRESHWINES (2018) that involves wineries in four warm areas in central and southern Spain. One of the selected strains shows a fermentative power in two triplicate tests of around 12% vol. This strain opens the door to the possibility of achieving fermentation of a must in single inoculation with LT.

## Selection and identification

As with many other non-*Saccharomyces* yeasts, LT is able to growth in lysine-selective media, which is an efficient way to isolate this species. The use of differential media such as CHROMagar© makes it easier to distinguish tentative LT colonies that later can be effectively identified by molecular techniques. In this media, LT colonies appear red-brown in colour (Fig. 2). Molecular identification can be done by sequencing the D1/D2 region of the 26S rDNA.

Another phenotypic confirmation method for identifying LT is to measure the production of lactic acid during fermentation. The best technique is to use an automatic analyzer to conduct enzymatic analysis. Alternatively, FTIR spectrophotometry is a faster but less reliable technique.

## Lactic acid production

Lactic acid production during fermentation by LT ranges from less than 1 g/L (Comitini et al, 2011) to more than 16 g/L (Banilas et al, 2016); however, suitable strains from an oenological point of view must be in the 3–7 g/L range (Morata et al, 2018). Metabolic production of lactic acid from sugars occurs thanks to the lactate dehydrogenase (LDH) enzyme (Fig 3). The presence of this enzyme is observed in several LT strains, with multiple sequence alignment of LDH sequences from different organisms, including *Lactococcus lactis*.

Maximum production of lactic acid during fermentation is correlated with higher populations and YAN levels of 200 mg/L. Other nutritional requirements are also essential for the formation of lactic acid. Usually the production of lactic acid is quite lower than expected for a strain if fermentation is done in model media or a diluted concentrated must. Maximum values are reached in fresh must. Maximum production is also correlated with a cell population higher than 6 log CFU/mL (4-5 $10^6$). When it is lower (5–6 log) lactic acid production decreases significantly.

The effectiveness of some strains at reducing wine pH during fermentation is remarkable. In real winemaking conditions with fermentation of crushed grapes, the LT L3.1 strain yields a reduction in pH, for example starting at 4–4.2 for the control (spontaneous fermentation) to 3.6

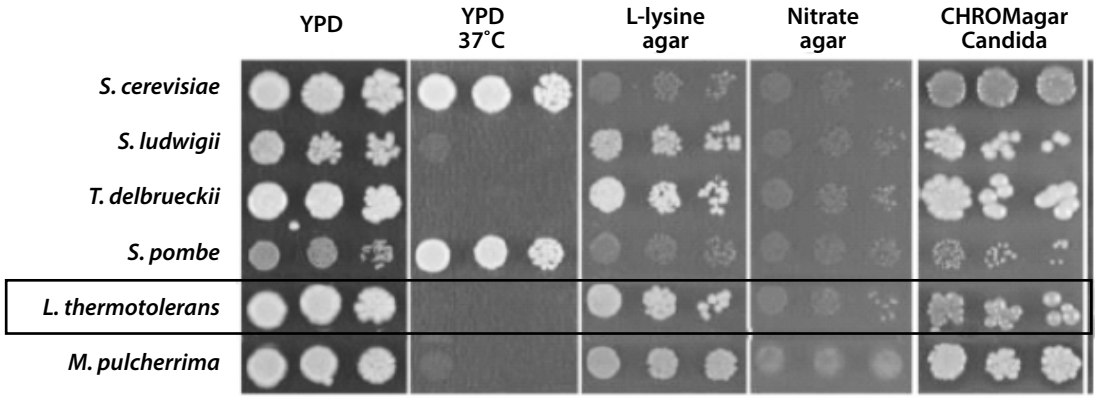


**Fig. 2**. Colony appearance in several general, selective or differential media; for *S. cerevisiae* and some non-*Saccharomyces* species.

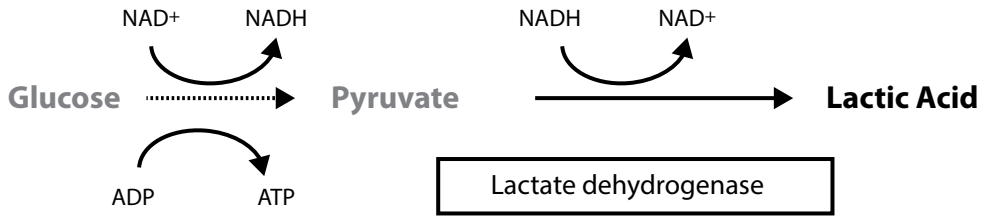


**Fig 3.** Tentative metabolic pathway for the formation of lactic acid from sugars.

(Morata et al, 2019; Fig 4). This biotechnology presents a new way to regulate pH in warm areas using a natural biological process. It should be noted that lactic acid is stable during winemaking and aging. Moreover, the fermentation is done with low levels of volatile acidity, frequently below 0.4 g/L. Simultaneous co-inoculation with *O. oenii* to produce concurrent malolactic fermentation (MLF) was even more effective, decreasing pH to 3.3. When LT is used and we decide to perform a subsequent MLF, lactic acid must be considered as inhibitory on this process at concentrations of around 4 g/L, which is easily within reach using this method. Inhibition of MLF at high concentrations of lactic acid can be considered as an interesting strategy to promote wine freshness.

From the point of view of implantation, it is quite relevant that most of the lactic acid production occurs at the beginning of fermentation. Therefore, it is easier to use LT with good results, even in crushed grapes with high initial wild yeast populations.

## Flavour and aroma

Concerning acidity, it is important to point out that the sensory profile of pure lactic acid is citric acidity without dairy taints. It stands to reason that high levels of lactic acid can produce excessively milky, cheesy or yogurt-like flavours as can happen with MLF; however, this sensory profile is due to some concomitant metabolites produced during the MLF, such as acetoin or diacetyl. These metabolites are produced in low values during fermentation by LT (Morata et al, 2019). The only compound that is increased together with lactic acid is the main alcohol ester: ethyl lactate (Morata et al, 2019), but its sensory threshold is very high. The production of other unpleasant or non-winey smells (such as ethyl acetate or acetaldehyde) or higher alcohol content is moderate with performance similar to *S. cerevisiae*. Conversely, positive esters such as 2-phenyl ethyl acetate are overproduced in single but also in sequential fermentations with LT.

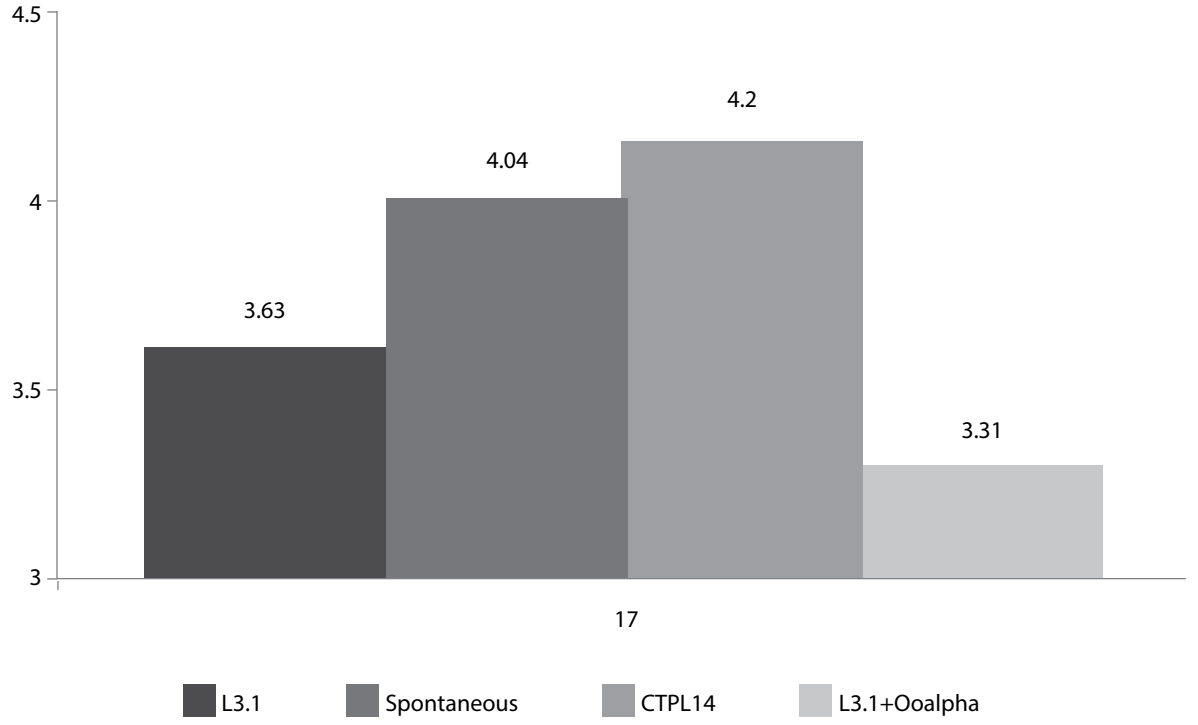


**Fig. 4**. pH levels after sequential fermentations with *L. thermotolerans* strain L3.1 and *S. cerevisiae* and controls. Also, in simultaneous coinoculation with *O. oeni* strain alpha.

## Colour

Non-significant effects have been observed with just LT in the formation of either vitisins, vinylphenolic adducts or polymeric pigments (Escott et al, 2016, 2018). Nevertheless, just a few strains have been tested in this issue and the application of a higher number could probably produce interesting results in the short run. The impact on colour and pigment stability is significant because the reduced pH produced by the formation of lactic acid has a hyperchromic effect in wine anthocyanins.

## Other applications

In beers we have used LT L3.1 in main fermentation and also later in bottle fermentation. pH control is even more effective in beer than in wine because of the lower buffer capacity of beer. We observe reductions in pH from 4 to 3 during fermentation alone (Callejo et al, 2017, 2019). Moreover, the acidity produced by LT helps to modulate bitterness when roasted malts are used. LT makes it easier to produce sour beers in absence of bacteria cultures.

In ciders the effectiveness is similar to beer. LT presents an opportunity to produce sour ciders even when using sweet apples with low levels of malic acid.

## Conclusions

*Lachancea thermotolerans* presents a new biotechnological opportunity in the production of many fermented beverages (wine, beer, ciders) in terms of improving pH, enhancing aroma and improving colour stability. Reducing pH makes it easier to use lower concentrations of sulfites while offering better microbial stabilities during aging. Some strains can allow single fermentation in the absence of *S. cerevisiae*.

## Acknowledgements

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# IMPACT OF MICRONUTRIENT LIMITATIONS AND NITROGENOUS STATUS ON YEAST CELL DEATH IN ALCOHOLIC FERMENTATIONS

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**Abstract.** Yeast cell death can occur during wine alcoholic fermentation and lead to sluggish or stuck fermentations. The mechanisms underlying cell death during yeast starvation in alcoholic fermentations remain unclear. In this work we addressed yeast cell death using a conceptual framework from aging studies showing that yeast resistance to starvation can be influenced by the nature of the nutrient limiting cell growth. We examined cell death occurrence considering yeast cells' ability to elicit an appropriate response to a set of nutrient limitations. We show that several micronutrient limitations (oleic acid, ergosterol, pantothenic acid and nicotinic acid) trigger cell death in a nitrogen-dependent manner. We provide evidence that the nitrogen Tor/Sch9 signaling pathway is involved in triggering cell death. In such conditions, yeast cells fail to acquire stress resistance given a restriction at a post-transcriptional level. We have examined the ability of different nitrogen sources to trigger cell death and show that they impact differentially on cell death and that NH4+ had a strong death inducing capacity. Finally, the QTLs approaches allowed the mapping of a set of loci controlling cell death under oleic acid and pantothenic acid starvation consistent with a multigenic control.

## 1. Introduction

During wine alcoholic fermentation, yeast cells can lose their viability, which leads to sluggish or stuck fermentations [1]. Loss of viability during alcoholic fermentation is usually attributed to an insufficient availability of lipids, specifically sterols or unsaturated fatty acids, given that a membrane deficiency in these compounds is thought to alter cell resistance to ethanol (2). Actually the mechanisms leading to cell death in wine alcoholic conditions are unclear. Recent studies on cell death have shown that yeast can die more or less rapidly depending on the conditions that trigger growth stop. An important conclusion from aging studies is that yeast resistance to starvation can be influenced by the nature of the nutrient limiting cell growth [3]. In this work we addressed yeast cell death using a conceptual framework from aging studies showing that yeast resistance to starvation can be influenced by the nature of the nutrient limiting cell growth. We show here that several micronutrient limitations (lipids and vitamins) lead to cell death and stuck fermentations in a nitrogen-dependent manner. We also show that yeast cell death under micronutrient limitation is controlled by the availability of residual nitrogen. Moreover, we observed that cell death is dependent on the nature of nitrogen sources and that some nitrogenous compounds, including ammonium salts, had a high capacity to trigger cell death while others had a reduced impact.

## 2. Materials and methods

### 2.1 Strains

We used the commercial wine yeast Lalvin EC1118®, a *Saccharomyces cerevisiae* strain.

### 2.2 Fermentation conditions

Alcoholic fermentations were carried on in a synthetic medium that mimics a natural grape must with a 230 g/L mix of glucose and fructose. The amount of assimilable nitrogen was provided by amino acids and/or $NH_4^+$ depending on fermentation experiments.

### 2.3 Determination of cell viability

Cell viability was determined by flow cytometry using a C6 cytometer and propidium iodine labeling and analysis using an Accuri cytometer.

## 3. Results and discussion

### 3.1. Micronutrient limitations can trigger cell death when associated with high nitrogen levels

In order to assess whether micronutrient limitations could trigger yeast cell death during alcoholic fermentation, strain EC1118® was set to ferment in a synthetic fermentation medium SM425 (containing 425 mg/L of yeast assimilable nitrogen) with various micronutrient limitations. We examined the response to limitations in ergosterol and oleic acid (given the yeast cell requirements for these two compounds in anaerobiosis), thiamin, biotin, inositol, pantothenic acid and nicotinic acid. The impact of these micronutrient limitations was examined using two levels of assimilable nitrogen. For each micronutrient limitation we used a low level of Nass that permits an identical biomass formation compared to the limiting micronutrient. As shown in Figure 1, when the ergosterol is limited in a high nitrogen level situation, significant cell death is observed. However, when the amount of assimilable nitrogen was adjusted to the minimum required to support growth to an identical degree, the viability of the yeast cells was restored. Cell death under ergosterol limitation is dependent on the level of nitrogen in the medium.

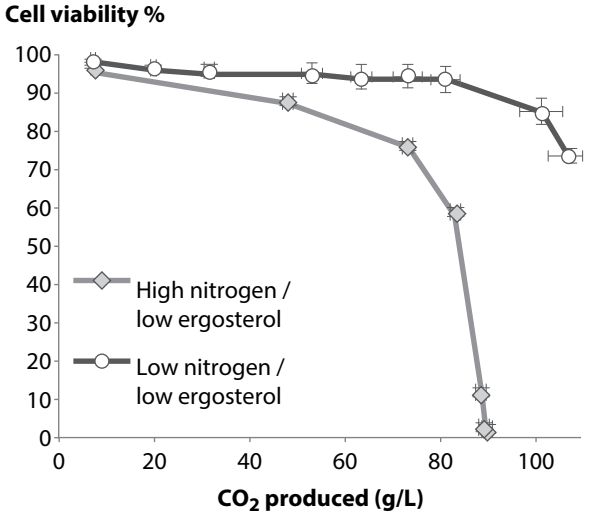


**Figure 1: Impact of nitrogen amount on cell viability in ergosterol-limited fermentation.** Ergosterol was 1.5 mg/L in each situation. Open circle: 71 mg/L of Nass; filled circle: 425 mg/L Nass.

Changes in cell viability were examined in fermentations with other micronutrient limitations. We observed a similar nitrogen-dependent yeast cell death in fermentations that were limited in oleic acid, pantothenic acid and nicotinic acid (data not shown). In each case, lowering the nitrogen level restored yeast viability, indicating that all these cell deaths follow a similar mechanism (4, 5). The amount of assimilable nitrogen modulates yeast cell death associated with micronutrient limitations. These results indicate that residual nitrogen sources are involved in triggering cell death when high nitrogen levels are used. In all these situations, we observed residual nitrogen in the fermentation media.

### 3.2 High nitrogen levels can lead to stuck fermentations in a micronutrient-limited medium

Cell viability has an impact on the fermentation capacity that can be observed on fermentation kinetics. As shown in Figure 2, an ergosterol-limited fermentation was slow when the level of nitrogen was low, but sugars were fermented to completion. However, when the level of Nass was high, the beginning of fermentation was quicker but it led to a stuck fermentation with 40 g/L of residual sugars. Therefore, in this situation a high nitrogen level favoured stuck fermentation. A similar response was observed for each micronutrient limitation leading to cell death.

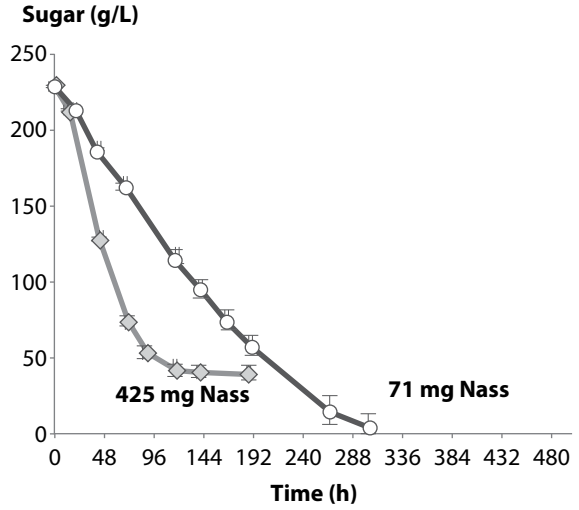


**Figure 2: Impact of nitrogen level on the fermentation kinetic of an ergosterol-limited fermentation.** Sugar profile with 71 and 425 mg/L Nass.

High levels of assimilable nitrogen clearly have a detrimental impact on cell death when associated with limiting the amount of a set of micronutrients. However, not all micronutrients do not have the capacity to trigger cell death in such situations. Biotin, thiamine and inositol limitation did not display such a nitrogen-dependent cell death (data not shown, *PLOS One*).

