02/21/2020 TWGGA

Managing New Winemaking Challenges with Non-Conventional Yeasts

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IMPROVEMENT THROUGH BIOTECHNOLOGY

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Scientific Adviser

Expertise : **Microbiology, Food Biotechnology, Fermentation Science**

Current Research Topics: **Indigenous Yeast, Wine Fermentation, Cell Physiology**

Education:

- **2018 - PhD** in **Wine Science** - **University of Adelaide, Wine Microbiology and Microbial Biotechnology Laboratory**
- **2014 - Master's degree** in **Industrial Biotechnologies** - **Heineken Netherlands / University of Milan-Bicocca**
- **2011** - **Bachelor's degree** in **Biotechnology** - **University of Milan - Bicocca**

Publications:

- Chen, L., Capone, D. L., **Tondini**, F., & Jeffery, D. W. (2018). Chiral Polyfunctional Thiols and Their Conjugated Precursors upon Winemaking with Five *Vitis vinifera* Sauvignon blanc Clones. Journal of Agricultural and Food Chemistry, 66(18), 4674-4682.
- **Tondini**, F., Jiranek, V., Grbin, P. R., & Onetto, C. A. (2018). Genome Sequence of Australian Indigenous Wine Yeast *Torulaspora delbrueckii* COFT1 Using Nanopore Sequencing. Genome Announcements, 6(17), e00321-18.
- **Tondini**, F., Lang, T., Chen, L., Herderich, M., & Jiranek, V. (2019). Linking gene expression and oenological traits: comparison between indigenous *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* strains. International Journal of Food Microbiology.

Introduction

No predictability

No reliability

Complexity

' Terroir expression'

Osmotic stress

Nutrient

Ethanol toxicity

Saccharomyces

Non-*Saccharomyces*

Assess the risk

Predict the outcome

Research & Industry objects

1. Monitor the sequential succession on the different yeast species

2. Evaluate the impact of different species metabolism on the final wine composition

1. Improve fermentation processes i.e. high sugar fermentations)

2. Rational development of new procedures (i.e. mixed fermentations).

IMPROVEMENT THROUGH BIOTECHNOLOGY

1- Non-*Saccharomyces* yeast selection

Where is it from?

• **Grapes** : main natural reserve of indigenous wine yeasts.

• The species of yeasts present on the surface of the berries are limited.

• Yeasts population evolves during grape ripening.

• The population present on the grapes in over-ripeness or on grapes affected by mold (eg Botrytis) are higher and can reach 10^5 and 10^7 CFU/g (Barata et al., 2008; Nisiotou & Nychas, 2007).

• Succession of yeast species:
- Increased surface of the grape
- Depending on the availability and quantity of nutrients
- Depending on the concentration of sugar, which increases with decreasing acidity

(Cadez et al., 2010; Combina et al., 2005).

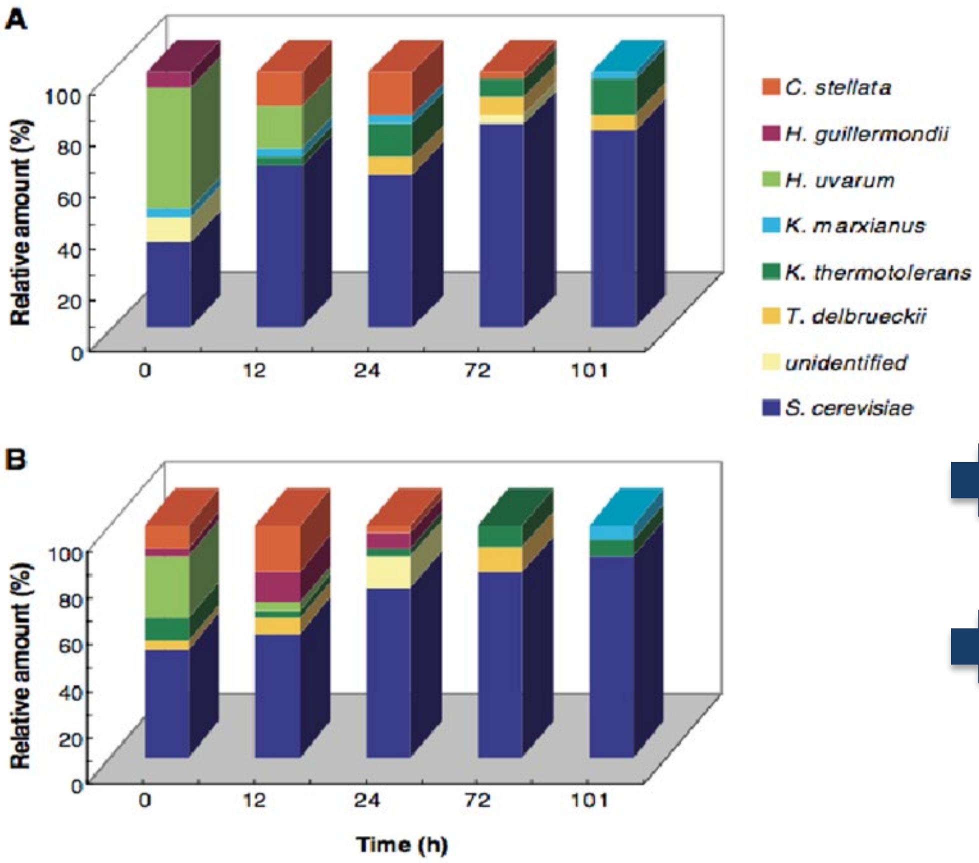
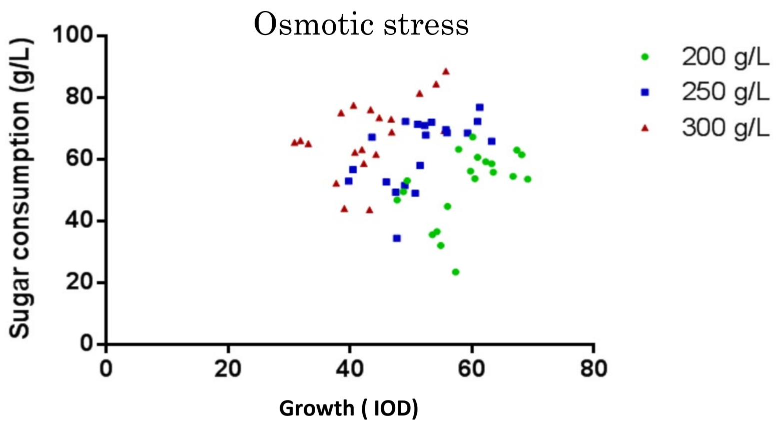


Figura 3 : Dynamics of yeast species during AF in White (A) and Red (B) (Xufre et al., 2006).

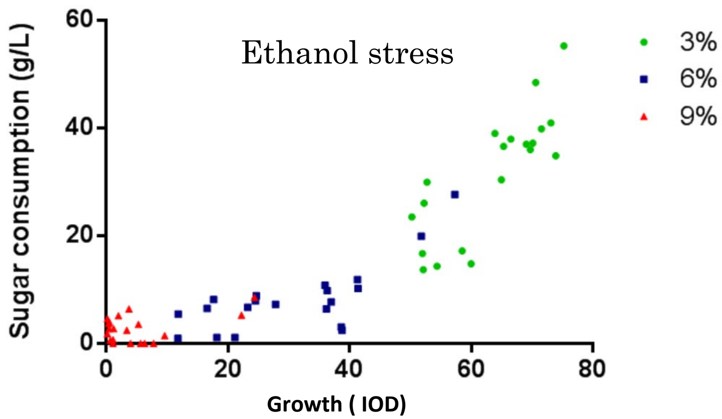
Yeast species succession during AF

- Common dynamics
- *Saccharomyces cerevisiae* takes dominance

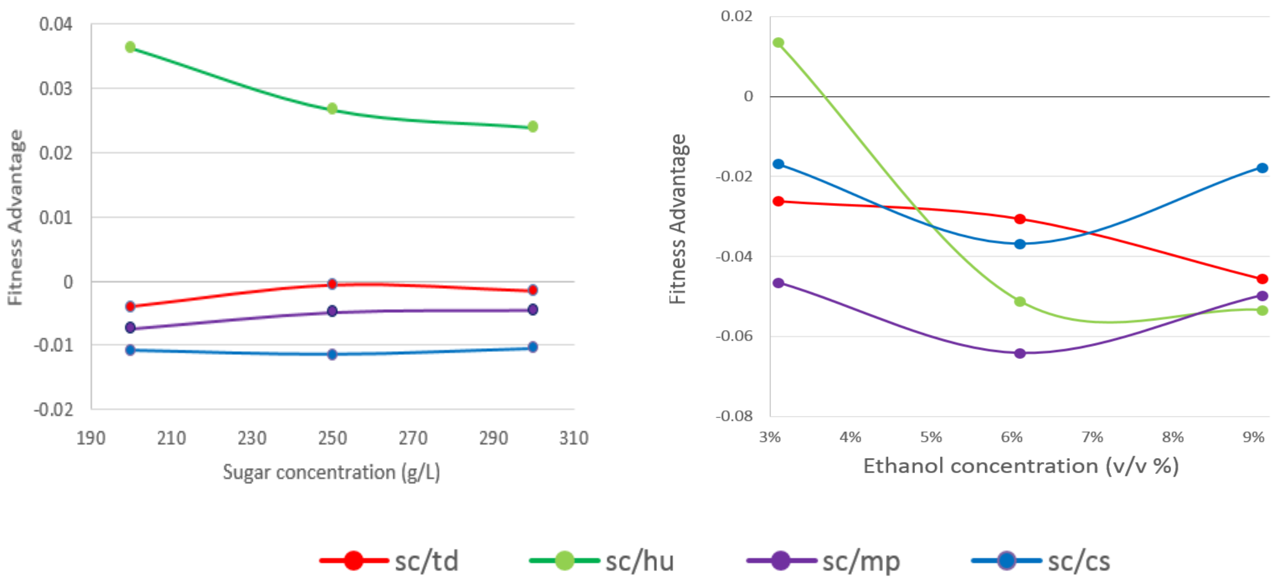
Increased sugar consumption under osmotic stress



No growth and limited sugar consumption under ethanol stress



Growth decreases – What are the effects on fitness?
Who is advantaged?



IMPACT OF NS YEASTS ON THE ORGANOLEPTIC CHARACTERISTICS OF WINES

- It increases the organoleptic potentials and qualities, confers greater aromatic complexity (Kim et al., 2008; Viana et al., 2008; Ciani et al., 2010; Comitini & Ciani, 2011; Medina et al., 2013) = increases the range of desirable flavors

- Some yeasts can be at the origin of olfactory defects produced during fermentation and / or during refining.

- detrimental yeasts: *Pichia membranifaciens, Candida, Zygosaccharomyces* and *Brettanomyces*, can produce compounds such as acetic acid, phenols, hydrogen sulfide (Chatonnet et al., 1995; Dias et al., 2003; Suárez et al., 2007).