### 3.3 Micronutrient limitations do not trigger a stress response

To specify the mechanisms involved in cell death we examined the impact of nutrient limitations on the setup of stress resistance in yeast cells. We measured the yeast's acquisition of stress resistance by checking its ability to resist a heat shock. We observed that while in nitrogen-limited fermentations yeast cells acquired a strong heat shock resistance upon entry into starvation, this resistance was not developed when micronutrients were limiting growth (data not shown, *PLOS One*). The lack of stress resistance acquisition overlapped perfectly with the yeast's ability to stay alive in fermentation, indicating that it was critical to fermentation outcome. The setup of stress resistance involves several steps that include both a transcriptional response (corresponding to enhanced expression of stress genes) and post-transcriptional events. Transcriptomic analyses revealed that stress response was correctly set up by all nutrient limitations, including micronutrients. This indicates that the lack of stress resistance in yeast in micronutrient-limited fermentations likely originates from a post-transcriptional control mechanism, which is dependent on the limiting nutrient.

The TOR pathway that senses the nitrogen status of the cell controls the stress response in yeast. Because nitrogen is available in the medium in micronutrient-limited fermentations, nitrogen activates the TOR pathway in these starved conditions. This signalling pathway activation prevents the setup of the stress response, as indicated by the fact that adding Rapamycin, a drug that blocks the TOR pathway, restored a high level of yeast viability in micronutrient-limited fermentations (data not shown, PLOS).

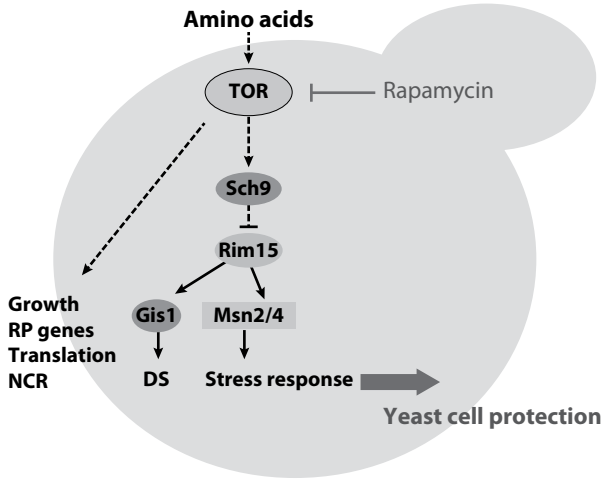


**Figure 3: Sensing nitrogen through TOR pathway and stress response**

### 3.5 Different nitrogen sources have different capacities for triggering cell death

We examined the ability of a large set of nitrogen sources to trigger cell death in micronutrient-limited fermentations. We used the fermentation medium containing a low level (71 mg/L) of assimilable nitrogen provided by a combination of amino acids and ammonium. This medium was used in conditions of oleic acid limitation. To assess the impact of individual nitrogen sources, each of the 19 amino acids or $NH_4^+$ were added to this medium in amounts corresponding to 354 mg/L assimilable nitrogen, for a total of 425 mgL Nass.

The nitrogen sources revealed different capacities for triggering cell death in these micronutrient-limited fermentations. The nitrogen sources were classified according to the cell death intensity capacity. A large group has only a weak ability to trigger cell death and contains the main amino acids available in a grape must in stationary phase (arginine). The two other groups have an average or high ability to induce cell death. The high capacity group likely reflects a toxicity of these amino acids at high levels in such conditions, the bases of which are unknown.

| Low ability | Average ability | High ability |
|---|---|---|
| Alanine | Cysteine | Lysine |
| Arginine | Histidine | Phenylalanine |
| Asparagine | Proline | Tryptophan |
| Aspartic acid | Tyrosine | |
| Glutamine | Ammonium | |
| Glutamic acid | | |
| Glycine | | |
| Isoleucine | | |
| Leucine | | |
| Methionine | | |
| Serine | | |
| Threonine | | |
| Valine | | |

**Table 1: Classification of nitrogen sources by their ability to trigger cell death in an oleic acid–limited fermentation.** The nitrogen sources were added at a level of 354 mg/L Nass to a basal medium 74 mg/L Nass.

The group with an average ability to trigger cell death may be of high relevance in alcoholic fermentation because several sources of these can be found at high levels. As shown in Figure 4, cysteine, histidine, proline and ammonium supplementation led to a loss of viability associated with stuck fermentations, with residual sugars varying from 13 g/L to 40 g/L. The control kinetic (without nitrogen addition) is slow but ferments all the sugars (squares).

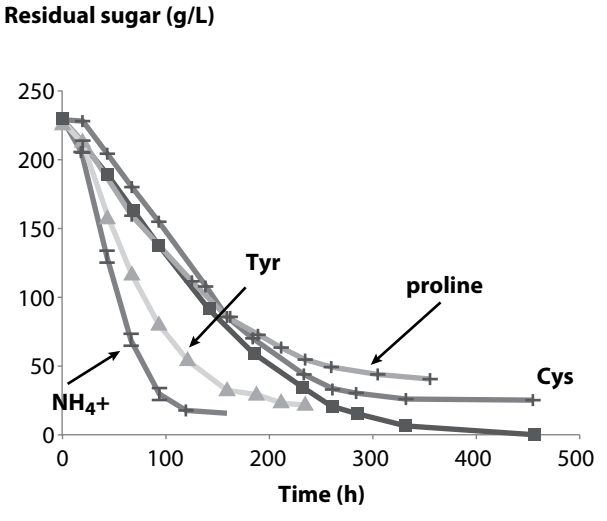


**Figure 4: Impact of nitrogen source on fermentation kinetics.** The control fermentation (squares) contained 71 mg/L Nass to which individual nitrogen source were added at 354 mg/L Nass.

Unexpectedly, proline belongs to the group of sources that can trigger cell death, even though this amino acid is not metabolized during alcoholic fermentation. Its impact may be of relevance given that this amino acid remains in the must throughout all fermentations.

Since we detected that $NH_4^+$ could trigger cell death, we examined whether this phenomenon was triggered in different micronutrient-limited situations. We monitored cells death and kinetics in conditions of ergosterol-, pantothenic acid- and nicotinic acid-limited fermentations. As shown in Figure 5 for ergosterol limitation, the addition of $NH_4^+$ to the must (before the fermentation) triggered strong cell death. A similar response was observed for pantothenic acid and nicotinic acid limitations. Therefore $NH_4^+$ clearly has a strong capacity for triggering cell death in various micronutrient-deficient situations in a nitrogen dependent way.

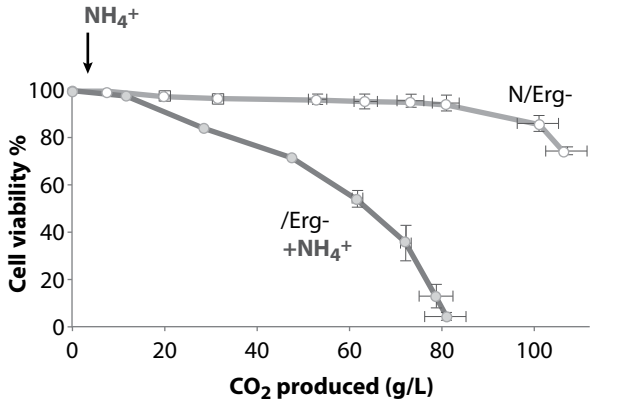


**Figure 5:** Impact of NH4+addition (354 mg/L Nass) to an ergosterol-limited must.

## 4. Conclusion

Understanding the mechanisms underlying yeast cell death is important to improving fermentation management and avoiding stuck or sluggish fermentations. We show that cell death can be triggered by a set of micronutrient limitations when associated with high nitrogen levels. The mechanisms identified in this work can likely explain the occurrence of cell death in many wine making conditions. For example, clarification of grape musts is known to deplete the medium of lipids and probably represents a classic situation of the kinds of micronutrient limitations (sterols, oleic acid–related) that yeast face rather frequently. The occurrence of other micronutrient limitations is less documented but has to be considered in connection with spontaneous microorganism development in must in the early phases of the process. Similarly, the impact of novel practices such as sequential inocula-

tion of yeasts may impact the availability of micronutrients in musts.

Our data shows that nitrogen plays a key role in the conditions that lead to cell death. This potential impact must be taken into consideration when increasing the nitrogen level in grape must. Several nitrogen sources, including $NH_4^+$, display an ability to trigger cell death. In some specific situations we could show that adding high amounts of $NH_4^+$ could have a detrimental impact on the fermentation kinetic. This capacity has to be taken into consideration for must supplementation. We have shown that several amino acids did not display such cell death–inducing capacity, suggesting that organic nitrogen may therefore be more suitable in some circumstances than $NH_4^+$. Our results instead highlight the need to take into account the status of micronutrients to manage nitrogen supplementation during wine fermentation so as to avoid stuck fermentations.

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# HOW TO PREVENT ALTERATIONS DURING VINIFICATION WHILE REDUCING THE USE OF $SO_2$ THROUGH BIOPROTECTION


## Pierre Martini

The studies were carried out by Carole Briffox, Isabelle Daventure, Vincent Gerbaux and Jérome Thomas from the IFV, Villefranche sur Saône Unit, France


### 1. Oenological incidence of grape microflora

Grape microflora is nebulous. We can't see it and it's not determined in practice. Terroir is characterized by a combination of grape variety, soil, exposure, climate conditions and practices. What about the microflora? What influence does it have? This question is increasingly important, especially as fewer sulfites are being added during vinification.

This experiment, carried out during the 2016 vintage on 80 plots from 14 *Domaines* between Dijon and Chagny, was supported by the IFV and the BIVB (interprofessional offices of Burgundy wines) under a regional program called "Au Coeur du vignoble." It aims to improve knowledge of grape microflora. The 80 plots are mainly planted with Pinot Noir (91%). The others are planted with Chardonnay or Aligoté. 14% are organic and the others are conventional.

Due to spring frost, the yield was very different from one plot to another. The sampling of 200 berries per plots was conducted about 7 days before harvest with a good sanitary state. During sampling and crushing, all equipment was disinfected to ensure there was no cross-contamination between batches.

As we can see in figures 1 and 2 (next page), the microflora is mainly composed of non-fermentative microorganisms. In fact, after 9 days, all batches were under 30%, and 60% of them were under 5% of AF, and most were still under 50% of AF after 18 days.

Moreover, 39% of the batches had mold on the surface after 4 to 15 days of incubation. All those batches were below 10% of AF after 18 days.

After 20 days, in order to be sure that the low AF (below 5%) was due to the microflora and not the medium, a yeasting with *Saccharomyces cerevisae* (Lalvin RC212™) was made on these 30 batches (batch 1 on Figure 3, page 49 – See also Table 1 on page 49).

In addition, no growth of *Brettanomyces* was identified up to 4 months after the end of sugar consumption.

This experiment shows that, with good sanitary conditions, grape microflora is contaminated with molds and different types of yeast, but virtually no *Brettanomyces*:

- Batch 2: Presence of yeasts that are very weakly fermentative and very weakly productive of acetic acid (especially *Metschnikowia*)
- Batch 3: Strong presence of yeasts that are weakly fermentative and strongly productive of acetic acid (especially *Hanseniaspora*)
- Batch 4: Succession of yeasts that are weakly fermentative and strongly productive of acetic acid (*Hanseniaspora*) and yeasts that are strongly fermentative and weakly productive of acetic acid (*Saccharomyces*)

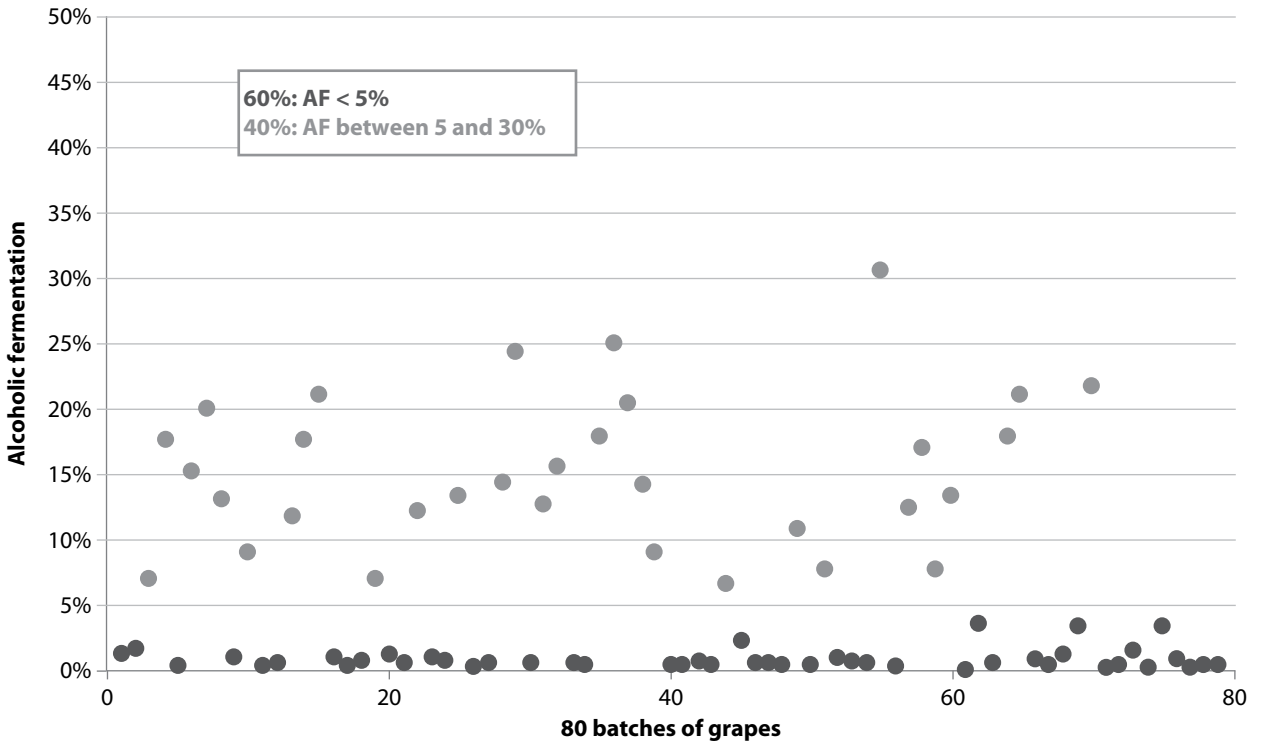


**Figure 1:** State of AF after 9 days at 20°C

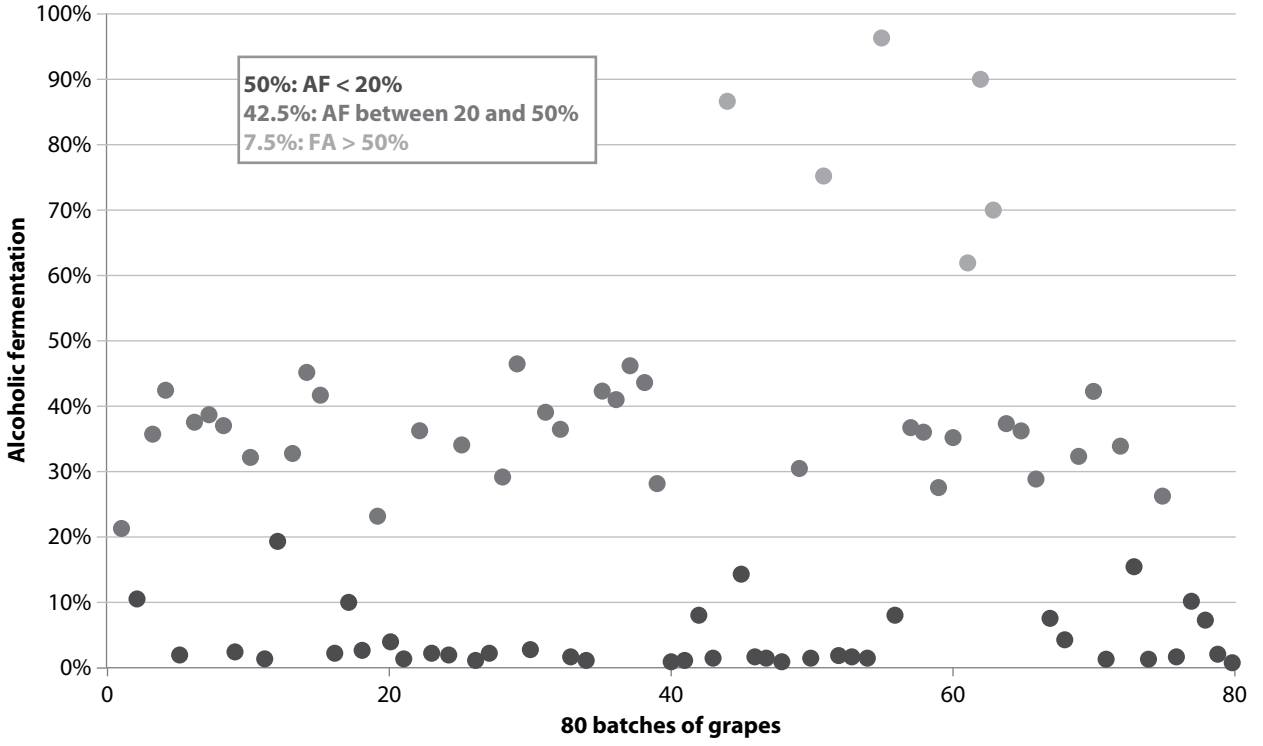


**Figure 2:** State of AF after 18 days at 20°C

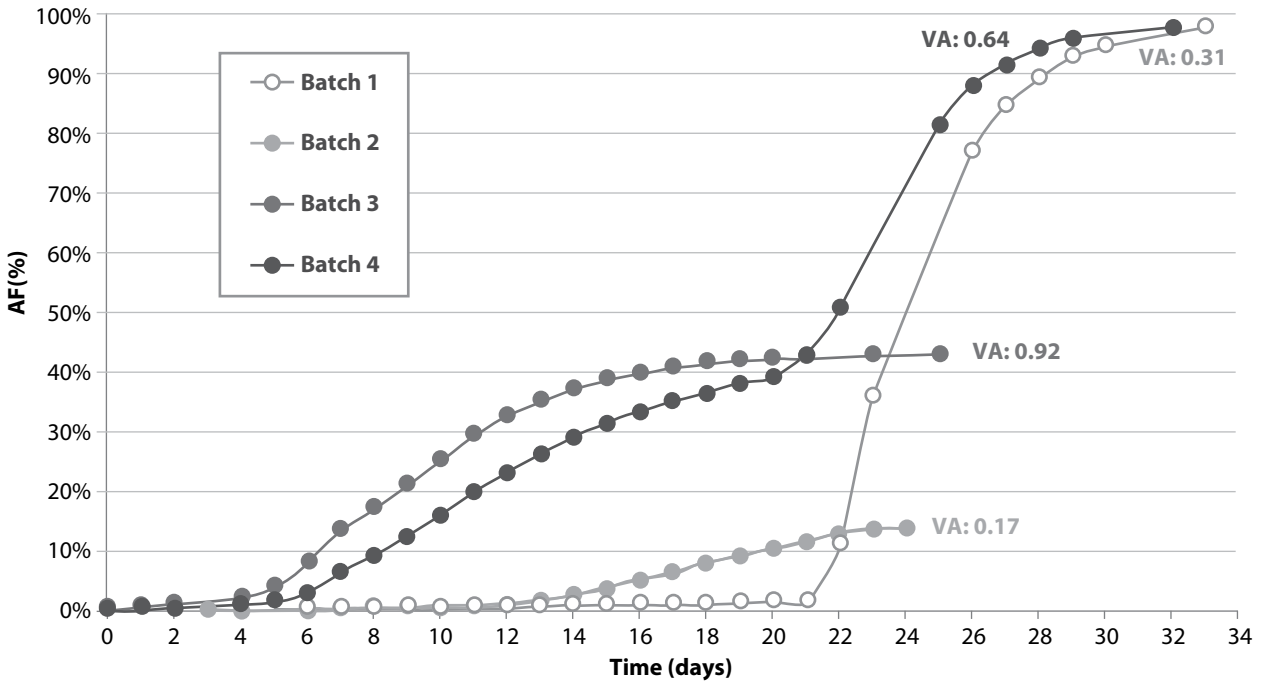


**Figure 3:** Evolution of AF and final acetic acid for 4 plots

Normally, viticulture is about producing the best possible grapes and oenology is about making the best possible wine with them. So there is no explanation why the very specific microorganisms necessary to make the best possible wine (oenology) should be introduced by the grape.