Need to study and select N-S yeasts with positive oenological characteristics

AEB
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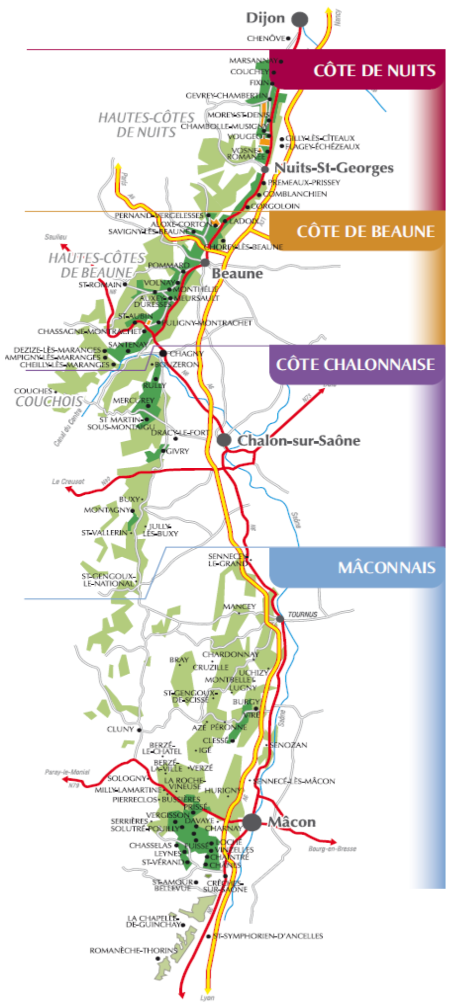
Isolation N-S



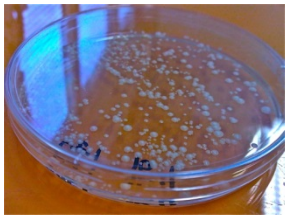
Samples of Pinot noir and Chardonnay grapes (12 sites located in Côte de Nuits, Côte Chalônnaise, Mâconnais et Center loire) - Grape harvest 2010

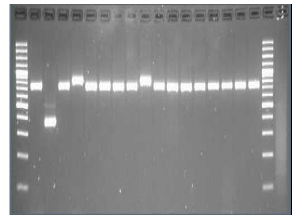
Total yeast isolation on YPD medium

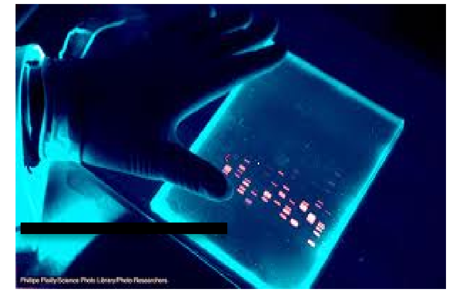
Isolation of non-Saccharomyces yeasts on Lysine

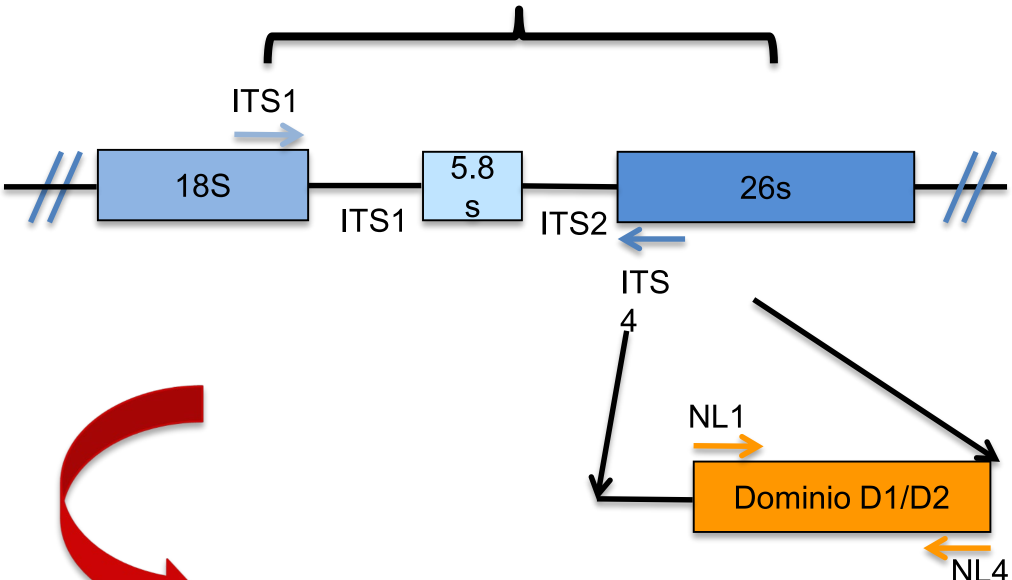
Colonies frozen at - 80 ° C on YPD-glycerol (50-50%)

PCR ITS-RFLP and sequencing region D1/D2 26S









Restriction profiles obtained after digestion with endonucleases: CfoI, HaeIII, HinfI or DdeI (Esteve-Zarzoso et al., 1999)

Identification of isolated strains confirmed by sequencing

Number of strains identified as a function of genus and species

Ceppi isolati ed identificati	Numero	%
Candida californica	05	0,5
Candida ishiwadae	02	0,2
Candida oleophila	12	1,2
Candida zemplinina	58	5,6
Cryptococcus flavescens	04	0,4
Hanseniaspora uvarum	658	64,0
Kluyveromyces thermotolerans	02	0,2
Metschnikowia aff. fructicola	03	0,3
Metschnikowia pulcherrima	02	0,2
Metschnikowia sp.	93	8,9
Metschnikowia viticola	01	0,1
Pichia fermentans	03	0,3
Pichia fluxuum	04	0,4
Pichia membranifaciens	28	2,7
Rhodotorula fijisanenesis	01	0,1
Torulaspora delbrueckii	07	0,7
Totale NS	**883**	85,9
Saccharomyces cerevisiae	145	14,1
Totale	**1028**	

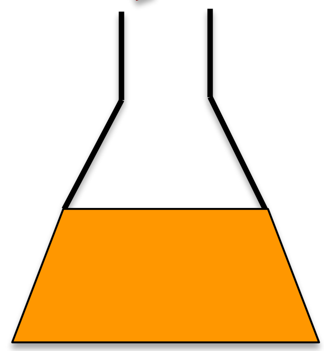
- 1028 strains isolated and identificated
  - 883 non-*Saccharomyces*
    - 16 genues and species

Microvinification

Time 48h

Non-*Saccharomyces*
10^7 cellule/mL

S. cerevisiae
10^6 cellule/mL



90 mL Standarized grape Juice

Fermentation

Analysis

Residual sugar, Etanhol, pH, TA, Acetic acid

Grape juice composition

Sugars (g/L)	**200**
pH	3,5
N (mg/L)	350
YAN (mg/L)	220
L-malic (g/L)	3,5

- Total population: YPD plate count
- non-*Saccharomyces* : plate Lys
- Sugars: glucose e fructose

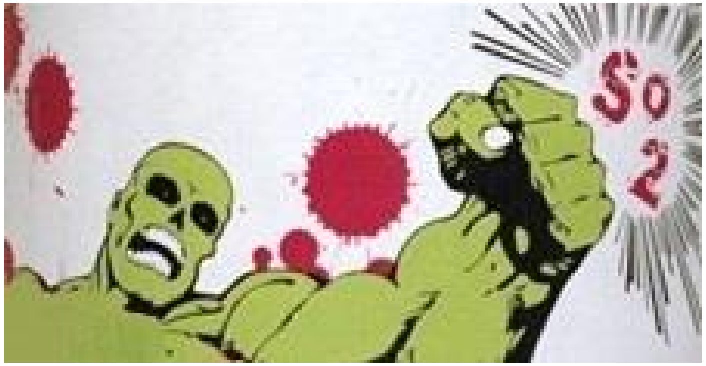
Triplicates

IMPROVEMENT THROUGH BIOTECHNOLOGY

PRIMAFLORA®

Bioprotection of the harvest, an alternative to SO2

Legislative rules, together with customer demands, have encouraged the production of “ecological wines” which have recently gained relevant interest. These are produced by environmentally friendly sustainable and integrated production methods, with lower concentration of S02



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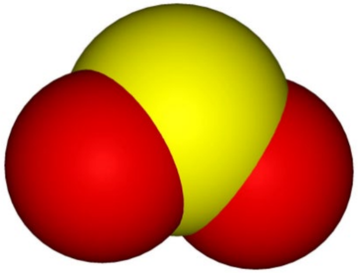
Sulphur dioxide (SO_2)



In few words…

Food additive: preservative
The oldest product used in oenology
Long spectrum of action (antioxidant, antiseptic, solvent) - precious product of the winemaker for its versatility
Oenological properties depending on different forms: total SO2, free SO2, active SO2 and combined SO2

It is also the most controversial and the most despised for many years.

Overuse: can hide aromas and flavours
Toxicity even in low doses

The alternatives

Ascorbic acid : Good antioxidant used with SO2, not alone.

Sorbic acid : Anti yeast action, in combination with SO2, stabilization of sweet wines, used for bottling.

Lysozyme : Enzyme extracted from egg white, degrades the wall of lactic bacteria, has no action on yeast, blocks FML and stabilizes wines.

Today, no substance can completely replace it, given its versatility.

Bioprotection, a new concept of wort protection

PRIMAFLORA® is a specific selection of microorganisms intended to be implanted in the must/ on the grapes from the first minute of harvest.

PRIMAFLORA® does not replace yeast, but replaces the must sulphite.

Bioprotection with PRIMAFLORA®

- Non-Saccharomyces yeasts Metschnikowia pulcherrima
- Saccharomyces Yeasts (VR)
- Organic nutrient

Organic Formula

- **Choose your formulation according to the type of winemaking**

Red wines
Primaflora® VR Bio
5-10 g/hL in the selling machine, in the vat

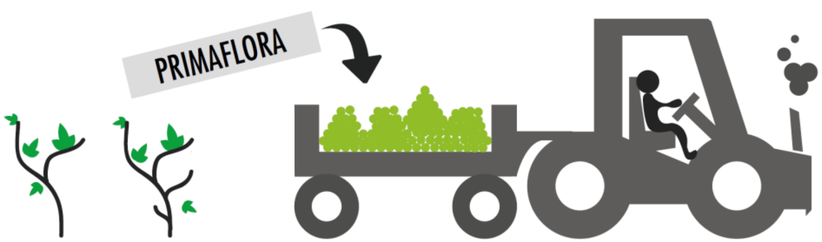
White wines
Primaflora® VB Bio
3-5 g/hL in the harvest machine, in the tread or in the press

For static sedimentation, flotation and pre-fermentation maceration, use PRIMAFLORA® VB BIO.

IMPROVEMENT THROUGH BIOTECHNOLOGY

Bioprotection with PRIMAFLORA®

- Principle of operation: establish dominance as quickly as possible of good microorganisms (with oenological interest) into the must and stop the development of unwanted indigenous microorganisms.
- When? As early as possible about the harvest.