In fact, there is no microbiological continuity between vine growing and oenology. For microorganisms, these are two different worlds. The microorganisms of interest in oenology are introduced either by the cellar and wine-making equipment (cross-contamination) or by inoculation. Cross-contamination could also increase unwanted populations such as *Brettanomyces*.

## 2. Bioprotection of the cold soak – Development of GAÏA™

Pre-fermentation cold maceration (also known as cold soak) is a technique widely used throughout the world for making red wines. The key factors directly influencing the microflora present are temperature and $SO_2$ addition. The temperature is usually 10–15°C, since the presence of solids causes heterogeneity, and current societal and political pressures are forcing producers to reduce their use of $SO_2$.

Furthermore, it has been clearly proved that the dominant grape yeast flora at this stage include yeast strains in the *Kloeckera apiculata* (or *Hanseniaspora uvarum*) species, which are characterized by their great ability to produce acetic acid and ethyl acetate.

Under these conditions, inoculating a non-fermenting yeast of known oenological suitability during cold soak was studied. This has a two-fold objective: to protect the must from undesirable yeast and promote aromatic expression. In order to do that, and after 6 years of study, the Beaune IFV selected a wine yeast *Metschnikowia fructicola*, isolated from Burgundy, for the cold soak of red: Gaïa™.

**Table 1:** Analytic results of batches at end of AF

| AF achievement | < 33% | 33 to 66% | >66% | |
|---|---|---|---|---|
| Batches concerned (total: 80) | 7.5% | 46% | 8.5% | 385 |
| Yeasting (SC) | No | No | No | Yes (at around 20 days) |
| Initial quantity of sugar (g/L) | 203 +/- 9 | 202 +/- 10 | 202 +/- 8 | 200 +/- 10 |
| Alcohol (v/v) | 2.8 +/- 0.4 | 5.1 +/- 0.7 | 11.8 +/- 07 | 11.6 +/- 1.1 |
| Acetic acid (g/L) | 0.19 +/- 0.4 | 0.822 +/- 0.4 | 0.34 +/- 0.4 | 0.38 +/- 0.4 |
| *Hanseniaspora* (Petri dish) | 0 on 6 | 35 on 37 | 0 on 7 | 0 on 30 |

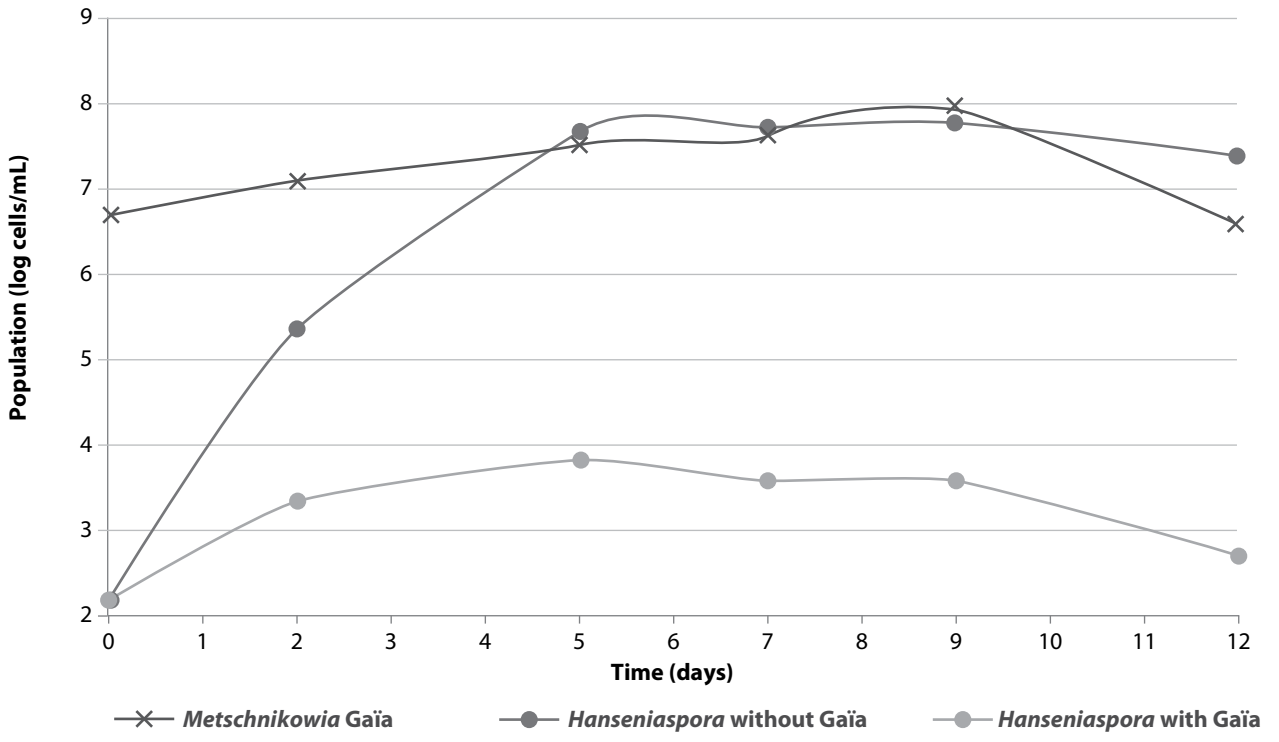


**Figure 4:** Evolution of Hanseniaspora with and without Gaïa™

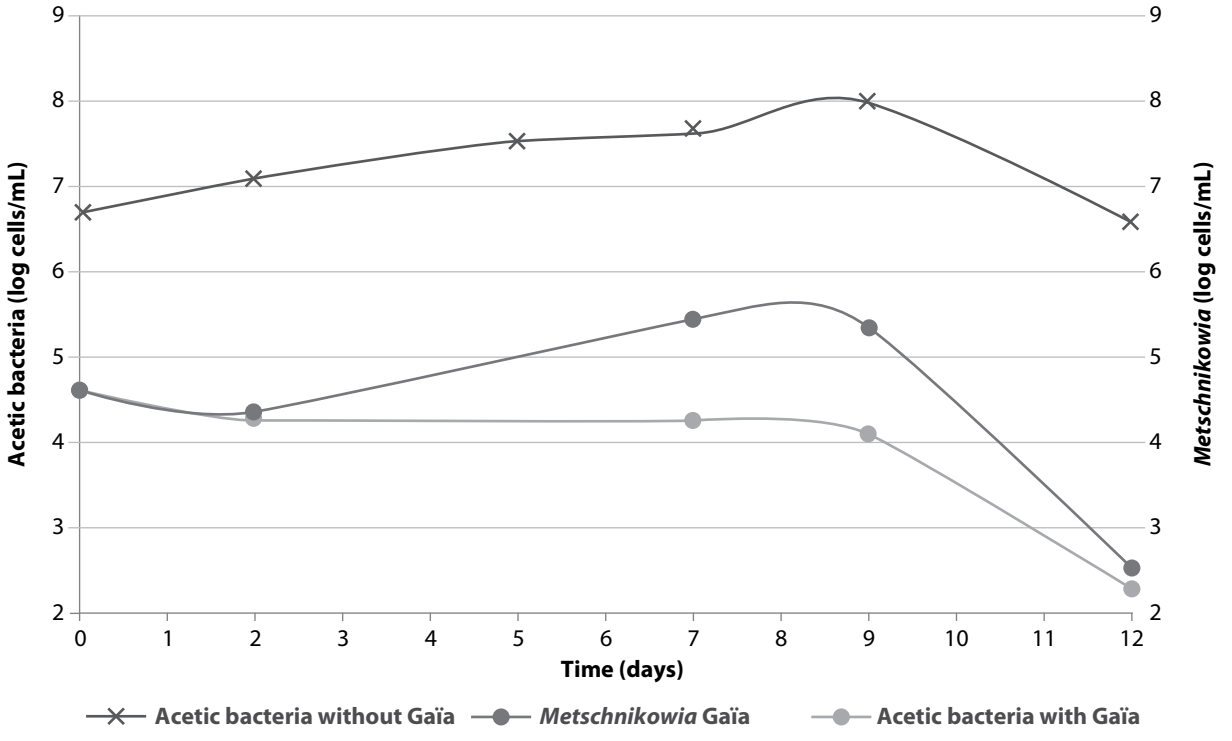


**Figure 5:** Evolution of acetic bacteria with or without Gaïa™

Gaïa™ was selected from a collection of 552 strains, isolated from grapes or unfermented musts between 2002 and 2009 in Burgundy. In 2011, studies were carried out on non-*Saccharomyces* in very small batches (250 mL) on Pinot Noir must. The selection was based on the following criteria:

- Good growth at low temperature
- Very low fermentative ability
- Very low ability to produce acetic acid
- Good aromatic balance

Once these criteria were met, it remained to be seen if this strain would protect must from undesirable yeast. For that, studies were carried out in the laboratory between 2012 and 2016 on pasteurized must of Pinot Noir (no microorganisms in the must at the start).

The study consisted of inoculating the must at T0 with *Hanseniaspora* in 100 cells/mL or with acetic bacteria (*acetobacter* and *gluconobacter*) in 10,000 cells/mL, with Gaïa™ bioprotection added in some batches but not in others. For all batches, after 7 days a yeasting was made with *Saccharomyces,* and the temperature was increased in order to start AF.

Results show an inhibition of *Hanseniaspora* and also of acetic bacteria by Gaïa™. For *Hanseniaspora*, the population was kept under 10,000 cells/L with bioprotection instead of 100,000 000 cells/mL. Similarly, the acetic bacteria population decreased by a lot thanks to bioprotection.

Gaïa™ is very effective at reducing volatile acidity, as shown in the next table. It also prevents the production of VA by *Sacharromyces*. This result was confirmed by another study conducted in 2018 where, after 19 days, the batch with no contamination and an addition of Gaïa™ presented 0.35 g/L of acetic acid at the end of AF versus 0.51 g/L for the batch without contamination and without Gaïa™.

**Table 2:** Concentration of volatile acidity at the end of AF

| Acetic acid (gL) at the end of the AF | Bioprotection with Gaia™ | |
|---|---|---|
| | Yes | No |
| No contamination | 0.31 | 0.49 |
| *Hanseniaspora uvarum* | 0.40 | 0.73* |
| Acetic bacteria | 3.30 | 0.59 |
| * Ethyl acetate: 388 mg/L (Threshold of perception = 140 mg/L) | | |

Finally, studies were conducted at an experimental winery where Gaïa™ was compared with other microorganisms.

Results show that only Gaïa™ allows an effective protection during cold soak. Moreover, after AF no problems or

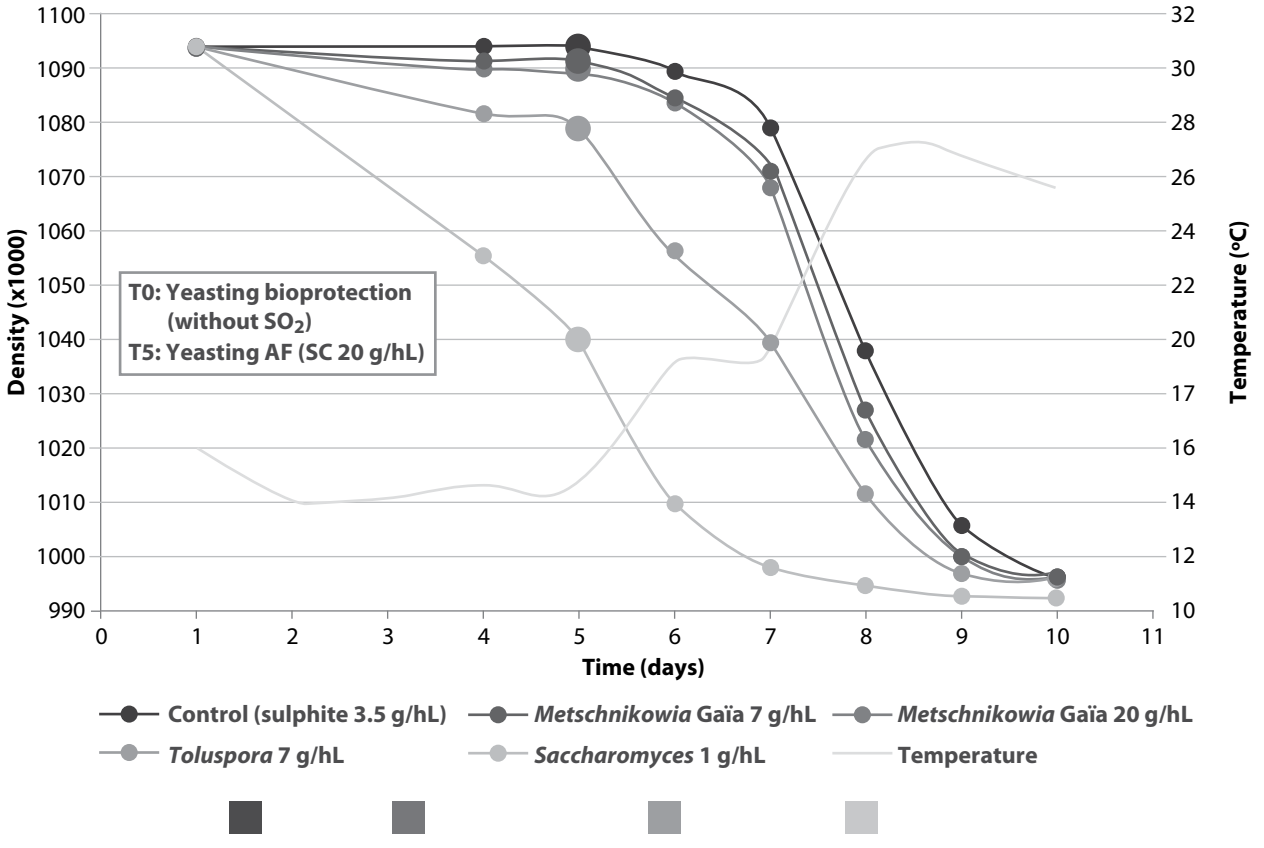


**Figure 6:** Evolution of AF in relation to the microorganism used

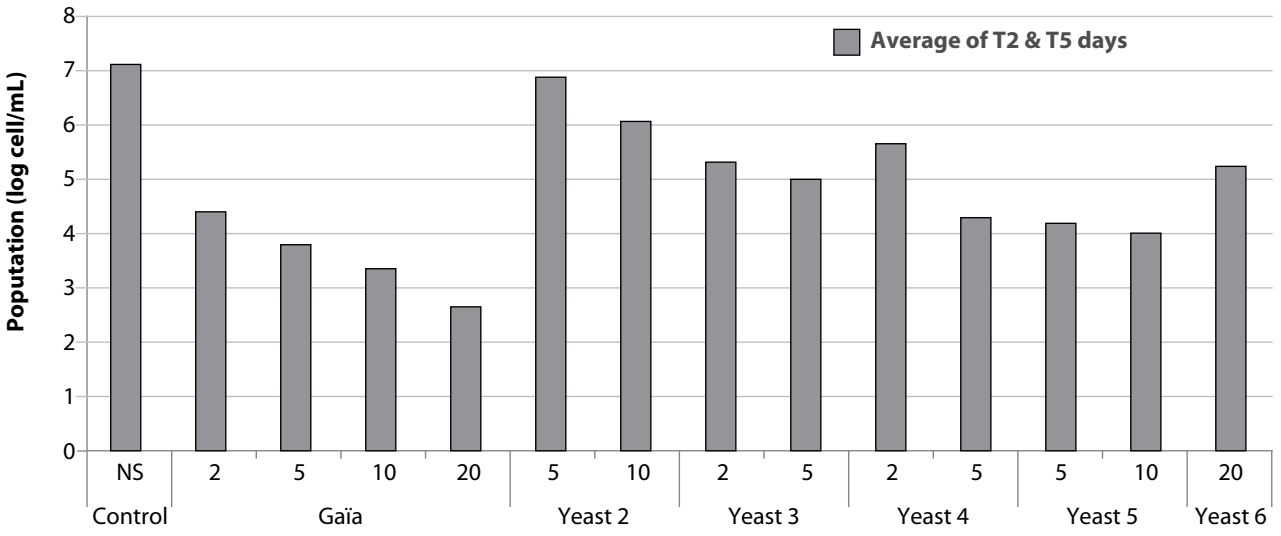


**Figure 7:** Population of *Hanseniaspora* in relation to the inoculation rate of the bioprotection used

analytic differences were noted after bioprotection with Gaïa™ compared to the control. Finally, Gaïa™ consumes very little nitrogen from the must. The nitrogen nutrition must be managed as usual.