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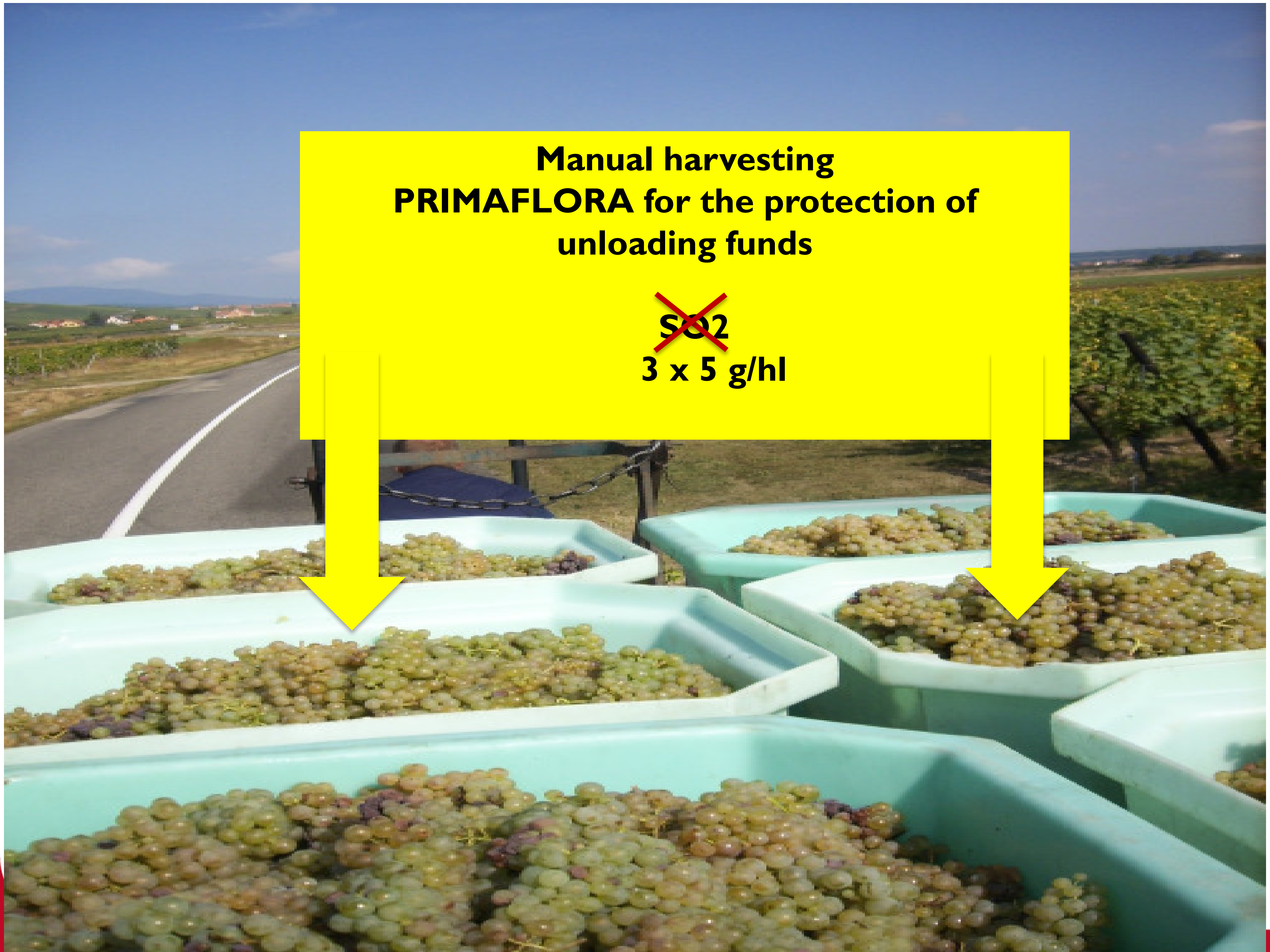
- Technical objectives: Limit the time of non-protection of grapes and must.


Manual harvesting
PRIMAFLORA for the protection of unloading funds
~~SO2~~
3 x 5 g/hl

Protocol:

Rehydrate 500g from PRIMAFLORA® in 10 liters of mineral water or unchlorinated water at 77-87 F and add 50g/l of sugar for 15 minutes.

Double the volume with unsulfured must to extend the life of the solution by 3 hours.

Quintuple volume with unsulfured wort to extend the life of the solution by 12 hours.

Raise your dose of PRIMAFLORA® as we would for SO_2

The higher the risk of contamination and proliferation of native flora, the higher the dose of PRIMAFLORA®.

The dose of PRIMAFLORA
It has to be increased according to the temperature of the wort
It needs to be increased according to the pH of the wort

It preserves some of the native flora

Schizosaccharomyces

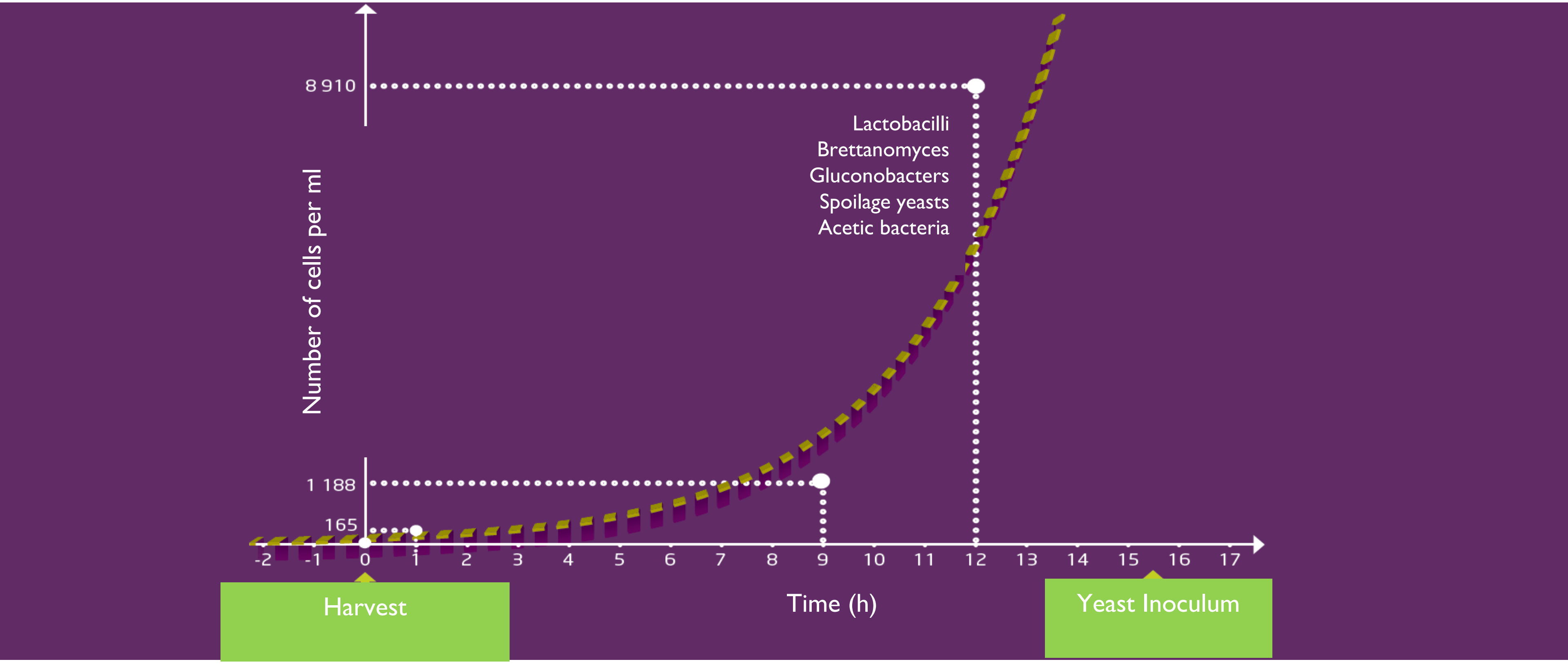
- *Cryptococcus*
- *Metschnikowia pulcherrima*
- *Brettanomyces*
- *Saccharomyces*
- *Hanseniaspora*
- *Rhodotorula*
- *Torulaspora delbrueckii*
- *Candida*
- *Pichia*

Study by Nicholas A. Bokulich of Davis University (published in 2013 , Journal of the US Academy of Sciences)

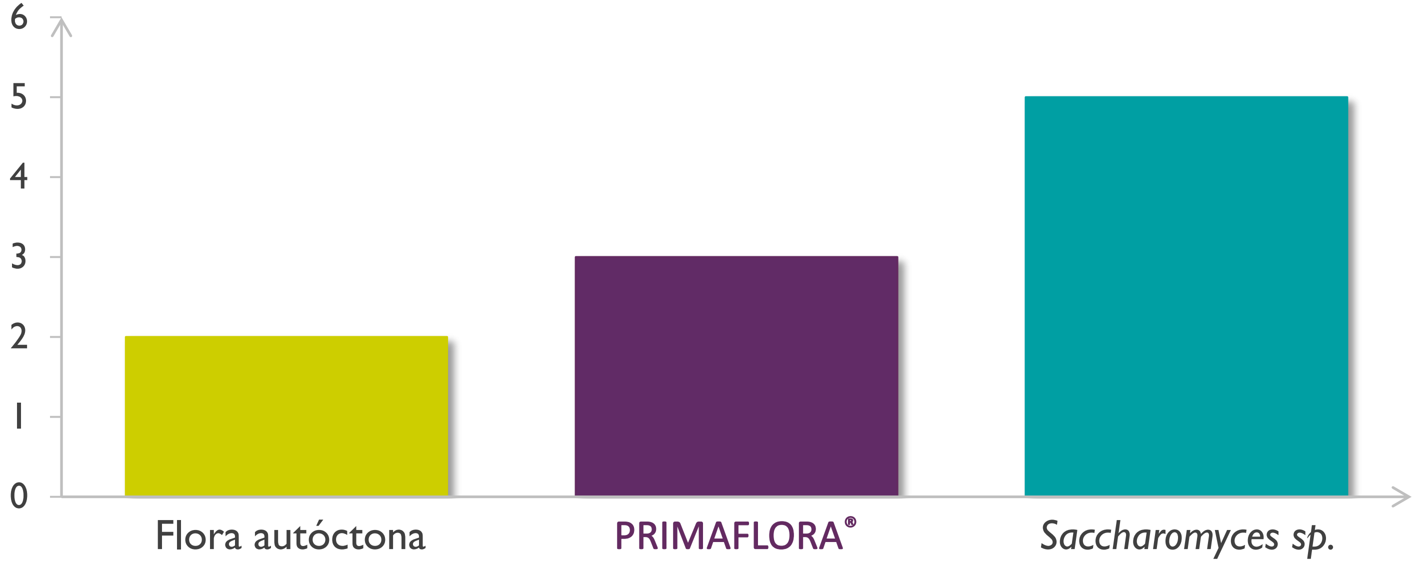
" Microbial biogeography of wine is grapes conditioned by cultivar, vintage, and climate "

- **The wines are also the product of a microbial soil, depending on the climate, the geography of the region, the vineyard and the grape variety. This study shows a composition of yeast, on the surface of the grapes, very characteristic of the terroir and the variety.**
- **The composition of the bacterial population is less typical of the terroir and variety.**
- **This study does not say that there is a strain of yeast specific to a terroir, but it shows that they are different genera and species depending on the origin (and that Saccharomyces cerevisae is a minority)**

Development of native flora after harvest



Succession of 3 populations of yeast



Depending on the nature and intensity of bioprotection, it is possible to leave more or less room for the expression of the native flora

PRIMAFLORA® is a microbiological protection

Why use Primaflora instead of SO2?

- Limits the combination of SO2 during fermentation.
- Avoid the selection of SO2-resistant microorganisms
- Add complexity of the wines and the aromatic quality: there is less production of H2S and natural enzyme systems are preserved.
- This biomass will provide nutrient to the yeast (e.g. sterols).
- Unlike SO2, PRIMAFLORA® does not participate in extractions of bitterness, vegetable flavor and, if the harvest is rotten, moldy or mushroom flavor. In addition, treatments are more effective.
- It facilitates co-inoculation and implantation of bacteria if you want malolactic co-fermentation.

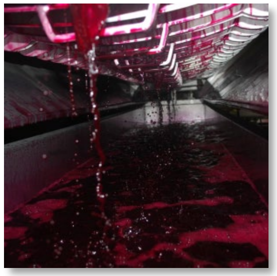
Is Primaflora as safe as SO2?

After several years of practice, we confirm that Primaflora® is so safe that SO_2 in the harvest.

His action against Brettanomyces, for example, is more effective than SO_2.

The question of oxygen and oxidation remains…

- Red wines need less antioxidant protection
- Most white musts tolerate oxidation
- Only musts with a low concentration in polyphenols, such as the « cuvés champenoises » need protection
- Use gallotannin (Gallovin) with Ascorbic acid (Gallovin C), yeast hulls rich in glutathione (Elevage Glu)



Bioprotection is a safe and approved method

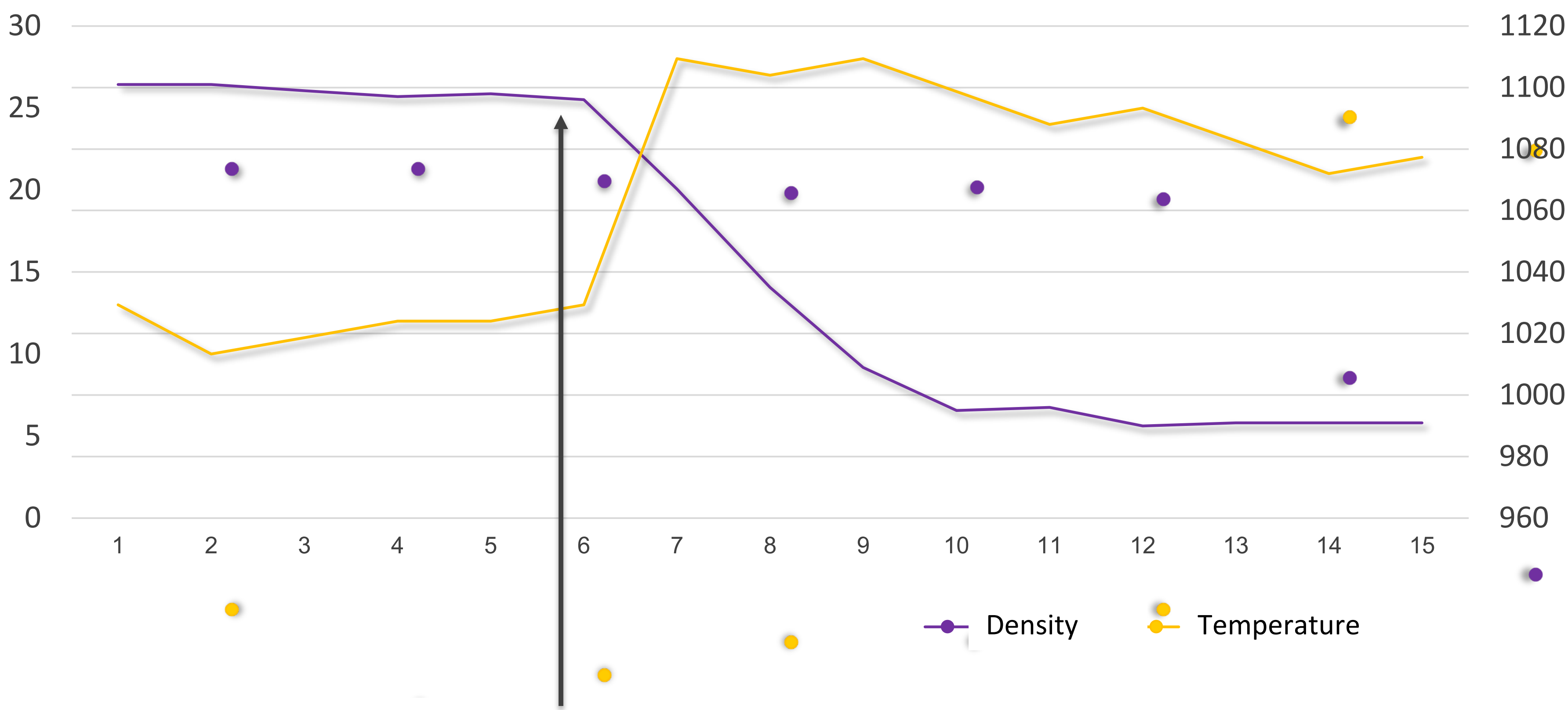
Going from « SO_2, without yeasts » to « no SO_2, with PRIMAFLORA® », there is always an improvement of safety and aromatic quality.

But bacteria can develop more easily at the end of fermentation if they take time to finish (the total SO_2 resulting from the harvest has an antibacterial action).

So be careful with malolactic fermentation for white wines.

Example

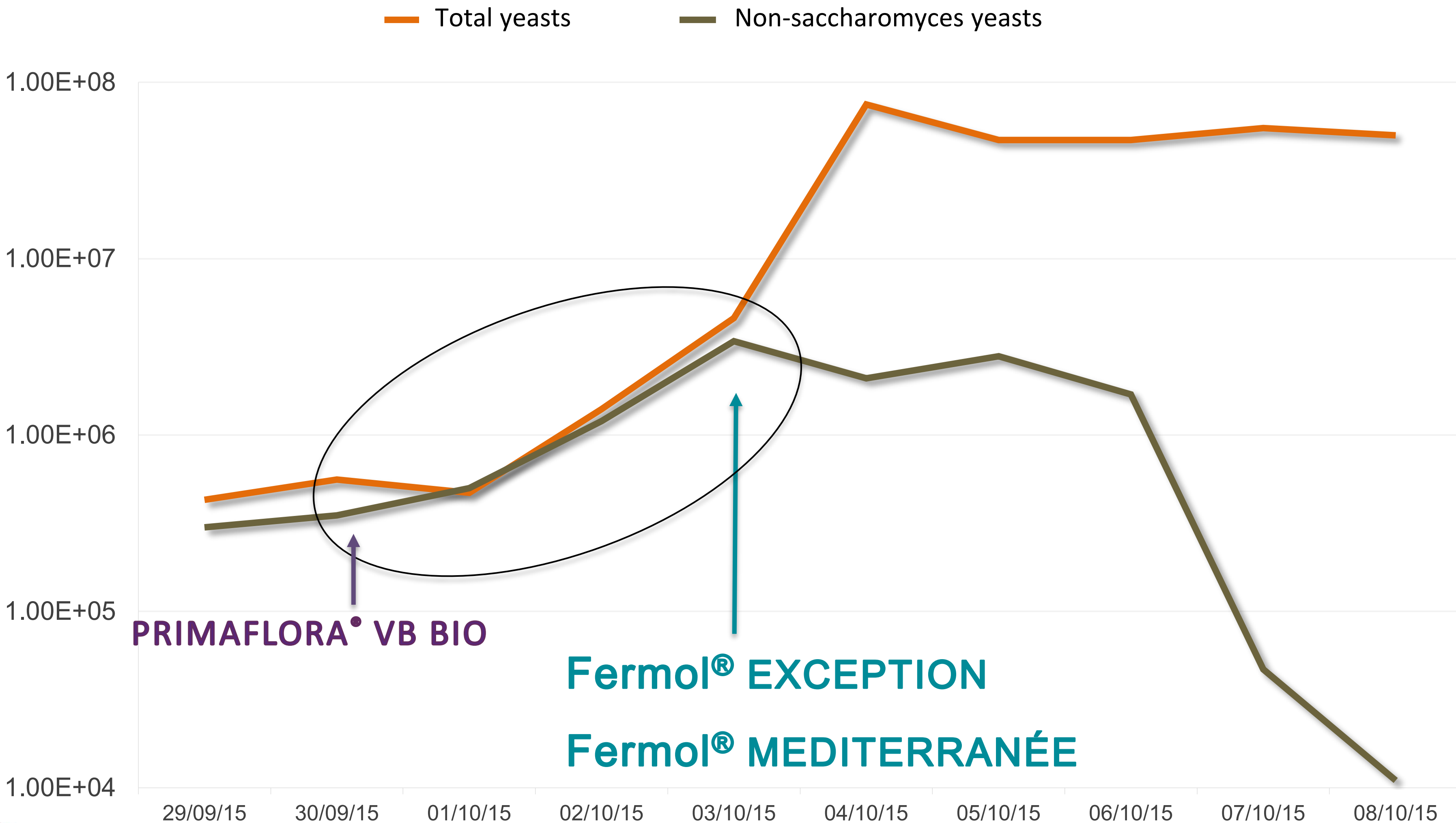
Figure 1 : alcoholic fermentation (tank 1).



Fermol® EXCEPTION / Fermol® MEDITERRANÉE

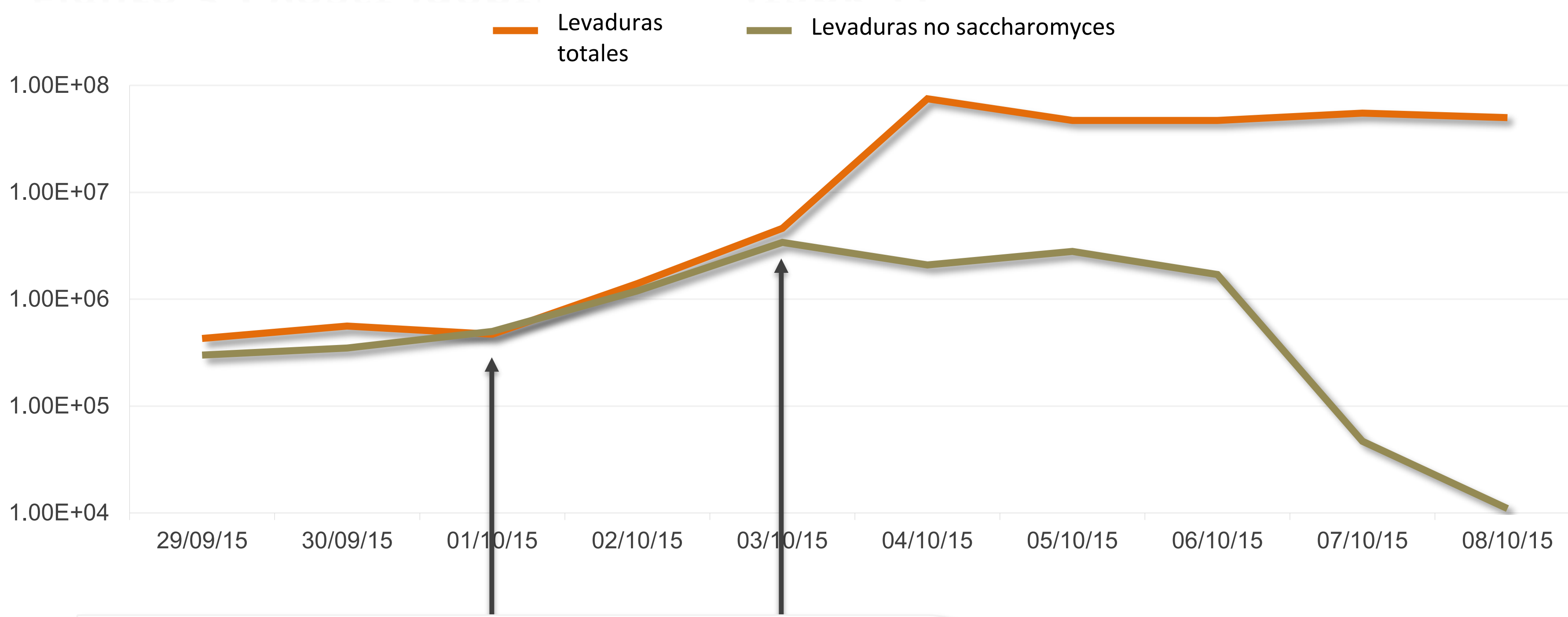
Duration of alcoholic fermentation: 8 days