In 2018, experiments were conducted to show the impact of the inoculation rate on the development of *Hanseniaspora*. Pasteurized Pinot Noir must was contaminated with *Hanseniaspora* (60 cells/mL) and bioprotection (Gaia™) was added 3 hours later.

As shown in Figure 7, the inoculation rate of Gaïa™ has a real effect on the population of *Hanseniaspora*. At equal inoculation rate, Gaïa™ is more effective compared to other microorganisms. In practice, the inoculation rate will depend of the sanitary level of the harvest.

To conclude, *Metschnikowia* is a grape yeast of interest for ensuring bioprotection during the pre-fermentative phase by inhibiting the development of *Hanseniaspora*, the dominant yeast found on grapes, which is a strong producer of acetic acid. Gaïa™ also prevented the proliferation of acetic bacteria and slowed down the growth of

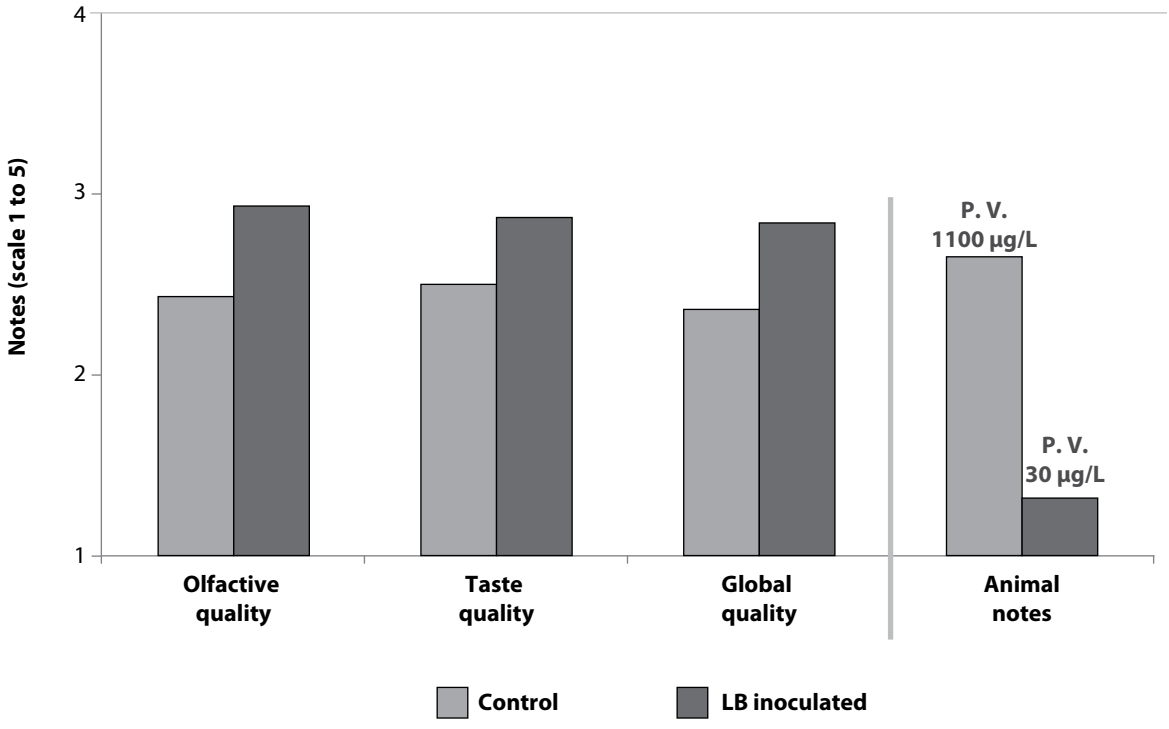


**Figure 8:** Tasting notes with and without addition of lactic bacteria (Pinot Noir contaminated with *Brettanomyces*)

*Saccharomyces cerevisiae* (not presented in results) until it was ready to start the alcoholic fermentation, therefore allowing for the full benefit of the cold soak.

Gaïa™ has good activity in the must at cool temperatures (10 to 15°C/50 to 59°F) and accepts sulphiting dose up to 50 mg/L. Since Gaïa™ is practically non fermentative, it allows for a true cold soak, while allowing winemakers to choose the desired yeast for AF.

Finally, the first objective of bioprotection with GAÏA™ is not to use a yeast to ferment the wine, but to express the best the quality of the grape and its terroir. To ensure this, tastings were conducted from 2013 to 2018, and the results showed no significant differences between the control and the batch protected by Gaïa™.

## 3. Bioprotection during aging – The action of lactic bacteria on *Brettanomyces*

The period between the end of AF and the end of malolactic fermentation (MLF) is critical when it comes to the development of *Brettanomyces*. To minimize this phase, inoculation with selected lactic bacteria is recommended. Today, the use of sulphites is on the decline, and pH values are increasing, which tends to favour *Brettanomyces* over lactic bacteria. Microorganisms are known to interact with each other. And interactions between *Oenococcus oeni* and *Brettanomyces* could be of interest for controling volatile phenols.

The inoculation of lactic bacteria secures the beginning of MLF and, as a result, reduces the occurrence of animal notes (volatile phenols). In other words, mastering MLF limits the amount of time left for *Brettanomyces* to develop and thus prevents the production of volatile phenols.

Between 2016 and 2018, experiments were carried out to understand more about the mechanism behind this. In these studies, only bacteria without cynamyl-esterase activity were selected in order to avoid unnecessary production of the precursors of volatile phenols. Some batches were inoculated one week before the lactic bacteria were inoculated with *Brettanomyces* at 1,000 cells/mL and the other, one week later at 50 cells/mL. Some controls were inoculated but no lactic bacteria were added.

Results show that when *Brettanomyces* are already in the wine in high concentrations (1,000 cells/mL), lactic bacteria do not inhibit the population, but it does help to keep it under a certain threshold (here, 100,000 cells/mL). On the contrary, when *Brettanomyces* are in low concentration (50 cells/mL) or come in after the population of lactic bacteria is established, lactic bacteria can reduce the population, keeping it at almost zero. In this case, the population of *Brettanomyces* declined by nearly 90% in one month.

More than a month after the end of MLF, batches that had been inoculated with a low concentration had no volatile phenol content, compared to the control, which exceeded the perception threshold. In addition, despite the lack of stabilization there was also no significant evolution of volatile acidity, which was limited to around 0.4 g/L $H_2SO_4$ in these batches.

To conclude, inoculation with *Oenococcus oeni* secures the beginning of MLF and helps prevent production of

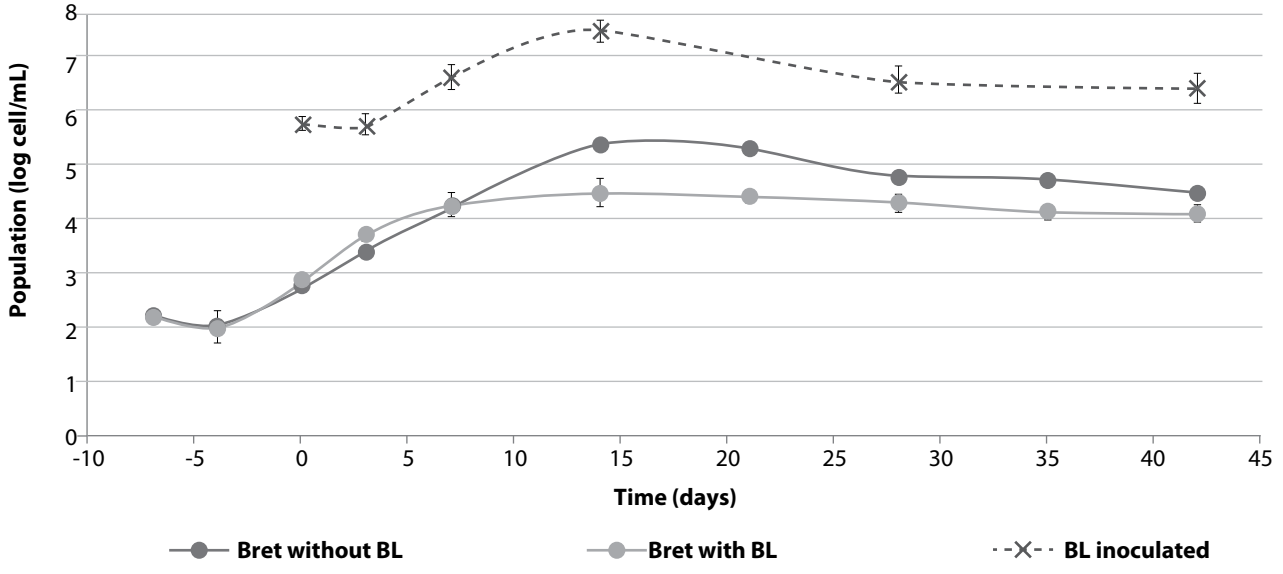


**Figure 9:** Evolution of a high concentration of *Brettanomyces* (Bret) with or without presence of lactic bacteria (Average of 9 strains of BL sowed at T0 with MLF done in less than 14 days)

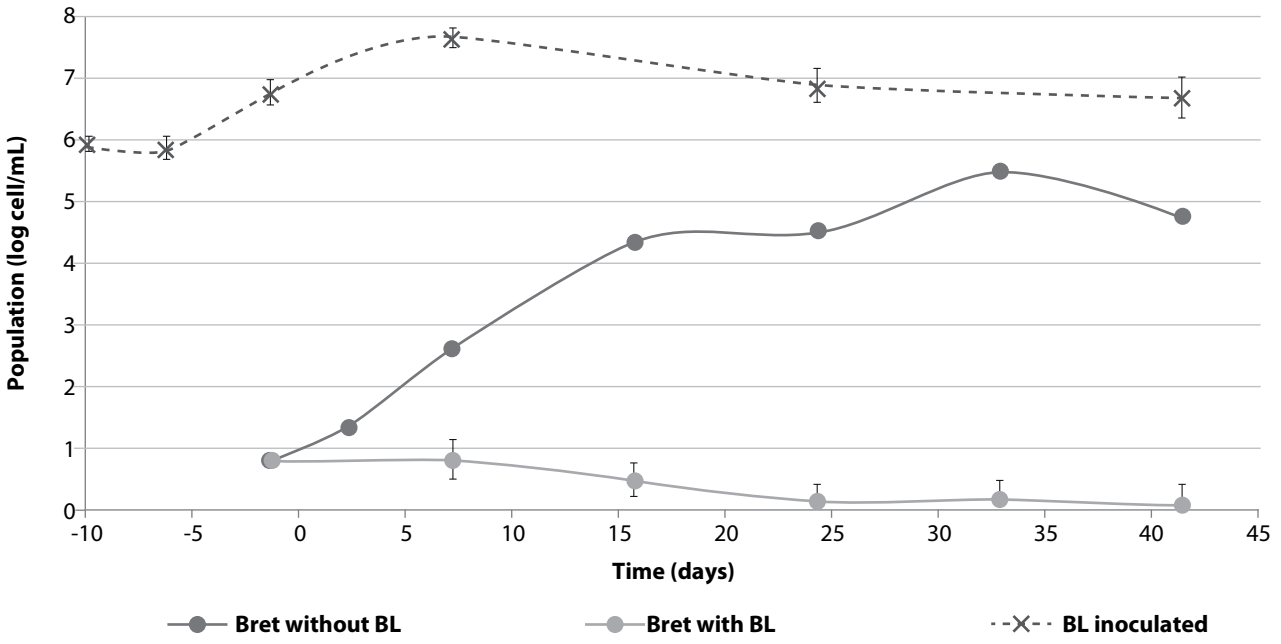


**Figure 10:** Evolution of a low concentration of *Brettanomyces* (Bret) with or without presence of lactic bacteria (Average of 9 strains of BL sowed at T0 with MLF done in less than 14 days)

volatile phenols by *Brettanomyces*. This is due to the fact that lactic bacteria can inhibit *Brettanomyces* populations especially when they are low (<100 cells/ml). No significant evolution of the AV is noticed when lactic bacteria stay in the wine after MLF.

In practice, if there is a high concentration of *Brettanomyces* before MLF, it's recommended to do some co-inoculation in order to limit the time between the end of AF and the beginning of MLF.

With low concentrations of *Brettanomyces*, lactic bacteria can be used for bioprotection (advantage for long aging). In this case, it's advised to work on perfectly dry wines (no residual sugars) at a low temperature (15°C) in order to have a longer MLF, which yields a higher population of lactic bacteria. Moreover, really good hygiene is required, and AV checking will help manage the aging.

# EVIDENCE BEHIND THE EFFECTIVENESS OF GLUTATHIONE-RICH SPECIFIC INACTIVATED YEAST IN PRESERVING WINE

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## Abstract

Glutathione is known as a key compound in the oxidative stability of wine. Scientists highlighted that glutathione-rich specific inactivated yeasts have a greater effect than only increasing the pool of available glutathione.

The recent application of liquid chromatography coupled with mass spectrometry in the wine industry helped paint a comprehensive picture of wine composition and thus the emergence of new evidence behind the use of specific inactivated dry yeasts (IDY). In this study, not only do we establish the difference between a wine with or without the use of IDY during alcoholic fermentation but also the specificity of one innovative glutathione-rich specific inactivated yeast ("GPlus-IDY") out of the IDYs studied.

The GPlus-IDY showed a higher diversity and relative abundance in potentially active compounds such as thiol or sulfhydryl compounds, and 45 unique peptides have been evidenced. We evaluated the efficiency of different IDYs both by the concentration of reductive compounds released and the ability of these compounds to catch oxidants. The aroma protection efficiency of the GPlus-IDY added before fermentation is also noticeable in real Sauvignon Blanc wine experiments in 2017 and 2018 vintages on 3-mercapto-hexanol (3MH) and its acetate (3MHA).

Finally, the GPlus-IDY showed high potential to improve wine's natural resistance to oxidative damage such as browning and aroma loss. This specific inactivated yeast naturally improves the pool of reductive compounds in wine and thus helps reduce the use of sulfites as a chemical antioxidant.

## 1. Introduction

### 1.1. Traditional wine preservation

Wine preservation can be defined as the wine's ability to maintain its genuine characteristics after bottling for a defined time. The composition of wine greatly impacts its aging potential, meaning the period of time in which a wine can be consumed without the appearance of olfactory or gustatory defects. Wine preservation is of interest to winemakers since it fits with consumer expectations. Traditional additives are commercially available to increase the aging potential of wine.

Sulfur dioxide ($SO_2$) is the most widely used additive in winemaking. This substance combines two important beneficial properties for winemakers: antimicrobial and antioxidant activity. The overall mechanism of action of $SO_2$ as an antioxidant has been studied for years to elucidate the exact way to protect wine. It is now known that not only oxygen, but also iron and copper are involved in oxidative reactions[1]. Briefly, the presence of iron and copper (average concentration in wine of 5.5 mg/L and 0.3 mg/L, respectively) leads to Fenton reactions, which produce hydrogen peroxide, a strong oxidant leading to acetaldehyde accumulation. Metals in the presence of oxygen can catalyze the oxidation of polyphenols containing a catechol system to a highly electrophilic quinone. Some of the more readily oxidizable polyphenols are caffeic acid, catechin and epicatechin. Oxidized polyphenols are apt to polymerize and cause browning (especially visible in white wines). Sulfites can both limit catechol oxidation and perform a nucleophilic addition to block the chain reaction of polymerization and browning (Figure 1).

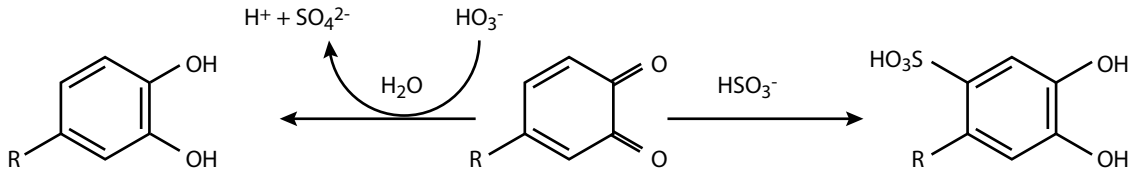


**Figure 1:** *Possible reactions of sulfite ions with quinones* [1]

The nucleophilic addition can also occur with numerous nucleophilic compounds such as thiol or amine, which are naturally present in wine. Unfortunately, thiol compounds are often strong varietal aromas such as 3-mercapto-hexanol (3MH) or 3-mercapto-hexyl-acetate (3MHA) in white Sauvignon wine. The nucleophilic addition of these compounds on quinone inhibits their aromatic properties and leads to an overall loss of aromas in wine.

## 1.2. How IDY could improve wine stability

Since many sulfur-containing compounds could act as reductants[2], the idea to improve the natural pool of these compounds in wine was considered. One of the most studied sulfur-containing compounds in cells is glutathione. This tri-peptide is present in almost all eukaryote organisms and thus in grapes and yeasts. Inside the cell, it acts as a strong reducing compound and helps maintain the cell's redox potential. Several studies have shown the ability of glutathione (and other sulfhydryl compounds) to react with electrophilic compounds in wine such as caftaric acid and coutaric acid[3,4]. The investigation of the impact of IDY (and especially IDY naturally rich in glutathione, GSH-IDY) on wine stability and aroma preservation is very recent. Andujar-Ortiz showed in 2010 the positive impact of soluble fraction from GSH-IDY on rosé wine volatile aromas and later in colour preservation, phenolic composition and sensory properties[5–7]. Beyond aroma preservation, it also showed that GSH-IDY led to a lower accumulation of furfural, an oxidative marker. This could be related to the higher amount of available glutathione in must and wine when GSH-IDY is used during fermentation[8]. The authors noticed that other compounds released by GSH-IDY (and not only GSH) could explain the action of this yeast derivative. Rodriguez-Bencomo highlighted the potential of many sulfur-containing compounds released by G-IDY as potential terpene protectors in model wine during accelerated aging[9]. Today, powerful analytical chemistry has shed light on the chemical composition of complex matrices and is thus a promising tool for discovering the soluble fraction of GSH-IDY, which is still poorly understood.