Figure 2 : yeast population (tank 1)



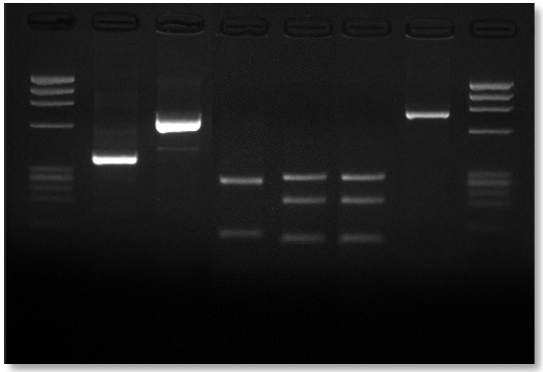
Ejemplo

Figure 3 : yeast identification (tank 1)



94% of non-Saccharomyces yeast flora is Primaflora® VB BIO

6% are native yeasts (Metschnikowia pulcherrima and Candida zemplimine).



Conclusion on effectiveness

Optimal protection: non-Saccharomyces yeast population > 10^6 CFU/mL

Dominance of Primaflora® (94%) over the native flora

Excellent fermentation kinetics : positive interaction with Saccharomyces

Balance

Bioprotection concept

Decrease 44%

	Harvest	+ Final MLF	+ Aging and bottling	= Total
Bioprotection concept	0 g/hL	5 g/hL	3,5 g/hL	8,5 g/hL
Conventional winemaking	5 g/hL	5 g/hL	5 g/hL	15 g/hL

Conventional winemaking

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Example

General conclusion of the winery

Perfect protection.
Regular alcoholic fermentation.
44% decrease in SO_2 used.
Obtaining qualitatively superior wines: more expression, purity, finesse and fruit.
No contamination with Brettanomyces since 3 years.

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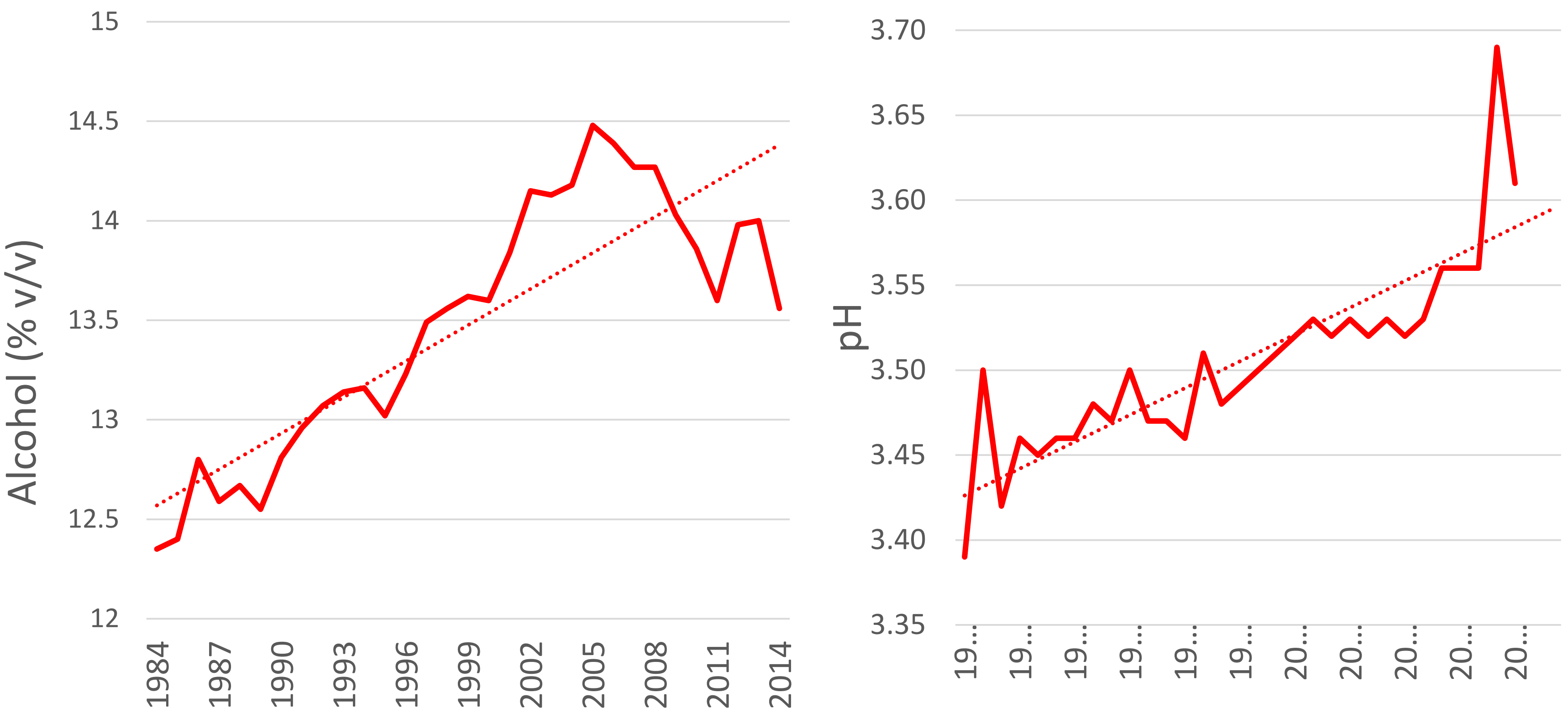
The use of heterofermentative yeast to lower pH and alcohol in must

Levulia Alcomeno

Ensenada 2019

Trends in wines



Australian reds; adapted from Godden et al. 2015

- Several ways can be used to control pH:

1. Direct addition of tartaric acid
2. Use of cation excanghers
3. Biological acidification

- Several ways can be used to control pH:

1. Direct addition of tartaric acid

NOT EFFICIENT ENOUGH because of the precipitation of potassium salt

Sensory profile is affected: NON NATURAL SOUR TASTE

- Several ways can be used to control pH:

2. Use of ion exchanger - AEB STABYMATIC

MORE EFFICIENT

CHEAPEST

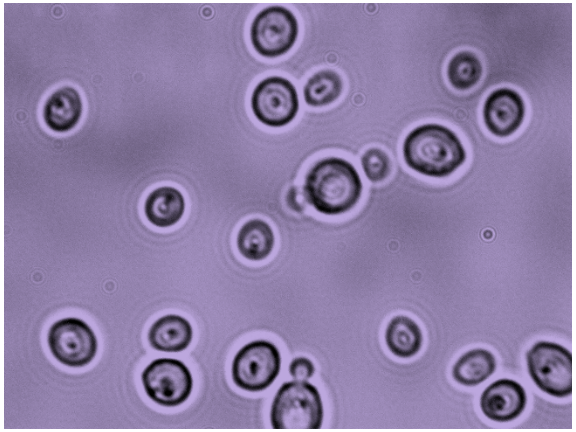
- Several ways can be used to control pH:

3. Biological acidification

USE OF YEAST to NATURALLY INCREASE ACIDITY

S. cerevisiae: selected strain can increase malic acid production of 0.5-1 g/L -> NOT ENOUGH to significantly affect pH and later DEGRADED by MLF.

AEB Solution:



Non-*Saccharomyces: Lachancea thermotolerans*

Interesting properties:

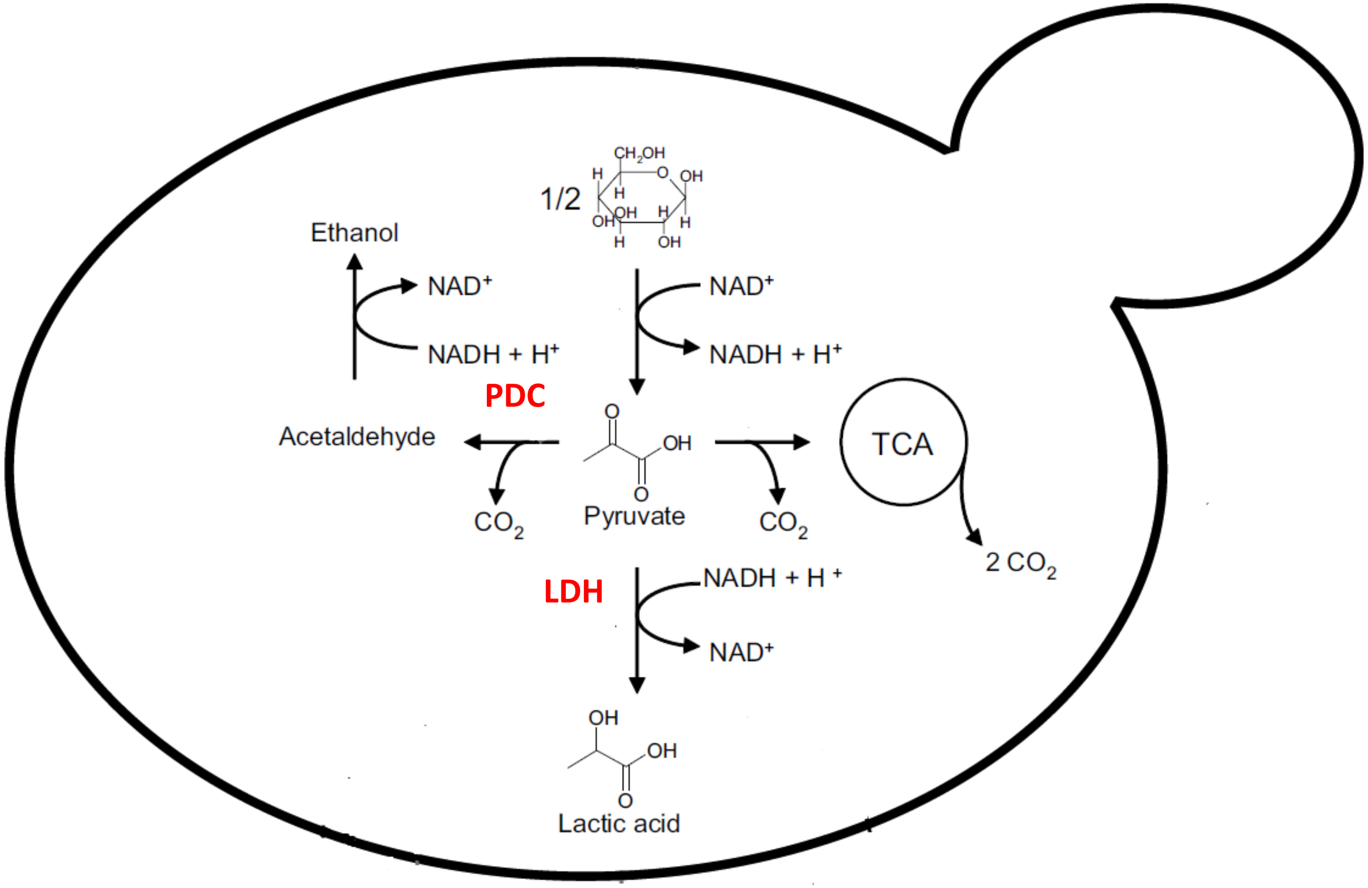
Softing and improving sensory qualities

Decreasing pH due to the production of lactic acid

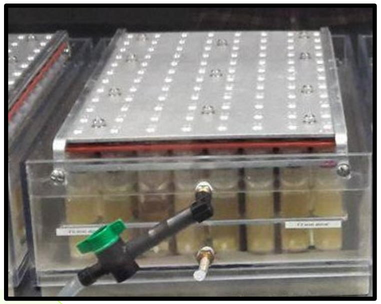
Lachancea Termotolerans ferments glucose and fructose