## 1.3. The potential of metabolomics for oenology

As defined by the metabolomics society, metabolomics is "concerned with the comprehensive characterization of the small molecule metabolites in biological samples. It can provide an overview of the metabolic status and global biochemical events associated with cellular or biological systems." One of the most commonly used instruments for metabolomics is mass spectrometry, which is increasingly trendy in non-medical sciences (especially as these instruments have become much more affordable). The main principle of mass spectrometry is to measure the intensity of the exact mass/charge ratio (m/z) of molecules ionized by different ionization sources. From the exact mass/charge ratio it is then possible to calculate raw formula and to annotate ions. The hyphenation of mass spectrometry with liquid chromatography makes it possible to separate isomeric compounds (same raw formula but different structure) and thus have access to the real diversity of compounds present in the sample.

In oenology, the use of metabolomics has become increasingly common, with several studies exploring the interest of untargeted analysis on characterization and discrimination of wines samples[10–12]. Metabolomics also enables the analysis of specific fractions such as the sulfur-containing fraction in wine[13] and in yeast derivatives[9], as well as correlations with sensory analysis[14]. This analytical tool is of great interest when looking at the characterization of inactivated yeasts, or their impact on the wine metabolome.

This study is aimed at characterizing the compounds released by three different inactivated yeasts, which differ in the concentration of glutathione released in solution. The characterization is performed from a chemical point of view in terms of the nature and diversity of the compounds released and in terms of chemical reactivity, looking at the potential relations between the chemical composition and the oxidative stability of the matrix. This scientific statement looks at the possibility of using inactivated yeast naturally rich in glutathione as a source of reductive compounds to help protect sensitive compounds in wine.

## 2. Material and methods

### 2.1. Material studied

Three specific inactivated dry yeasts were obtained from Lallemand SAS (Blagnac, France). These products were produced at a laboratory scale to optimize the chemical, physical and nutritional features of the bio-process in order to maximize the intracellular concentration of metabolites, notably glutathione. Two products were specifically produced from different yeast strains to increase the bioavailability of glutathione (G-IDY and Gplus-IDY, which release 17 mg and 25 mg of glutathione per gram of IDY, respectively). The third product (N-IDY) was produced with the same strain as G-IDY without following the specific process of GSH accumulation and releasing 6 mg of glutathione per gram of IDY. IDYs were resuspended at 4 g/L in 12% (*v/v*) ethanol in ultrapure water with 0.01% (*v/v*) formic acid at pH 3.2, and soluble fractions were obtained after 1 hr. of stirring at room temperature in the dark. Samples were then centrifuged (12,000 g, 5 min, 4°C) and the supernatants were aliquoted and stored under nitrogen at 4°C until analysis. All samples were prepared in triplicate.

Other yeast derivatives (namely RO1, RO2, RO3, RO4), differing in production process, solubility, glutathione concentration, etc., were used in order to compare yeast derivative products in a chemical stability assay. The soluble fractions from these products were obtained exactly in the same manner as the previous inactivated dry yeasts.

### 2.2. Metabolomic analysis

Ultra-high-resolution mass spectra were acquired in negative mode on a Bruker SolariX Ion Cyclotron Resonance Fourier Transform Mass Spectrometer ((-)FT-ICR-MS) (BrukerDaltonics GmbH, Bremen, Germany) equipped with a 12 Tesla superconducting magnet (Magnex Scientific Inc., Yarnton, GB) and an APOLO II ESI source (BrukerDaltonics GmbH, Bremen, Germany). 20 µL IDY-soluble fraction was diluted in 1mL of pure methanol and then injected at a flow rate of 120 µL/h into the microelectrospray. Spectra were acquired with a time-domain of 4 megawords over a mass range of *m/z* 147 to 2000. A total of 300 scans were accumulated for each sample. All samples were injected randomly in the same batch to avoid batch variability. External calibration was done with clusters of arginine (10 mg/L in methanol). Internal calibration was performed for each sample by using yeast ubiquitous compounds for negative mode[15]. External and internal calibration led to a day-to-day mass accuracy better than 0.1 ppm. (-)FT-ICR-MS data was processed with DataAnalysis (v. 4.3, Bruker Daltonik GmbH). Calibrated data was filtered to keep only m/z peaks with a signal to noise (S/N) ratio above 10 and an absolute intensity higher than $2.0x10^6$. Peak alignment was performed by Matrix Generator software (v. 0.4, Helmholtz-Zentrum Muenchen) with a mass accuracy window of 1 ppm[16]. Peaks with intensity equal to 0 in more than 80% of samples were removed from the analysis. Finally, the in-house software NetCalc 2015 (v. 1.1a, Helmholtz-Zentrum Muenchen) was used to annotate peaks[17]. 46% of the initially aligned peaks were annotated by NetCalc and used for this study. Van Krevelen diagrams, which plot the H/C against the O/C ratio of annotated metabolites, were generated by an Excel file, providing instantaneous chemical pictures of metabolite diversity[18,19]. The OligoNet webserver was also used to annotate potential peptides with a maximum error of 1 ppm[20].

### 2.3. Preservation capacity assay

#### *2.3.1. Radical scavenging capacity*

The radical scavenging activity assay was specifically adapted for the wine conditions described in a recent publication[2]. Briefly, a solution of stable 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH) was prepared at 25 mg/L in a specific buffer at pH 3.6. The methanol buffer was a mix of 60:40 (*v/v*) methanol in aqueous mixture of 0.1 M citric acid and 0.2 M phosphate disodium.

100µL of the sample at different concentrations (diluted with ultrapure water) was spiked in 3.9mL of the DPPH methanol buffer solution in an oxygen-free atmosphere. After 240 min, the absorbance of the solution was read at 525 nm via spectrophotometer and compared to the absorbance of 3.9 mL of DPPH spiked with 100 µL of water (blank sample). Depending on the nature of the sample, the result can be expressed as the mass ratio (equation 1) or volume ratio (equation 2) needed to decrease the absorbance of the blank sample to 20% ($Rm_{20\%}$ and $Rv_{20\%}$, respectively).

$$Rm_{20\%} = \frac{m_{DPPH}}{m_{Sample}} = \frac{Abs_{100\%} - Abs_{80\%}}{K} = \frac{20}{K} \quad (1)$$

$$Rv_{20\%} = \frac{V_{DPPH}}{V_{Sample}} = \frac{Abs_{100\%} - Abs_{80\%}}{K} = \frac{20}{K} \quad (2)$$

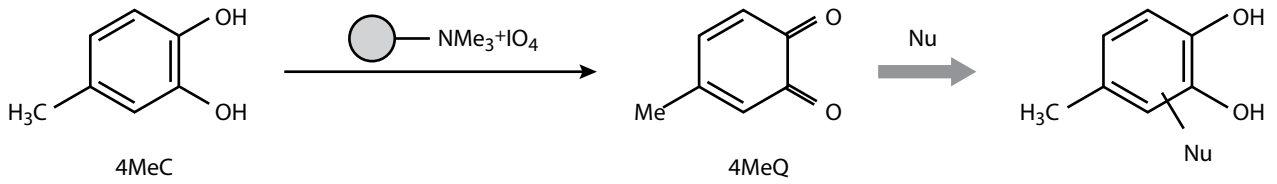


**Figure 2:** Oxidation of catechol by periodate resin leading to quinone production and the results after nucleophilic addition.

Where K is the linear regression coefficient between the absorbance (expressed in % of the initial absorbance) and the corresponding Rm (or Rv).

### *2.3.2. Nucleophilic activity*

The nucleophilic activity assay is based on the ability of the compounds from the IDY-soluble fraction to perform nucleophilic addition on specific electrophilic compounds. This reaction (called derivatization) was performed using an adaptation of the protocol described by Nikolantonaki et al[21]. The electrophile was produced by mixing 4-methyl-catechol (4MeC) with periodate resin, which produces *o*-quinone: 4-methyl-1,2-benzoquinone (4MeQ) (Figure 2).

50µL of 4MeQ was added to 1 mL of sample to reach a final concentration of 1 mM 4MeQ. After 30 min of reaction at room temperature, 1.5 mM of $SO_2$ was added to reduce the remaining 4MeQ in the sample. The addition of *o*-quinone in excess makes it possible to derivatize all the nucleophilic compounds present in the soluble fraction of the yeast derivatives. Samples were then analyzed by high resolution UHPLC-Q-ToF-MS as follows: separation was performed with ultra-high-performance liquid chromatography (Dionex Ultimate 3000, ThermoFischer) coupled to a MaXis plus MQ ESI-Q-ToF mass spectrometer (Bruker, Bremen, Germany). The non-polar and low polar metabolites were separated in reversed phase liquid chromatography by injecting 5 µL in an Acquity UPLC BEH $C_{18}$ 1.7 µm column 100 x 2.1 mm (Waters, Guyancourt, France). Elution was performed at 40°C by (A) acidified water with 0.1% (*v/v*) of formic acid and (B) acetonitrile with 0.1% (*v/v*) of formic acid with the following gradient: 0–1.10 min 5% (*v/v*) of B and 95% (*v/v*) of B at 6.40 min. The flow rate was set at 400 µL/min and maintained for 5 min. at initial conditions before each injection. Solvent and analytes were ionized with an electrospray (Nebulizer pressure = 2 bars and nitrogen dry gas flow = 10 L/min). Ion transfer was done with an end plate offset at 500 V and transfer capillary voltage at 4500 V. A divert valve was used to inject four times diluted ESI-L Low Concentration Tuning Mix (Agilent, Les Ulis, France) at the beginning of each run, allowing a recalibration of each spectrum. The mass spectrometer was calibrated with undiluted Tuning Mix before batch analysis in enhanced quadratic mode, with less than 0.5 ppm errors after calibration. Spectra were acquired on the 100 to 1500 *m/z* mass range, in positive ionization mode.

Two sets of samples were prepared and analyzed in a random order, one set with the native samples and a second set with samples where electrophilic compounds were added. After acquisition, calibration of each spectrum, peak picking and alignment of the features (meaning couple of *m/z* and retention time), supervised analyses were performed. New features found after addition of electrophile potentially corresponded to the results of nucleophilic addition in 4MeQ. These compounds were then specifically extracted and compared in diversity and abundance between samples.

## 2.4. Wine fermentation

### *2.4.1 Laboratory-scale Chardonnay fermentation*

250 mL of Chardonnay must from the Languedoc area (Montpellier, France) harvested in 2017 was used to perform fermentations with or without IDY. N-IDY, G-IDY and Gplus-IDY used directly at 4g/L (total fraction), or the soluble fraction corresponding to 4g/L was added to the must. After 1 hr. of contact, selected active dry yeasts (Lalvin QA23™, Lallemand) were inoculated to perform the alcoholic fermentation.

The weight of each fermenter was monitored twice a day until reaching constant for 2 days. At this time, Foss instruments were used to check the residual concentration of sugars and the classic oenological parameters (pH, volatile acidity, total acidity, malic acid, sugars) before malolactic bacteria inoculation (Lalvin VP41™, Lallemand). Malolactic fermentation was performed in closed 200 mL Schott with small head space. The decrease of malic acid was first monitored with Foss instruments, then with enzymatic kits once the Foss limits of quantification were reached. At the end of fermentation, the wines were stored in 20 mL in a dark, oxygen-free environment until analysis. All these conditions were made in triplicate, and

metabolomic profiles were compared to one of the control wines without any IDY addition.

#### *2.4.2 Pilot-scale Sauvignon Blanc fermentation*

Trials comparing G-IDY and Gplus-IDY treatments to a control tank were made in 2018 in the Val de Loire area. In this trial, 40 g/hL of G-IDY or Gplus-IDY was added during settling (12 hr., 5°C) and then inoculated with selected active dry yeast. After fermentation, the wines were bottled after addition of sulfites, and aromatic thiols (3-mercapto-hexanol, 3MH; 3-mercapto-hexyl acetate, 3MHA) were analyzed after bottling for the control and the two wines treated with IDY. In addition, DPPH activity was also assessed after bottling.

## 3. Results & discussion

### 3.1. Metabolomic diversification

Metabolomic analysis is a powerful tool for observing metabolic modifications of organisms under different environments. In our conditions, it enables us to see the impact of the bio-process leading to glutathione accumulation in the global metabolism of the yeast cell. This approach doesn't give any clues about genetic expression modification during the process or the impact of the inactivation and drying processes, only the final metabolic fingerprint is observed. Since the process to inactivate and dry the yeast cells is the same for the three products, we assume that all the metabolic differences are due to the bioaccumulation process or the yeast strains. Nevertheless, it is likely that some of the observed metabolites appear during the inactivation and drying steps of the production process.

The ultra-high accuracy of (-)FT-ICR-MS makes it possible to determine the exact mass of ionizable compounds present in complex matrices, including wine, which provides a more comprehensive picture of the chemical diversity present in the sample[10,13]. Network annotation of the *m/z* ions present in IDY-soluble fraction by NetCalc software gave access to the molecular formulas of 53% of the 1,674 ions detected. The extensive chemical differences between G-IDY, Gplus-IDY and N-IDY were clearly visible from the histograms depicting the distribution of elemental compositions (CHO, CHON, CHOS, and CHONS), along with Van Krevelen diagrams of the (-)FT-ICR-MS derived molecular formulas (Figure 3). Figure 3A presents the 379 annotated *m/z* ions (42.7%) common to the three IDY-soluble fractions. These ions could be considered as representative of extractable metabolites from IDYs, whatever the strain or production process. Common ions were found in different chemical spaces such as lipid-like, peptide-like and saccharide-like domains, which is in line with a glutathione accumulation bioprocess that preserves the yeast's basic metabolism during production.

Figure 3B, 3C and 3D show annotated masses unique to each IDY, coloured according to their chemical compositions and sized according to their mass peak relative intensity. An overview of the Van Krevelen diagrams reveals significant differences between the samples in terms of the number of unique formulas and chemical families. N-IDY (Figure 3D) appears much richer in unique CHO-containing formulas (16) than G-IDY (3) and Gplus-IDY (0). These formulas are mainly in peptide-like and lipid-like domains, which could correspond to short-chain fatty acids, for example. In contrast, Gplus-IDY (Figure 3B) is characterized by a significantly higher number of unique CHONS-containing formulas compared to G-IDY and N-IDY (36 vs. 3 and 1, respectively). These formulas, mainly located in the peptide-like domain, could correspond to peptides with sulfur-containing amino acid residues, such as methionine and cysteine. The high diversity of sulfhydryl-containing compounds (-SH group) could explain the relative activity of these products against oxidation, as it is known that peptides and thiols could have antioxidant properties in wine[22–24]. It is remarkable to note how the glutathione enrichment process, which is designed to accumulate intracellular glutathione, is actually accompanied by an overall increase of the CHONS/CHO ratio when going from N-IDY to Gplus-IDY, with G-IDY potentially releasing 3 times more CHONS compounds than N-IDY, and Gplus-IDY releasing more than 10 times more compounds than N-IDY. With a moderate hypothesis of 3 isomers per (-)FT-ICR-MS ion, these results together show that Gplus-IDYs would be discriminated by more than 100 different N,S-containing compounds, compared to G-IDY and N-IDY, thus providing an unprecedented molecular representation of the actual metabolic response of glutathione enrichment. The relatively low number of unique compounds released from G-IDY is not surprising since it is obtained from the same strain as N-IDY, and it follows the same bio-process as Gplus-IDY, thus most of the released compounds are likely shared with at least one other IDY. Although it was not the aim of this study, it is further interesting to note the strain-dependency of the glutathione enrichment process, with Gplus-IDY releasing nearly 4 times more CHONS compounds than G-IDY, while the released glutathione is increased by roughly 2.