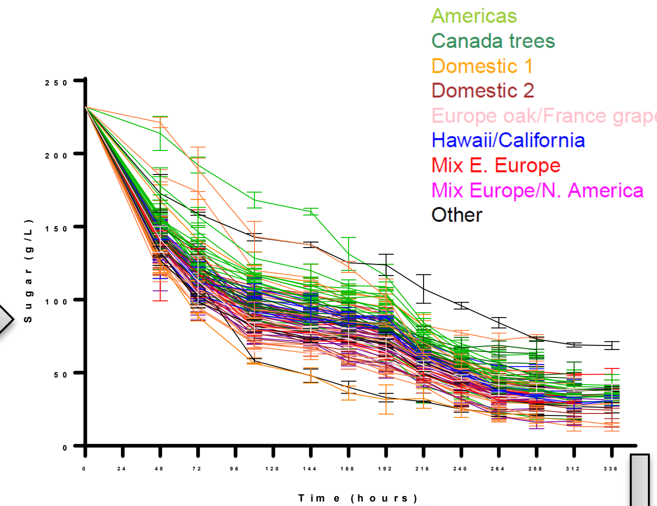
Improves wine acidity by the production of lactic acid

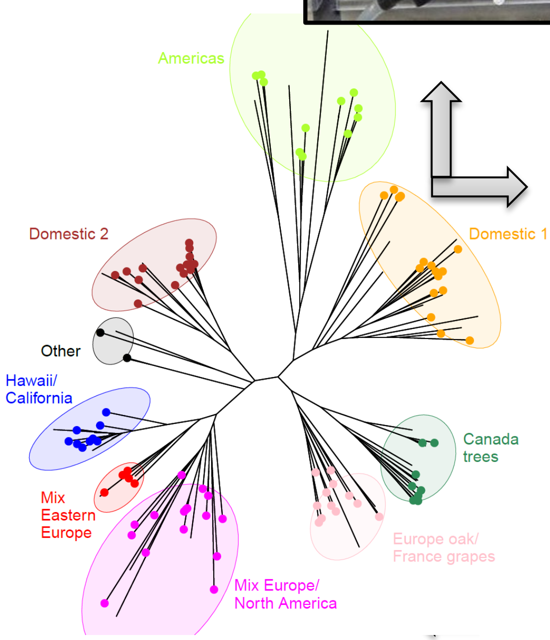
Enzyme lactate dehydrogenases: converts pyruvate to lactic acid (with NAD+ regeneration)

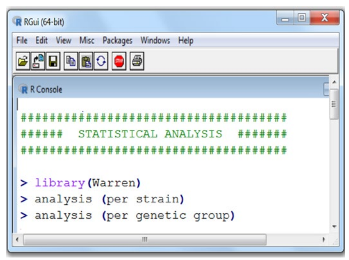


Oenological characterisation of 94 LT strains in Chardonnay ferments (236 g/L sugar; pH 3.5)

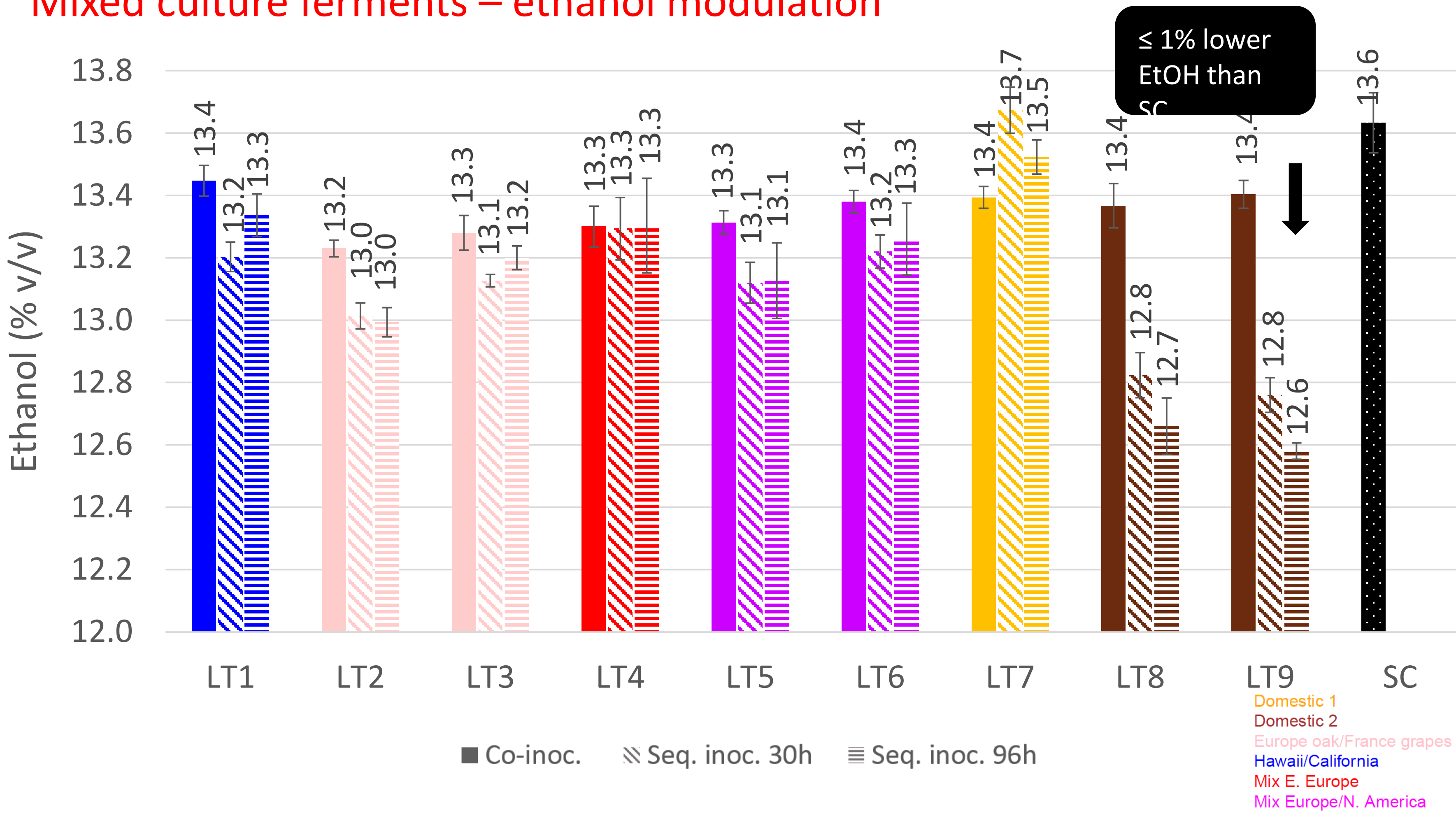








Mixed culture ferments – ethanol modulation



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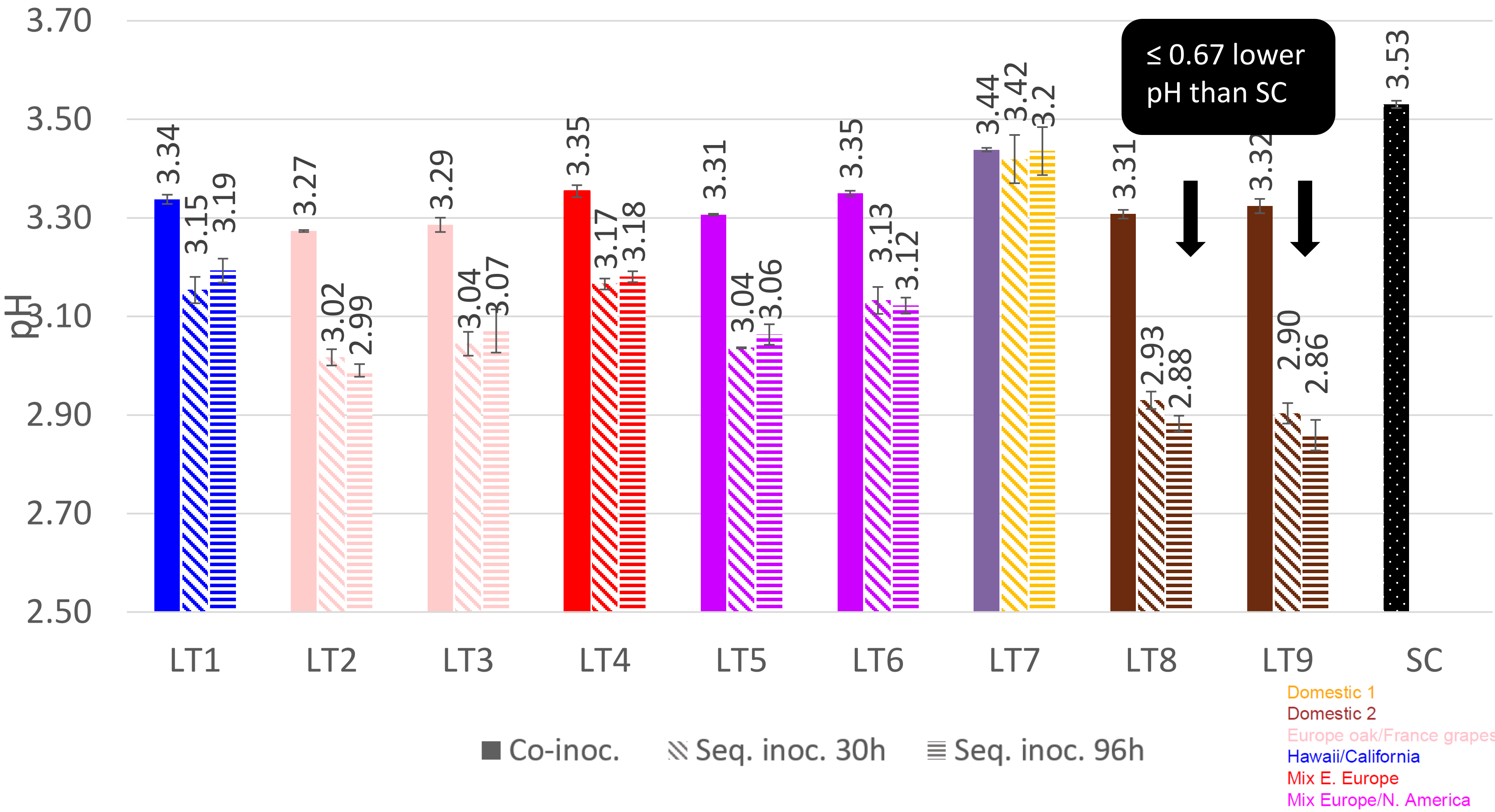
Mixed culture ferments – pH modulation