The presence of cysteine in the growth medium during IDY GSH accumulation[25] modifies the global metabolism of sulfur amino acids and leads to overrepresentation of

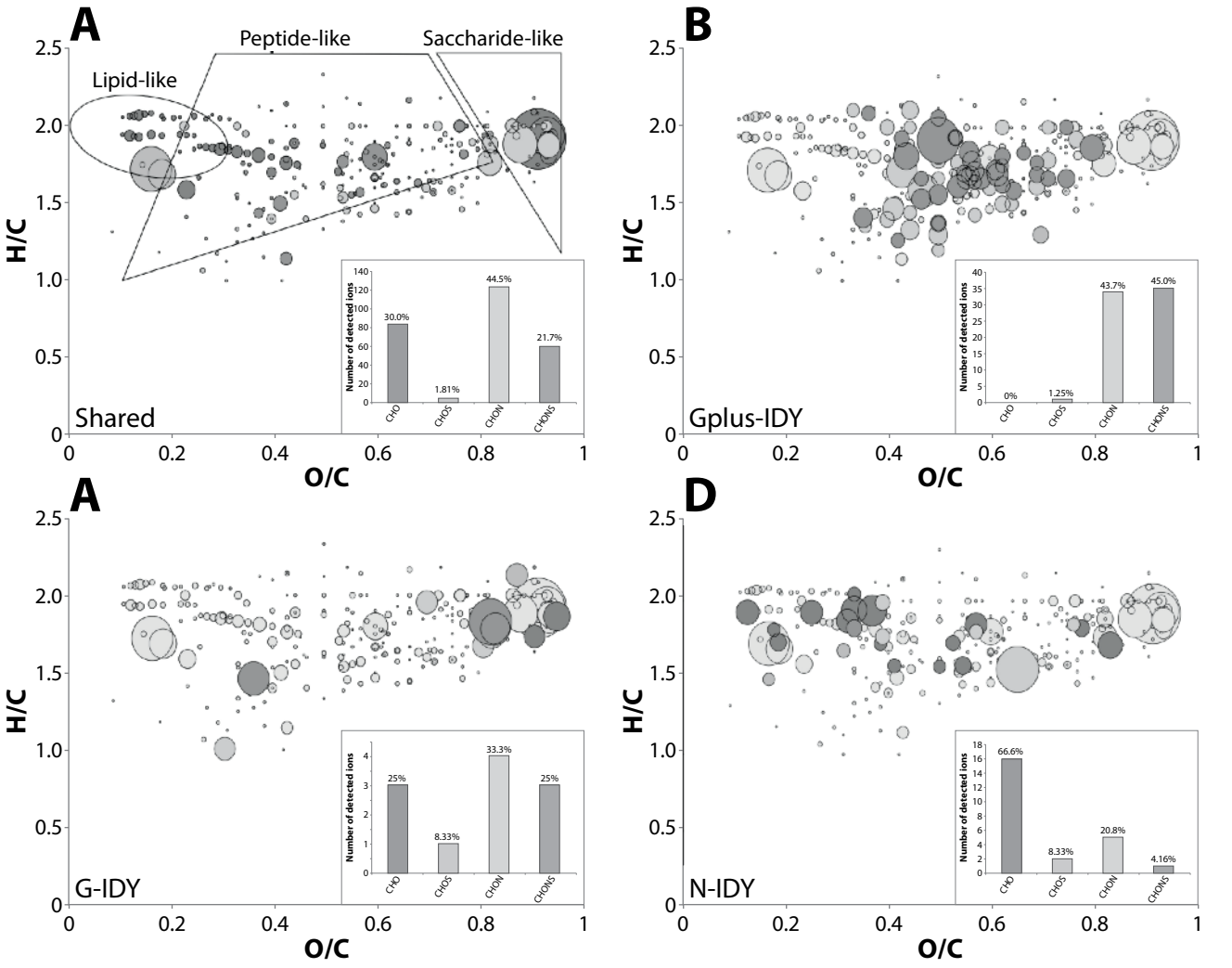


**Figure 3:** Van Krevelen representation of annotated masses from (-)FT-ICR-MS analyses of the MWSFs (Model Wine Soluble Fraction) of Gplus-IDY, G-IDY and N-IDY. (A) masses shared by Gplus-IDY, G-IDY and N-IDY MWSFs. (B) Specific m/z ions to Gplus-IDY , (C) specific m/z ions to G-IDY, and (D) specific m/z ions to N-IDY. In B, C and D, common m/z ions were depicted in grey. Van Krevelen plots were coloured according to molecular classes i.e., CHO ( ), CHOS ( ), CHON ( ), CHONS ( ). Bubble sizes correspond to the triplicate averaged relative intensities of m/z peaks. Bar histograms indicate the number (and corresponding percentage at the bar-top) of molecular formulae presented in the Van Krevelen diagrams.

sulfur-containing metabolites[26]. As the Van Krevelen diagrams highlight the increasing diversity of CHON- and CHONS-containing compounds along with the enrichment process, this diversity could be putatively attributed to peptides containing cysteine or methionine residues. Of the 1674 *m/z* submitted to the OligoNet webserver, 193 were annotated as potential peptides (from 2 to 5 residues) with an error below 1ppm. Within the 193 annotated ions, 132 have a Multiple Amino Acids Combination (MAAC) and 61 a Unique Amino Acids Combination (UAAC) (Figure 3). Most of the peptides (144) are common to at least two out of three IDYs, whereas Gplus-IDY presents the greatest diversity with 40 unique peptides versus 7 and 2 for G-IDY and N-IDY, respectively. Among the UAAC, 26 out of 65 contain a cysteinyl residue. These unambiguous annotations of peptides made it possible to determine all possible connections between these peptides. The Gplus-IDY clearly released more unique peptides and more peptides with a cysteinyl residue. Nevertheless, most of the UAAC are shared between the IDYs (regardless of their relative concentrations). The global similarity between our samples analyzed by (-)FT-ICR-MS allowed us to compare the absolute intensity between samples, providing an indication of the abundance of each compound released. Within the 61 UAAC released by the IDY, Gplus-IDY released 28 peptides significantly more intense than G- and N-IDY (3 peptides and 1 peptide, respectively). These results are in accordance with the Van Krevelen diagram (Figure 3B), showing a higher diversity of compounds in the peptide-like domain for Gplus-IDY.

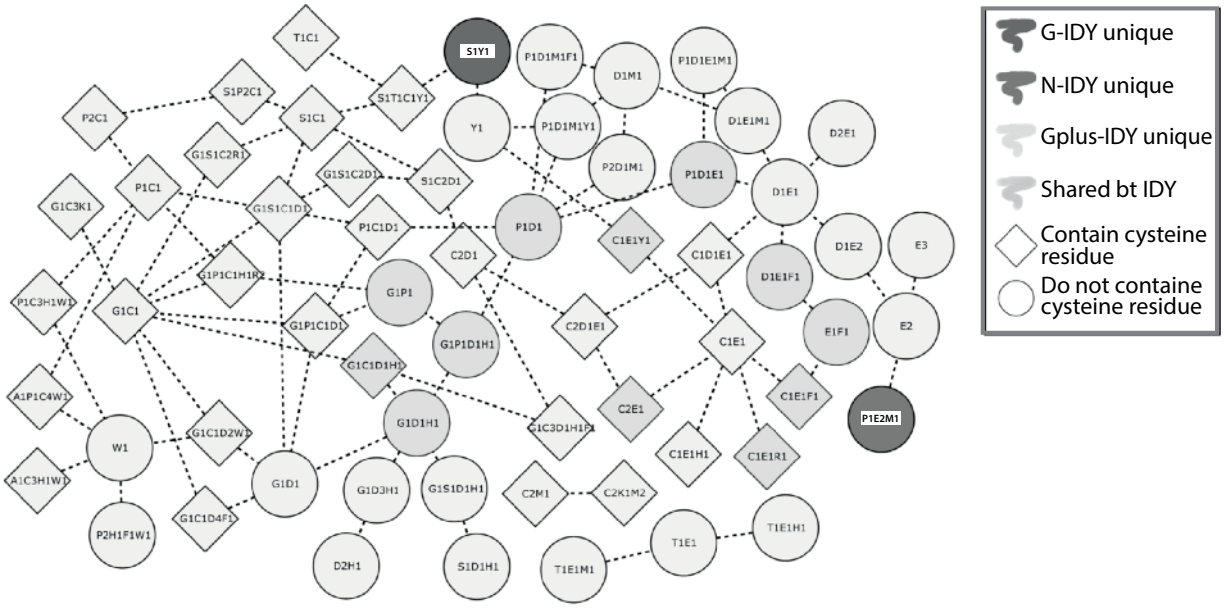


**Figure 4:** Network visualization of the 61 unique amino acid combinations (UAAC) annotated by the OligoNet webserver from the 1,674 m/z ions detected by (-)FT-ICR-MS. The circles correspond to peptides without cysteinyl residue, and the squares correspond to peptides with a cysteinyl residue. The colour indicates the IDY to which the peptide is related: the blue for G-IDY, the red for N-IDY and the green for Gplus-IDY. The light grey is for peptides shared by at least two different IDY.

Gplus-IDY, which results from the combination of a new strain of yeast with an efficient bio-process, not only shows an increased natural accumulation of glutathione but also a greatly improved diversity and abundance of new compounds, notably peptides, containing reductive residue. Gplus-IDY appears to provide an efficient way to naturally supplement the wine with potential reductive compounds. The second step of this work is to study the potential of Gplus-IDY, in comparison with other yeast derivatives, for specific stabilization reaction such as radical scavenging activity and nucleophilic addition on a specific electrophile.

## 3.2. Nucleophilic activity

Radical scavenging activity is based on the ability of a sample to reduce a radical. In this case we used DPPH radical, which is not naturally present in wine. Nevertheless, this assay is widely used to investigate the antioxidant properties of many food products and polyphenols. Since the result of the DPPH assay is expressed as the ratio of the mass of DPPH against the mass of the yeast derivatives needed to decrease the initial absorbance of 20% ($Rm_{20\%}$), the bigger the value, the better the scavenging activity from the sample. Figure 5 shows the results from the 3 initial IDY (N-, G- and Gplus-IDY) and 4 other yeast derivatives differing in solubility, glutathione concentration, process of production, and so on. Gplus-IDY and G-IDY clearly have a greater effect than the other products, with Gplus-IDY almost 50% higher than G-IDY. These results tend to show the potential relation between abundance in reductive compounds (showed in the first part) with the effective ability to reduce radical compounds.

It is therefore possible to take this further by investigating the real potential of reductive compounds in wine-like conditions, in other words with electrophile function potentially found in wine, such as quinone group. An excess of 4-Methylquinone (4MeQ) was added to the 7 yeast derivatives soluble fraction, and the reaction was maintained for 30 min. Then, sulfites were added to reduce remaining quinone and stabilize the whole solution until UHPLC-Q-ToF-MS analysis. Differences observed between pre- and post-addition of 4MeQ made it possible to extract the dimers nucleophile-electrophile (Nu-4MeQ). Between 85 and 132 dimers were found within the 7 samples, all were used in the principal component analysis shown in Figure 6. This 2D plot shows the proximity between samples in terms of Nu-4MeQ abundance and diversity. If we exclude RO4 sample, which appear really different from other samples (only 88 ± 3 dimers), we can see a kind of linear relation from Gplus-IDY to RO2. The order of the sample is exactly the same as with the DPPH activity assay: Gplus-IDY > G-IDY > RO3 > RO1 > N-IDY > RO2.

These two assays clearly show the potential of IDY naturally rich in glutathione (G-IDY and Gplus-IDY) to protect against oxidative damage in the matrix. Both the radical scavenging activity and the nucleophilic addition are of

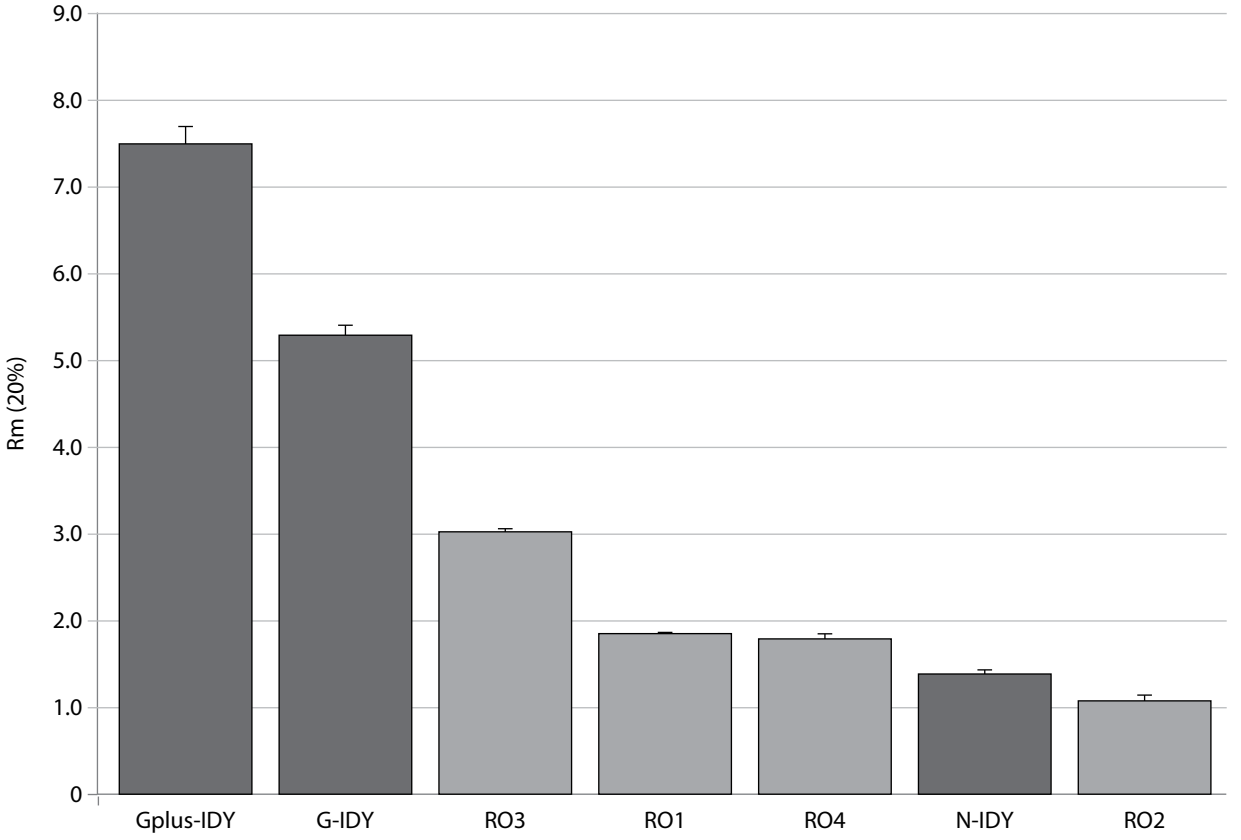


**Figure 5:** DPPH scavenging activity of N-IDY, G-IDY and Gplus-IDY compared with 4 other yeast derivatives. Results are expressed in averaged $Rm_{20\%}$ of 3 replicates and the error bars correspond to the standard deviation.

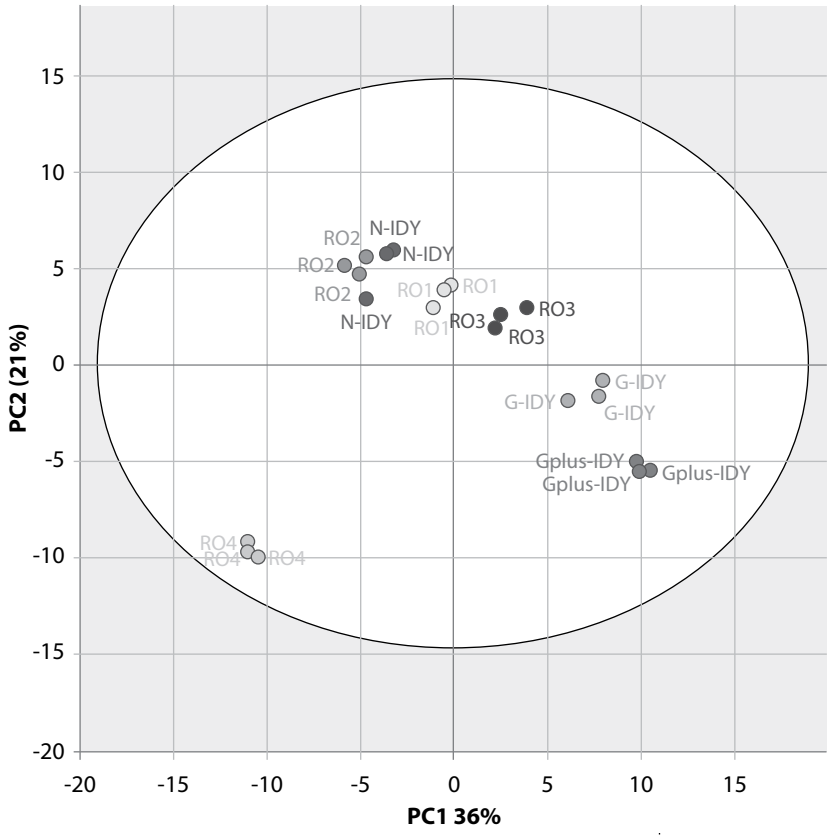


**Figure 6:** Principal components analysis of metabolites from soluble fraction of different yeast derivatives (3 replicates) after 4-Methylquinone derivatization. PC1 and PC2 explain 57% of the total variability of the dataset.

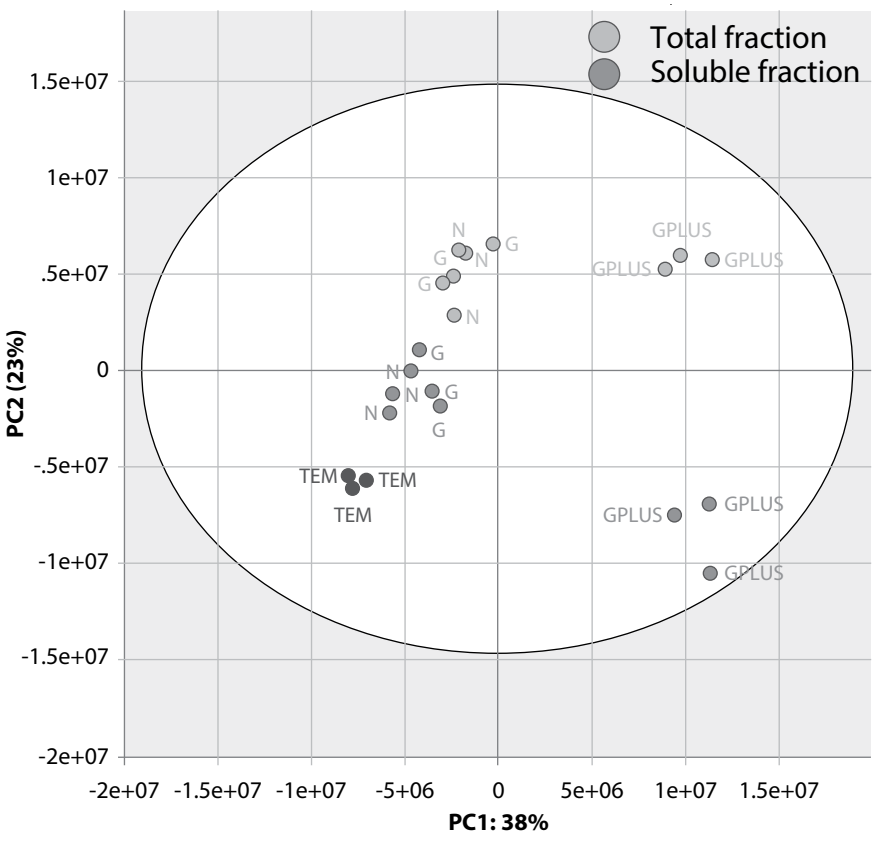


**Figure 7:** Principal components analysis of metabolites from wine fermented with G-IDY (G), N-IDY (N), Gplus-IDY (GPLUS) or nothing (TEM) after 1 year in 20 mL vials. The colour of the point indicates if only IDY-soluble fractions were added during fermentation (blue) or soluble and insoluble fractions were present (yellow). PC1 and PC2 explain 61% of the total variability of the dataset.

great importance in estimating wine longevity. In all cases, the Gplus-IDY looks to have a better effect than other yeast derivatives and thus could more efficiently protect the wine. Since the previous experiments were performed in model solution, these laboratory results must be contrasted with results in real wine, especially in terms of sensitive volatile aroma and overall wine chemistry.