LEVULIA ALCOLMENO

Wild Isolate from Burgundy

1% reduction alcohol v/v

8 g/L Lactic acid

- **COMPOSITION AND TECHNICAL CHARACTERISTICS**
- Strain: *LACHANCEA THERMOTOLERANS*
- Alcohol tolerance: 7% vol.
- GMO-free and not subjected to ionizing treatments

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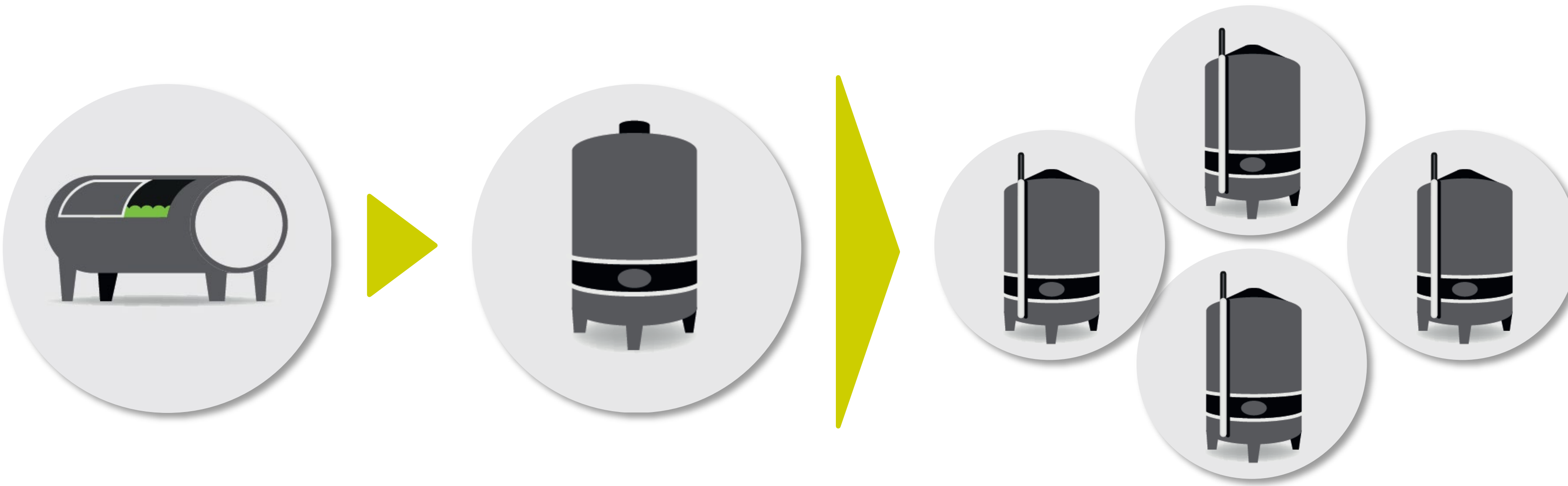
Vintage test benches

Technical Protocol Grape variety: Pinot Auxerrois



Open-cage pneumatic press

Capacity of 30 hL

Static settling thermoregulated tank

10 ° c

Homogeneous distribution of the must

4 stainless steel tanks of 1hL

Method

Control

Inoculation after settling with yeast of the species S. cerevisiae (LEVULIA® ESPERIDE) at a dose of 20 g/hL.

L. T (*Lachancea thermotolerans*)

Inoculation after settling with yeast of the species Lanchacea thermotolerans (LEVULIA® ALCOMENO) at 30 g/hL.
Then after 72 hours, inoculation with yeast S. cerevisiae (LEVULIA® ESPERIDE) at 20 g/hL.

Characteristics of the grape must of Pinot Auxerrois

Sucres (g/L)	213
TAP (% Vol.)	12,67
pH	3,41
AT (g/L H_2SO_4)	3,43
Acide malique (g/L)	2,02
Azote assimilable (mg/L)	181

Duration of alcoholic fermentation

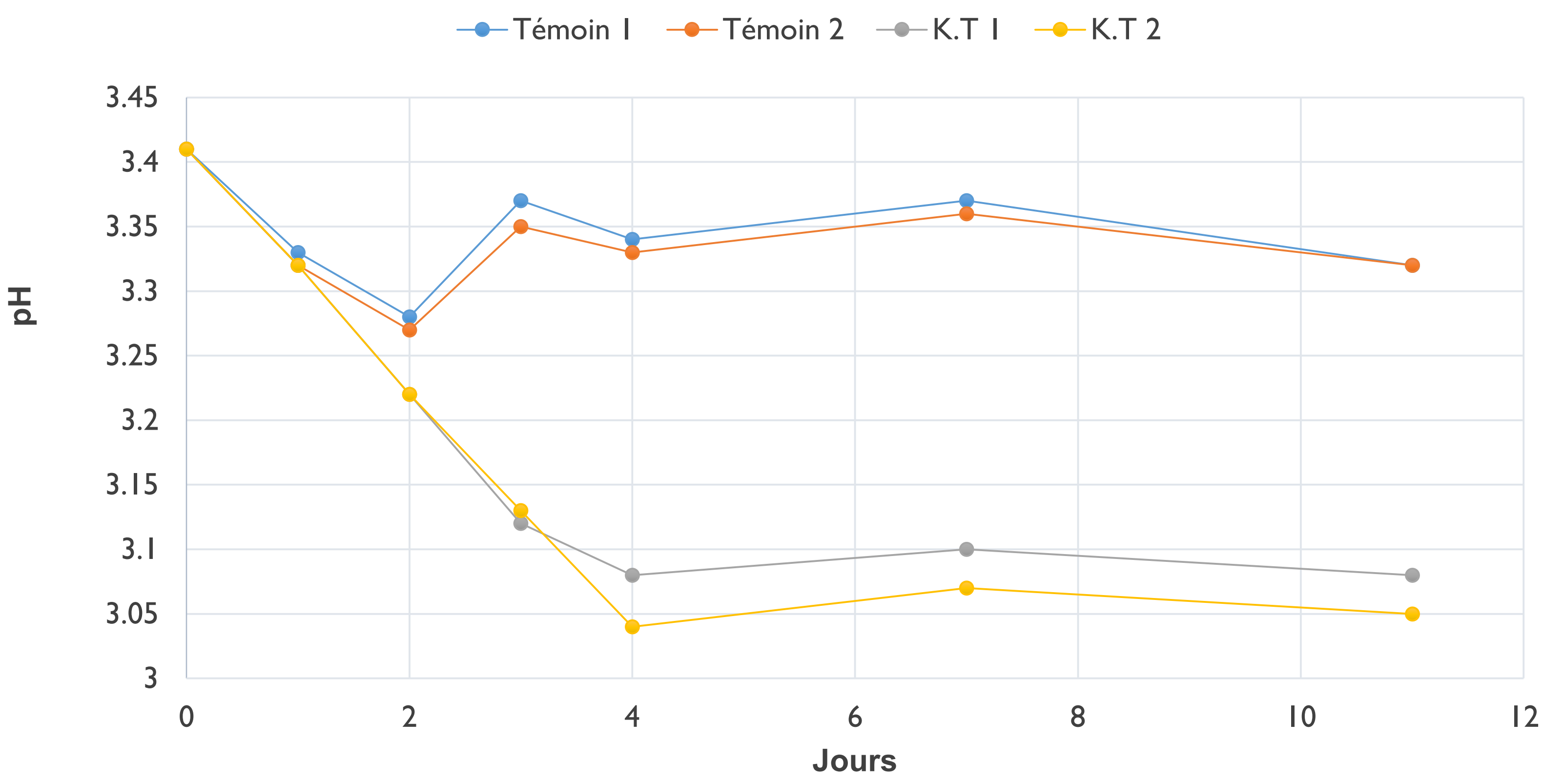
Control: 14 days
L. T: 17 days.

Difference due to the sequential inoculation of 72 h with yeast S. cerevisiae in the control modality.

Test results

Dosage of lactic acid on Pinot Auxerrois

PH measurement on Pinot Auxerrois



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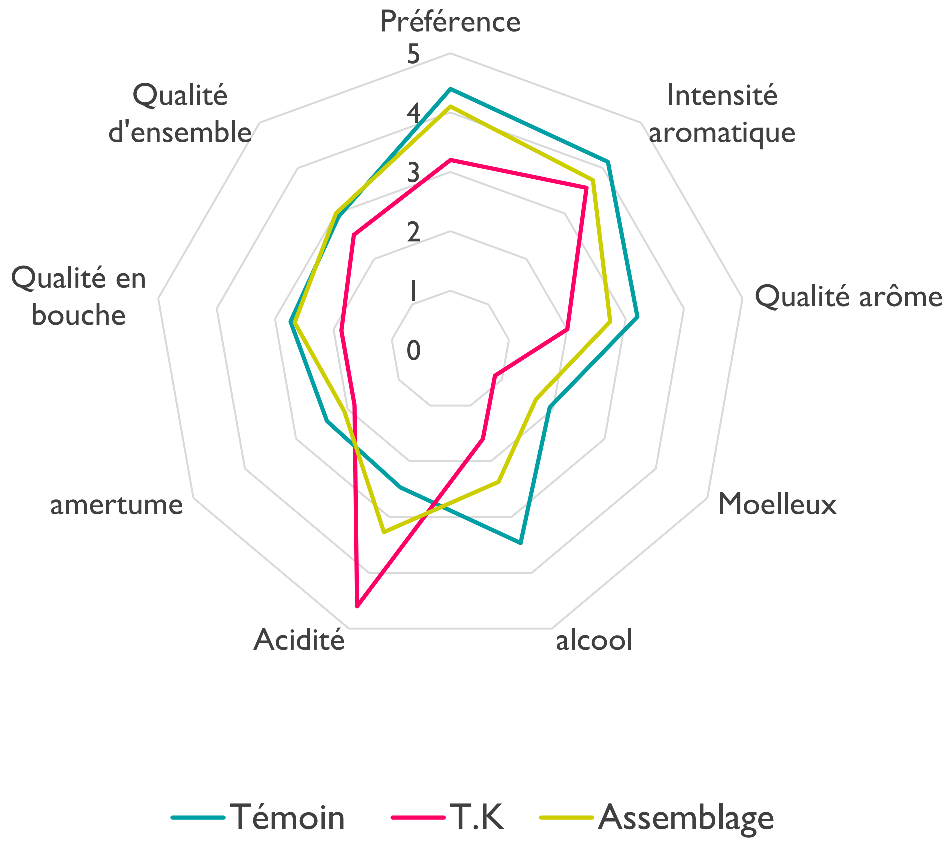
Analytical assessment of wines after bottling

	T- 1	T- 2	T.K 1	T.K 2	Assemblage
Acidité totale (g/L H_2SO_4)	**3,84**	**3,72**	**7,79**	**8,12**	5,23
pH	3,27	3,28	3,01	2,99	3,13
Glucose Fructose (g/L)	0,0	0,0	0,0	0,0	0
Titre Alcoométrique Volumique (% Vol.)	**13,87**	**13,81**	**12,46**	**12,48**	13,16
Acidité volatile (g/L H_2SO_4)	0,20	0,20	0,52	0,58	0,32
Acide malique (g/L)	2,04	2,01	1,33	1,28	1,76
Acide lactique (g/L)	0,01	0,01	7,12	7,43	2,49

Sensory analysis of wines after bottling

Sensory analysis by tasting

Jury of Oenologists composed of 15 people in order to obtain sensory descriptors for each of them.