### 3.3. Wine assay

Untargeted chemical analysis of wine fermented with or without the inactivated yeast is an opportunity to observe the impact of these oenological products without any a priori on wine sensory property.

Figure 7 shows the principal component analysis of metabolites analyzed by UHPLC-Q-ToF-MS. It is much easier to assess the chemical proximity of wines fermented in different conditions than compare the thousands of molecules detected. Firstly, we clearly see the impact of the fraction used during fermentation since there is a vertical separation between wines fermented with total fraction or soluble fraction only. The wines fermented with the total fraction are much more different from the wines fermented with soluble fraction or without IDY. It is known that the presence of cell walls (polysaccharides and mannoproteins) impacts the composition of wine at the early stages of fermentation, and that could explain these differences. Moreover, the separation between N-, G- and Gplus-IDY follows the same pattern as with the soluble fraction. This could be attributed to a specific action of the cell wall of the IDY, and not be related to the product itself. A second separation was made between wine fermented with G-IDY, N-IDY or no IDY and those fermented with Gplus-IDY. It did not give any clues about wine quality, which only shows that wines treated with Gplus-IDY are chemically different from wine fermented without IDY addition.

To delve deeper in terms of wine quality, we must focus on the specific compounds related to sensory quality, aromas for example. 3-mercapto-hexanol (3MH) and its acetate (3MHA) are known for their grapefruit and passionfruit notes and are thus involved in the fruitiness of Sauvignon Blanc wine. The control of their concentration during storage is a good indication of the freshness of the wine. Figure 8A shows the concentration of these two compounds in Sauvignon Blanc after sulfiting and bottling. Gplus-IDY leads to a better preservation of these two aromas in the wine compared to the control wine. 3MH and 3MHA are 100 ng/L and 10 ng/L higher in Gplus-IDY wine, respectively.

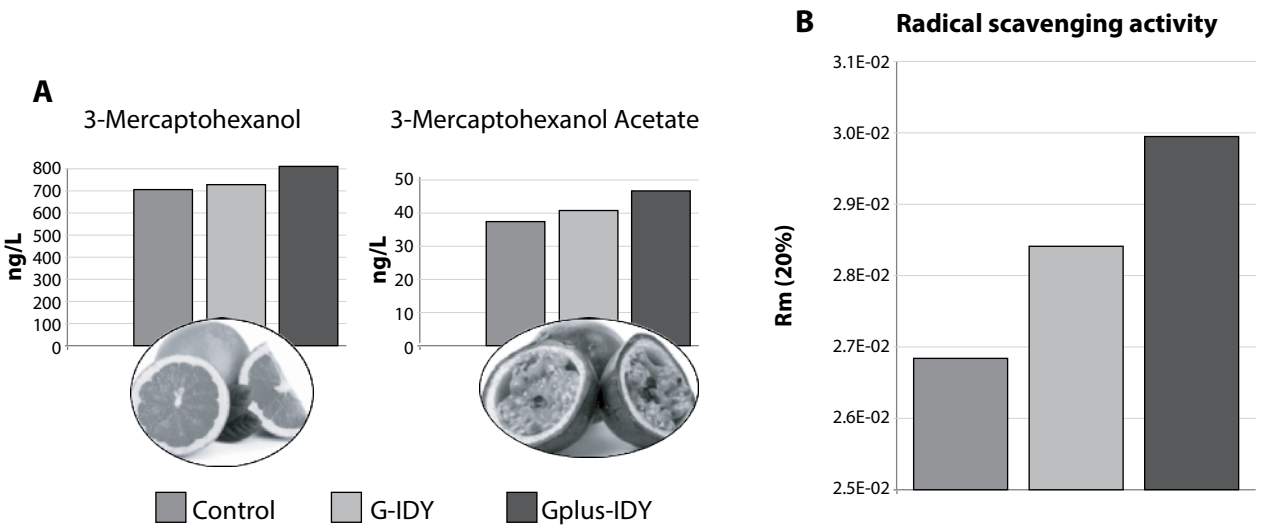


**Figure 8:** Analysis of Sauvignon Blanc wines produced in the Val de Loire area (France), 2018 vintage after bottling:
(A) Volatile thiols analysis in the control condition in comparison with G-IDY and Gplus-IDY added at 40g/hL.
(B) DPPH radical scavenging activity of control condition in comparison with G-IDY and Gplus-IDY added at 40g/hL.

In addition to these targeted aromatic thiols analyses, the radical scavenging activity was assayed (Figure 8B). The wine fermented with Gplus-IDY has a greater anti-radical capacity, which again shows the potential for this wine to age longer and retain freshness against oxidative stress. The potential of this wine to react with radicals could explain the maintenance of sensitive thiols such as 3MH and 3MHA.

## 4. Conclusion

In this study, untargeted metabolomics analysis and classical chemistry are combined to illustrate the potential of specific yeast derivatives to complement sulfites addition. Inactivated dry yeast specifically developed and produced to naturally accumulate and release high amounts of glutathione are compared to other yeast derivatives. Both the model solution and real wine were tested to offer a good overview of the potential of these products in terms of stabilization properties.

The bio-process leading to glutathione enrichment goes beyond simply increasing the glutathione concentration. This bio-process makes it possible to modulate the whole pathway of peptides in the cells and the increase in reductive amino acid integration. The metabolomic approach adopted highlights the originality of GPlus-IDY, which attests to a higher diversity and abundance of reducing peptides. The specificity of this chemical composition explains the higher impact of GPlus compared to other yeast derivatives on the improved preservation of wine quality throughout aging.

These chemical hypotheses are validated in the model solution by the classical anti-radical scavenging assay involving DPPH-stable radical, which confirms the greater impact of Gplus-IDY for stabilized radical in solution. In a second step, the use of quinone (which occurs naturally in wine) allows us to extract specific compounds with nucleophilic activity. The two glutathione-rich inactivated yeasts have the best nucleophilic activity, showing the potential of these product to partially replace other chemical nucleophiles such as sulfites.

Finally, real wine experiments allow us to see the impact, after bottling, of using of these IDYs. 3-mercapto-hexanol and its acetate are widely used as quality markers because of their sensitivity against oxidation. In the experiment led in Val de Loire in 2018, the wine resulting from an early pre-fermentative addition of Gplus-IDY kept a higher concentration of these two compounds after bottling when compared to control modality (all others parameters being the same between the two conditions). Glutathione-rich inactivated dry yeasts, and Gplus-IDY in particular, seem to hold great potential for wine preservation. Although this study is not dedicated solely to comparing sulfites and Gplus-IDY, it is clear that these two products contribute to stabilize wine during aging. In this way, Gplus-IDY could be an opportunity to partially replace sulfites naturally.

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# SWEET WINE FERMENTATION – IMPROVING QUALITY THROUGH OPTIMIZED FERMENTATION PROCESSES

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Sweet wines may well only be a niche in the German winegrowing sector, yet they generate a lot of interest and perform particularly well in terms of worldwide exports. Producing high quality sweet wines is no small feat for German winegrowers, from both vinicultural and oenological perspectives. For many years now, climate change has made it almost impossible to harvest the right *botrytized* or dried grapes for producing *Beerenauslese* or *Trockenbeerenauslese* wines. This type of production requires an enormous amount of work. Producing *Eiswein* (or ice wine) is not getting any easier either due to climate change. In recent years, there have been fewer and fewer days with the right freezing temperatures. On top of this, the frost has generally arrived so late in the season that the grapes are no longer healthy enough to produce clean sweet wines. Climate experts project that days and/or nights ideal for ice wine harvests will become ever rarer in the next few years and decades. In the long term this means that the days for *Eiswein* production in Germany are numbered.

An alternative for producing sweet wines might be to make wine out of dried grapes. Ripe and healthy grapes could be harvested and laid out to dry on straw mats or in fruit boxes. This would reduce the risks and allow the grapes to be dried in a controlled way. This wine production method is known in other regions of Europe under protected designations of origin, such as *Strohwein*, *Vin Santo*, *Passito* and so on.

Sweet wines have their own distinct sensory profile. They are often characterized by exotic notes, such as peach, apricot and passionfruit, and above all by a more or less prominent note of honey. But sweet wines can also have more negative attributes, such as dull musty notes or volatile acidity. While dull musty aromas can unfailingly be traced back to the grape harvest, volatile acidity seldom comes from the grapes themselves during fermentation. These notes of acidity are most certainly seen as an intrusion and are not considered an aroma associated with noble sweet wines. In fact, solutions for keeping volatile acidity content under control have yet to be found in some areas. As a result, every year several noble sweet wines are produced that cannot be marketed because the volatile acidity levels exceed the set limits. Although in Germany the regulatory limit for noble sweet wines is significantly higher than for conventional winemaking, in some cases it is still not enough (the limit for volatile acidity is 1.8 g per litre for *Beerenauslese* wines and *Eiswein*, and 2.1 g per litre for *Trockenbeerenauslese* wines).

Acetic acid, the main component of volatile acidity, is formed during the fermentation process due to high osmotic pressure. This is caused by the high sugar content in the musts, which puts yeast cells under extreme pressure. The sugars dissolved in the must – glucose and fructose – are osmotic agents and cause water to be extracted from the yeast cells.

The unidirectional movement of a substance (usually water) through a semipermeable membrane is called osmosis (from the Greek *osmos* = penetration). A semipermeable membrane is only permeable to certain substances. Water can pass through the membrane, but the substances dissolved in it (solutes), such as sugar or salt, cannot.

Osmosis is basically a process of balancing out the concentration of solutes between the two sides of the membrane. Water always moves from where there is a lower concentration of solutes to where the concentration is higher. Osmotic pressure persists until the concentration on both sides of the membrane has been balanced out. The difference in sugar concentration between the yeast cells and the must draws water out of the yeast cells. To counteract this, the yeast cells store glycerol, which reduces the difference in concentration. If must density is high, the yeast will produce glycerol to compensate for the loss of water. Glycerol can bind to water and therefore prevents yeast cells from dehydrating. This enables the yeast to continue breaking down sugars, even when conditions are less favourable. The higher the must density is, the higher the glycerol content in the finished wine. However, the production of glycerol is also associated with the production of acetic acid.

During fermentation, yeast cells never quite manage to strike the right concentration balance. A yeast that is exposed to a must weight of over 120 °Oechsle (Oe) is put under extreme pressure and produces three to four times more acetic acid than under normal fermentation conditions. Yeast growth and the quantity of live yeast are significantly reduced with must density of 100 °Oe and above. This shows the increasingly hostile environment associated with high must density. This effect also explains why sugar is added to fruit when preserves or jams are made: it prevents micro-organisms from developing.

At the Regional Institute for Viticulture and Horticulture in Veitshöchheim, experiments were carried out between 2014 and 2017 to investigate the various factors that influence the amount of volatile acid produced during fermentation for wines with extremely high sugar contents. The musts used for the experiments included ice wine must, must from dried grapes or musts enriched with rectified grape must concentrate. 0.6 mg/L of thiamine, 50 g/hL of DAP and 15 g/hL of a yeast cell wall preparation were added to make sure the yeasts had enough nutrients.

## The influence of yeast

There are very few yeasts specifically recommended for fermenting musts with extremely high sugar content. Manufacturers were asked which of their yeasts they would recommend and could provide for the trial. For the experiment, a must with a must weight of 204 °Oe was used, and seven different pure culture yeasts were tested: six *Saccharomyces cerevisiae/bayanus* and one *Torulaspora delbrueckii*. As expected, despite having added an initial yeast quantity of 50 g/hL, fermentation was slow. It was clear that with such a high level of osmotic pressure, the yeast cells could hardly multiply at all and remained small. As a result, fermentation stopped for all the yeast strains at about 5% ABV. Only the *Torulaspora delbrueckii* (Lalvin Biodiva™) fermented to about 6% ABV.

Otherwise, during fermentation, all of the types of *Saccharomyces* tested produced 1.9 to 2.4 g/L of volatile acid, whereas *Torulaspora delbrueckii* produced only 0.8 g/L.

This finding was confirmed in all the experiments carried out over the four years. Figure 4 illustrates this clearly, with a graph that compares volatile acid production and ABV production. The two yeasts that are most frequently used for noble sweet wines, Lalvin EC1118 and Zymaflore ST, show a sharp increase in volatile acid production at the start of fermentation. However, as the osmotic pressure decreases due to the breakdown of sugar during fermentation, the production of volatile acid slows down. All the *Saccharomyces* tested in the experiment showed a similar result and were only removed from the graph to make it less cluttered. In contrast, when *Torulaspora delbrueckii* reached 5% ABV, the volatile acid content was only 0.2 g/L.

In other fermentation experiments, *Saccharomyces* often produced a significantly higher ABV level, whereas in fermentation using *Torulaspora delbrueckii*, alcohol production stagnated at 6–7% ABV. But this can be considered as one of the advantages of this yeast, given that an ABV level of 6–7% for noble sweet wines is more than sufficient. Quite often, fermentation is not stopped early enough, or it cannot be stopped at all, and the wine has an unwelcomely high alcohol content and thereby a residual sugar content that is too low. As a rule, this cannot happen when a pure culture *Torulaspora delbrueckii* is used in fermentation. To obtain a higher alcohol content, however, sequential inoculation with a *Saccharomyces cerevisiae/bayanus* yeast is required once the *Torulaspora delbrueckii* has broken down the must density enough for the *Saccharomyces* to tolerate the osmotic pressure exerted. Experiments conducted in 2015 showed that with this method, the volatile acid content increases only slightly compared with *Torulaspora delbrueckii*-only fermentation.

## The influence of yeast nutrients

Through the use of yeast nutrients, particularly inactive yeasts and yeast autolysates, the yeast cell wall is said to become more stable. The idea is to obtain a higher final degree of fermentation.

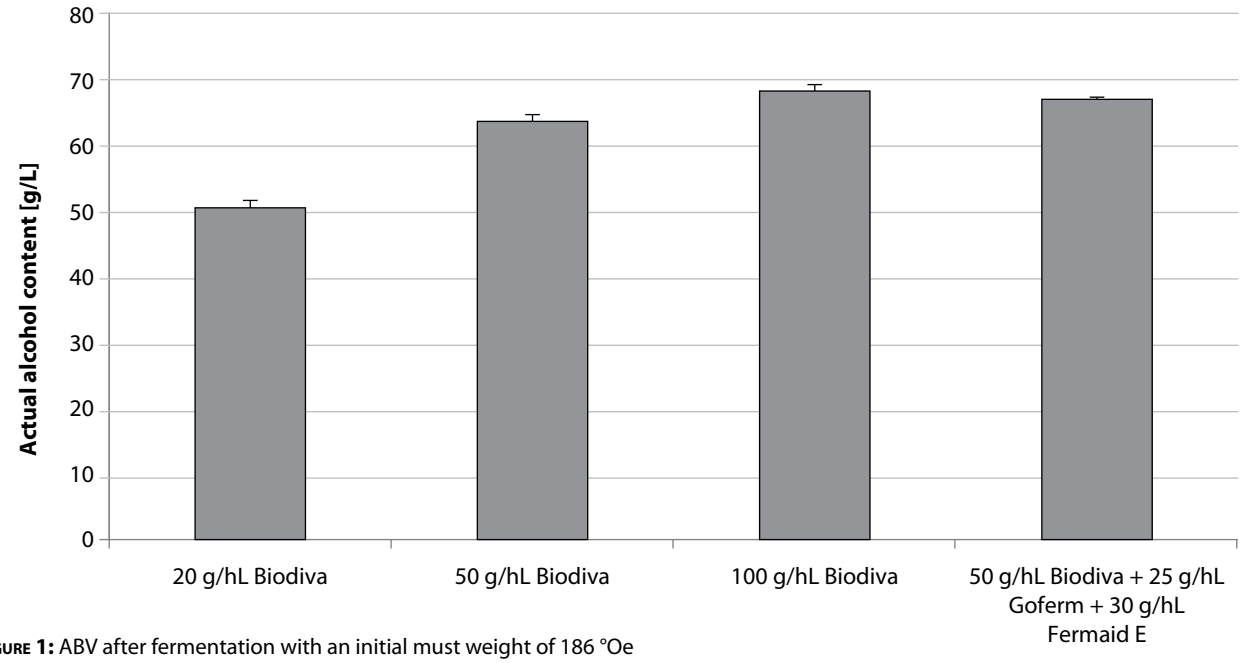


**Figure 1:** ABV after fermentation with an initial must weight of 186 °Oe

However, in musts with high osmotic pressure, the experiments showed that the final degree of fermentation increased only slightly when nutrients were added. In contrast, the quantity of initial yeast added had a significant influence on the level of alcohol produced. By increasing the amount of yeast added, a higher alcohol content was obtained (Fig. 1).

That said, it is also clear that a larger amount of yeast leads to a higher level of volatile acid. Increasing the number of yeast cells under high osmotic pressure also increases volatile acid content, as the production of this acidity is linked to glycerol production. The use of yeast nutrients did not reduce the production of volatile acid (Fig. 2).

## The influence of fermentation temperature

Yeast prefers warmer fermentation temperatures to cool ones. Fermentation temperatures under 16°C usually lead to more fruity and aromatic wines. However, this puts stress on the yeast. At higher temperatures, the yeast multiplies more easily and quickly, resulting in faster and more reliable fermentation. A Silvaner must was used to test whether the same happened for musts with high osmotic pressure or whether, as with high osmotic pres-

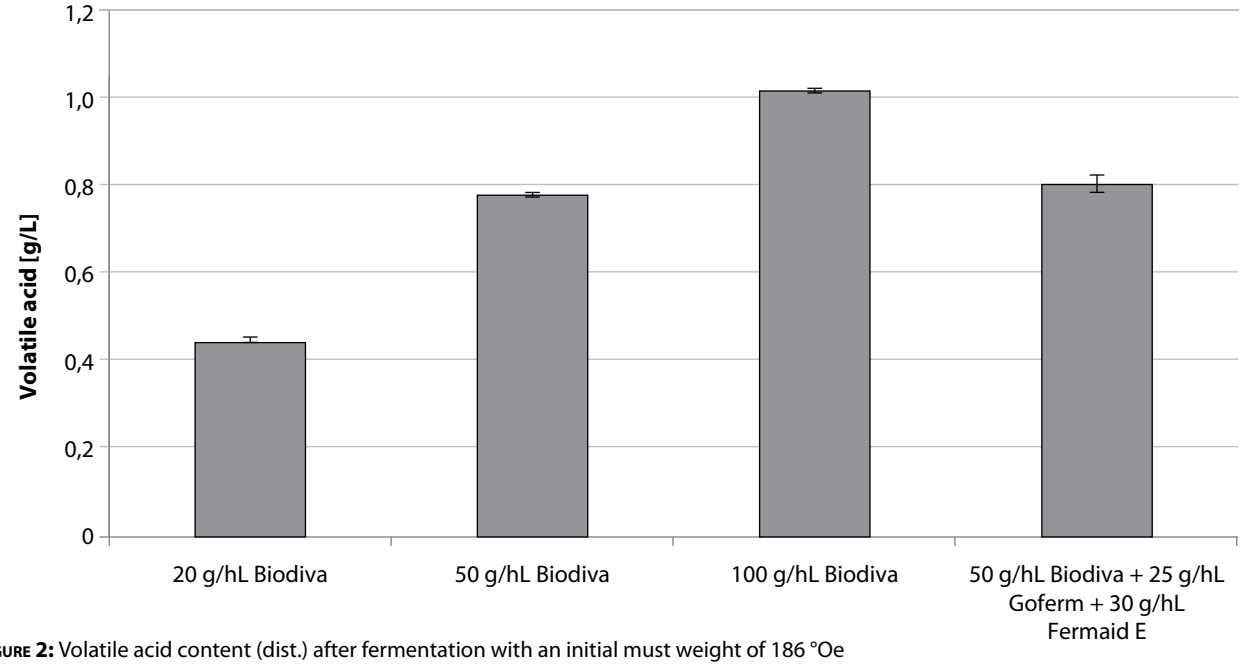


**Figure 2:** Volatile acid content (dist.) after fermentation with an initial must weight of 186 °Oe

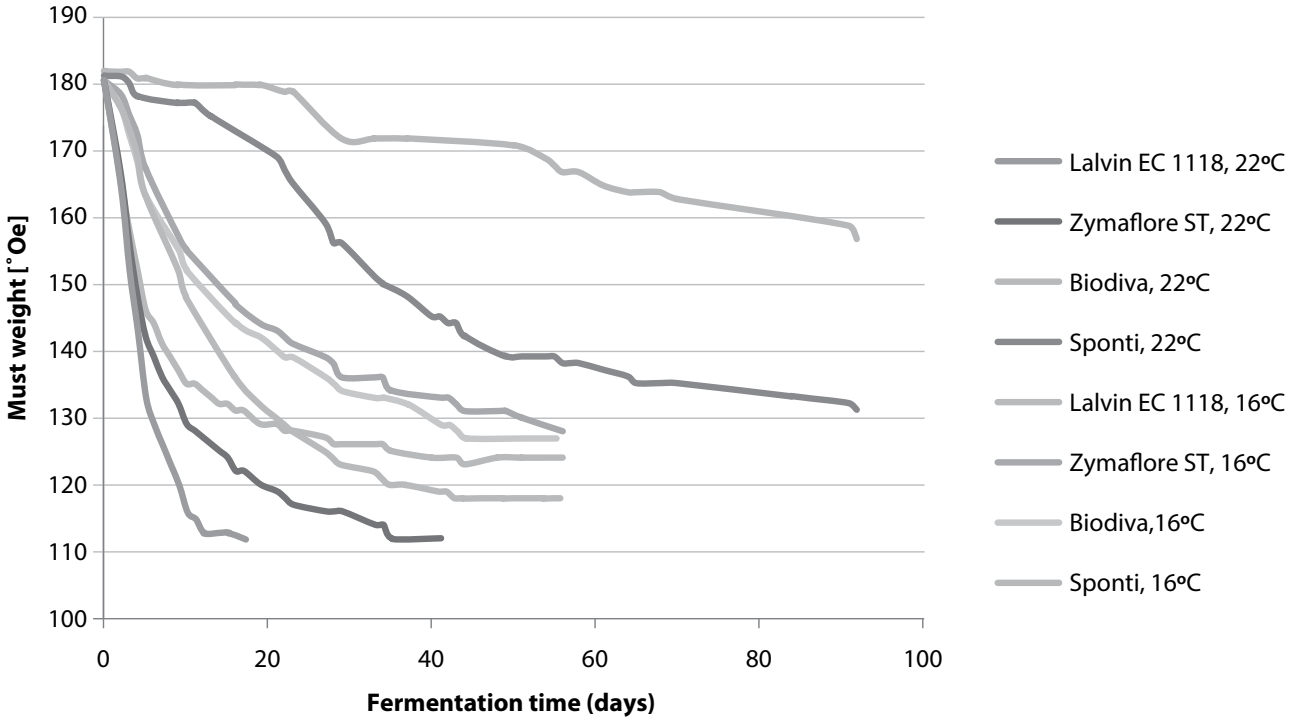


**Figure 3:** Fermentation process for the different yeasts tested in a 2016 Silvaner must (180 °Oe) at 16°C and 22°C

sure, the stress produced by the temperature had a negative effect on volatile acidity levels. The Silvaner must was enriched with rectified grape must concentrate to a must weight of 180 °Oe and fermented at 16°C and 22°C. In each case, three pure culture yeasts (two *Saccharomyces* and one *Torulaspora delbrueckii*) were used; one strain

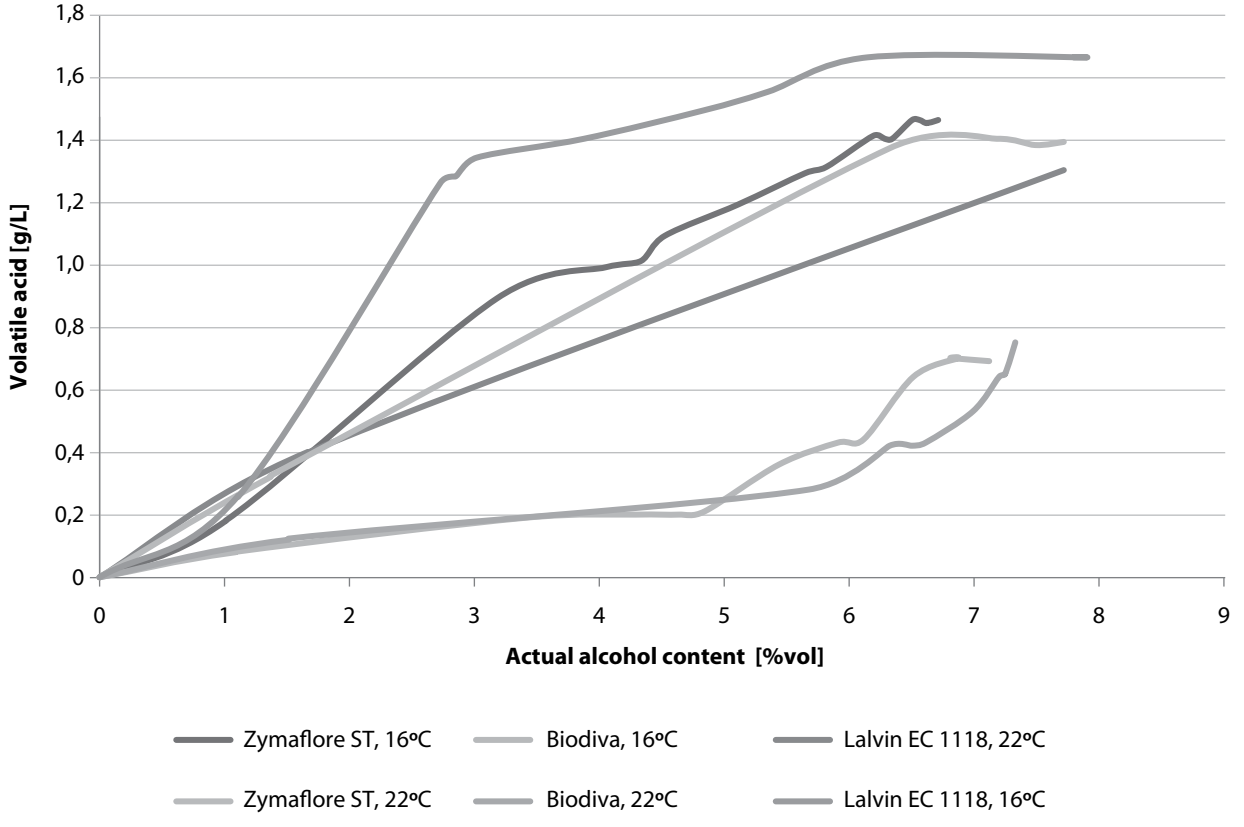


**Figure 4:** Production of volatile acid during fermentation at 16°C and 22°C (2016 Silvaner must, 180 °Oe)

was spontaneously fermented. The must was preclarified with a chamber filter and the usual yeast nutrients were added.

As expected, the fermentation started faster at 22°C for all strains and the total fermentation time was significantly shorter, with a final degree of fermentation that was much higher in the *Saccharomyces cerevisiae/bayanus* fermentations and for the spontaneous fermentation. *Torulaspora delbrueckii* fermented a lot more slowly at 16°C, but had a similar final degree of fermentation (Figure 3). Spontaneous fermentation is practically impossible for musts with high must density because the small amount of yeast present in the must can hardly multiply due to the high osmotic pressure exerted. At 16°C, spontaneous fermentation cannot produce any reasonable amount of alcohol.

The amount of volatile acid produced depended on the yeast used. For example, fermentation for Biodiva™ and Zymaflore ST was almost the same at 16°C and 22°C, whereas Lalvin EC1118™ produced 0.3 g/L more volatile acid at 16°C than at 22°C. This was of course in tandem with each respective ABV (Figure 4). From a general point of view, volatile acid content is higher for all yeasts when fermentation takes place at 16°C than when it takes place at 22°C. This is because higher temperatures provide better conditions for yeasts.

## The influence of initial must density

A high must density means that yeasts are put under higher osmotic pressure during fermentation. With must densitydensity of 100 °Oe, the osmotic pull of sugar, which draws water from the yeast cells, is quite weak. However, with must density of 150 °Oe or more, this pull effect is much stronger. And with must density of around 250 °Oe, the sugar draws so much water from the yeasts that they can no longer function. These types of must, with a high sugar content, cannot ferment at all.

### The level of sugar in them is almost the same as that in rectified grape must concentrate or jam!

To test the effect of high osmotic pressure and its direct influence on the production of volatile acid, a 2016 ice wine (140 °Oe) was gradually enriched up to 200 °Oe with rectified grape must concentrate. A *Saccharomyces cerevisiae* (Zymaflore ST) and a *Torulaspora delbrueckii* (Biodiva) were again selected for this experiment, to investigate the difference in fermentation behaviour and volatile acid production.

Figure 5 shows the volatile acid content after fermentation. We can clearly see that during fermentation using *Saccharomyces cerevisiae*, volatile acid content directly correlates with the initial must weight. Although a volatile acid content of 1.3 g/L for an initial must weight of 140 °Oe is totally acceptable for an ice wine, a wine with a volatile acid content of 2.3 g/L for an initial must weight of 200 °Oe is simply unmarketable!

The experiment also revealed that high osmotic pressure was less of an issue for the Biodiva yeast (*Torulaspora delbrueckii*) and that the production of volatile acid was not dependent on the must weight. Figure 5 shows that during fermentation with must density of 140 °Oe and 200 °Oe, Biodiva™ produced only 0.8 g/L of volatile acid. This is a very important finding for optimizing the production of noble sweet wines, as they are often rejected, or even become unmarketable, due to high volatile acidity.

In this experiment, despite the fact that fermentation took place using a non-*Saccharomyces* yeast, Lalvin Biodiva™, an alcohol content that went up to as much as 15.2% ABV was recorded. This raised the question of whether *Torulaspora delbrueckii* alone was responsible for the fermentation, or whether there had been an unwelcome visitor. To investigate this issue, sterile samples were taken at the end of fermentation and put under the microscope. As all the yeast cells were small and round, no differences were observed. Therefore, a PCR analysis (ITS 1/ITS 2, RFLP) was performed to see whether there was any yeast present other than the initial pure yeast strain used. The results showed that only *Saccharomyces cerevisiae* was present at the end of fermentation in the samples in which initial must densities were 140 °Oe and 160 °Oe. We can therefore deduce that the Biodiva yeast inoculated in the must was outgrown during the fermentation process by an existing wild *Saccharomyces*, as the osmotic pressure was within the tolerance range of genuine wine yeast. This was the reason why the alcohol content was so high and the wine was completely fermented even with a must weight of 140 °Oe. When the initial must weight was 180 °Oe, a mixture of *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* was present in the wine. In the sample where the initial must weight was 200 °Oe, only the *Torulaspora delbrueckii* yeast could be detected (see Table 1). This shows that for must density of over 180 °Oe, *Saccharomyces* have almost no chance of survival because of the high osmotic pressure, while the *Torulaspora delbrueckii* was able to ferment the must clean to 6.8% ABV.

**Table 1:** Yeast strain after Biodiva fermentation, in relation to initial must weight

| Final must weight | % ABV after fermentation | Yeast strain |
|---|---|---|
| 140 °Oe | 15.2 | *Saccharomyces cerevisiae* |
| 160 °Oe | 10.7 | *Saccharomyces cerevisiae* |
| 180 °Oe | 8.8 | *Saccharomyces cerevisiae and Torulaspora delbrueckii* |
| 200 °Oe | 6.9 | *Torulaspora delbrueckii* |

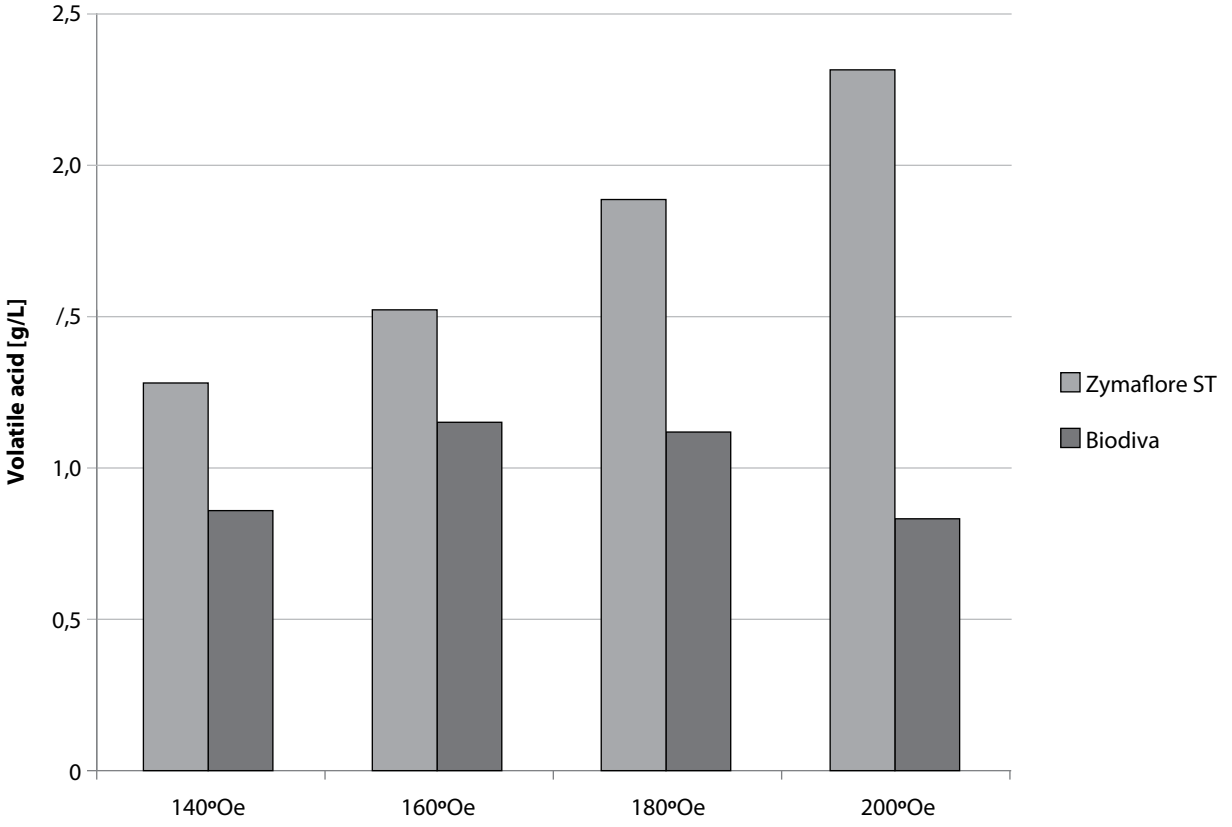


**Figure 5:** Volatile acid content after fermentation, in relation to initial must weight and yeast strain (2016 Silvaner must)

## Conclusion

Noble sweet wines are, and always will be, an all-out speciality, as producing them requires several different factors to come together at the right time, in the right way and under the right conditions. The high levels of volatile acidity in such wines can usually be traced back to the fermentation process. Obtaining lower levels of volatile acidity would be an important milestone in the development of the style of sweet wines. The volatile acidity in wines produced late in the season increases due to the initial must weight. *Saccharomyces cerevisiae/bayanus* produce greater amounts of acetic acid due to the high osmotic pressure exerted on the yeast cells. Obtaining significantly lower levels of volatile acidity under optimal fermentation conditions involves using *Torulaspora delbrueckii* yeast, possibly even combined with a *Saccharomyces cerevisiae* yeast. The conditions for optimal fermentation are temperatures above 20°C, a good nutrient supply and a sufficient quantity of initial yeast (at least 50 g/hL).

Another great advantage of using a *Torulaspora delbrueckii* for the fermentation of noble sweet wines is the fact that this yeast only produces an alcohol content of about 6–7%. It avoids the issue of high alcohol levels being produced due to fermentation not being stopped at the right time.

Although the price of *Torulaspora delbrueckii*, which can be up to four times that of a standard *Saccharomyces* yeast, may be a little off-putting at first, using it is highly recommended. The lower levels of volatile acid produced and the ensuing greatly improved quality (and even mar-

ketability) of the wine would enable winegrowers to obtain a significantly higher market price.

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