Conclusion

- The use of a non-Saccharomyces yeast with a particular metabolism is now an interesting technological breakthrough.
- Lachancea (Kluyveromyces) thermotolerans allowed to obtain a wine with a decrease in the alcohol content of 1.37% vol.
- Uses some of the glucose available in the must to produce lactic acid (lactic fermentation) which is a particularity peculiar to this non-Saccharomyces yeast.
- Very significant increase in the total acidity of the wine of 4.1 g/L H_2SO_4 in favor of the L.T. wines
- Complementary aromatic signature of S. cerevisiae.
- Vintage 2019: Fermentation trials in red wines with ROC, Uni Fresno, UoBC, Casa Madero

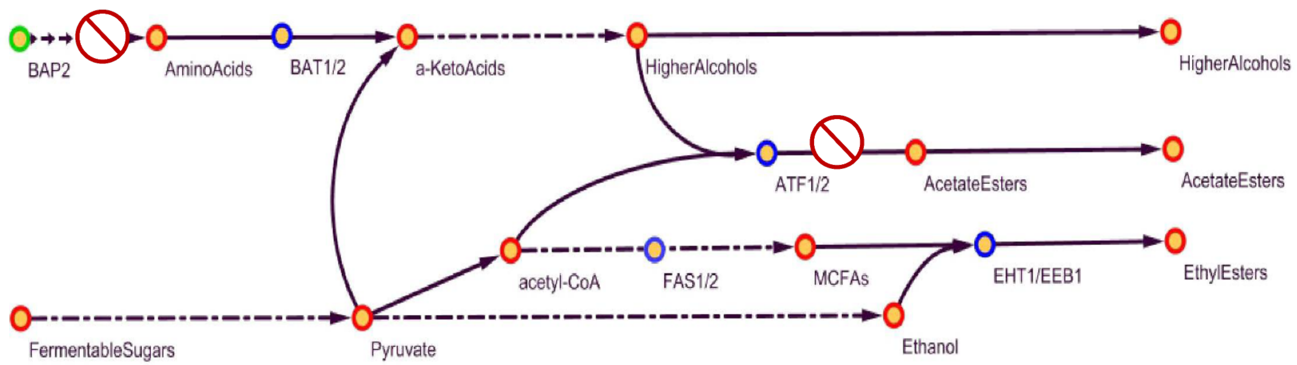
AEB®
IMPROVEMENT THROUGH BIOTECHNOLOGY

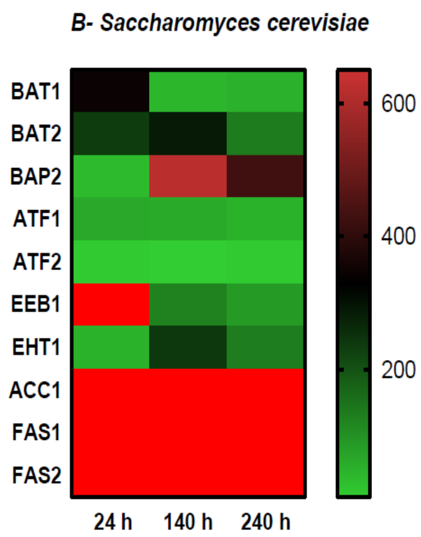
Yeasts co-fermentation to increasy aromatic complexity and mouthfell

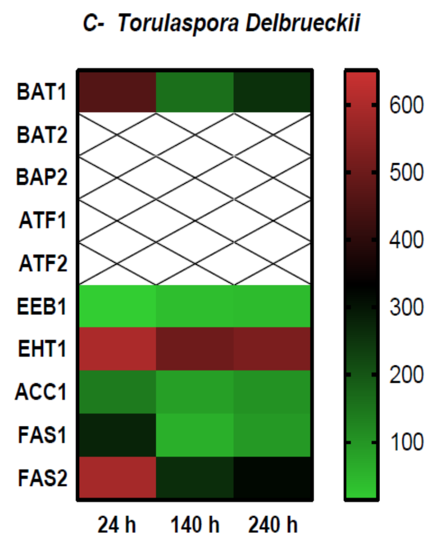
Levulia Torula

Ensenada 2019

- LEVULIA® TORULA is a yeast strain belonging to the species Torulaspora delbrueckii. It contributes positively to the organoleptic complexity of the wine while limiting the production of volatile acidity.
- It contributes to reduce the sensations of astringency in the mouth by the release of polysaccharides.
- LEVULIA® TORULA is suitable for all types of grape varieties, rich in terpenes and / or thiols (Sauvignon Blanc, Chardonnay, Gewurztraminer, Colombard, Riesling, Muscat, Sémillon, etc.) because of its high enzymes production (glucosidase and sulfur-lyase). + TERPENES +THIOLS
- LEVULIA® Torula can ensure the alcoholic fermentation at least up to 9% of the volume and can be used alone, in co-inoculation or sequential inoculation (24 to 48h) with the desired S. cerevisiae.
- *Tips&Tricks: Levulia Torula has very low acetic acid production in high sugar must making it ideal for sweet/late harvest wines.*

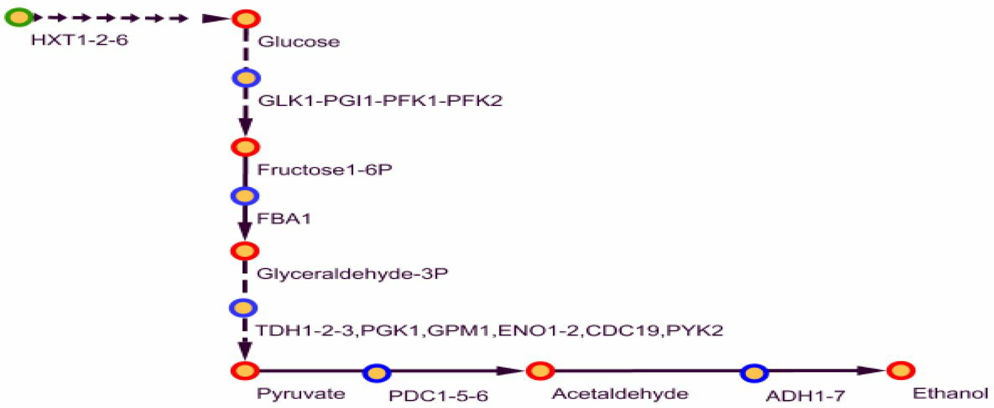


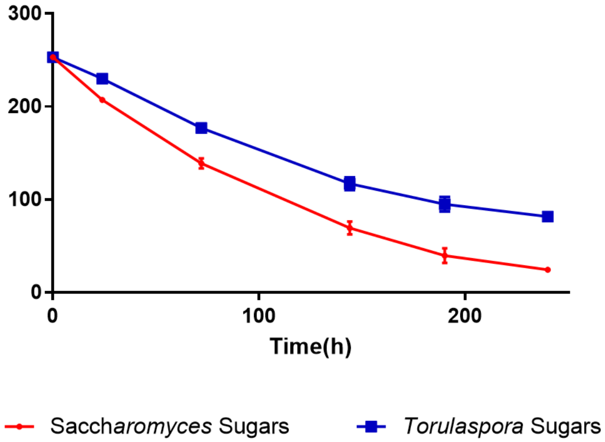




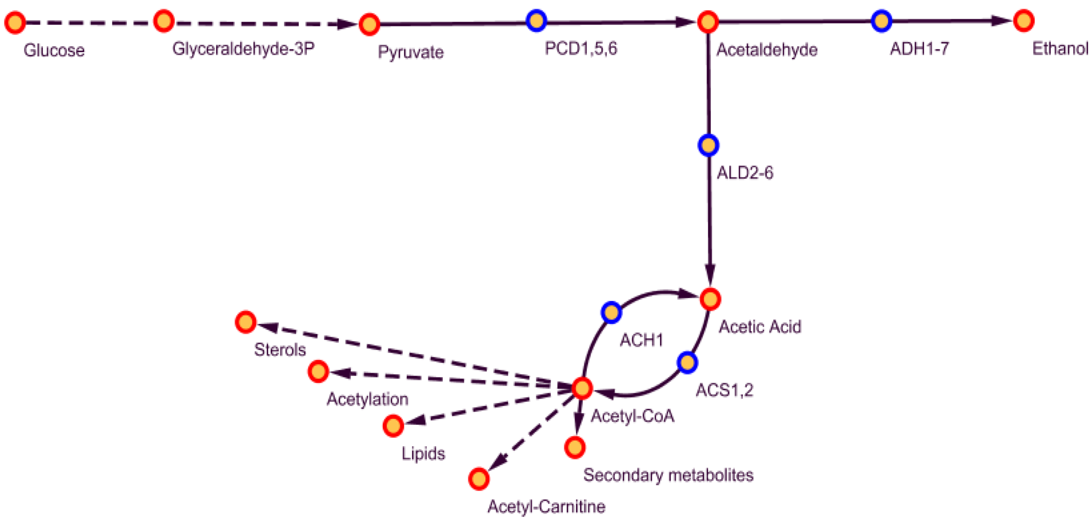
T. delbrueckii is missing multiple genes (higher alcohols, Acetate esters)

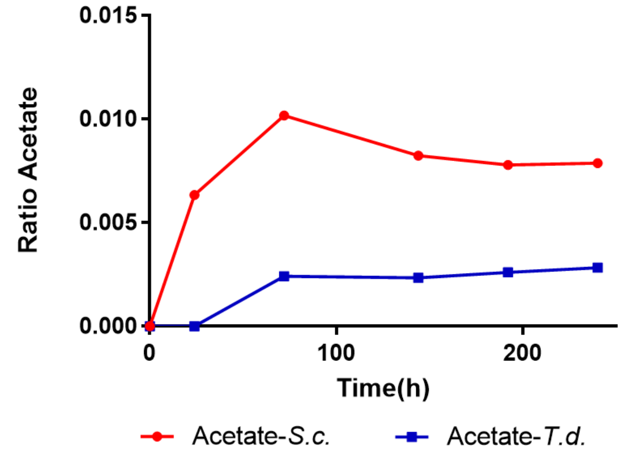
FAS1/2 transcripts expression lower (Ethyl esters)

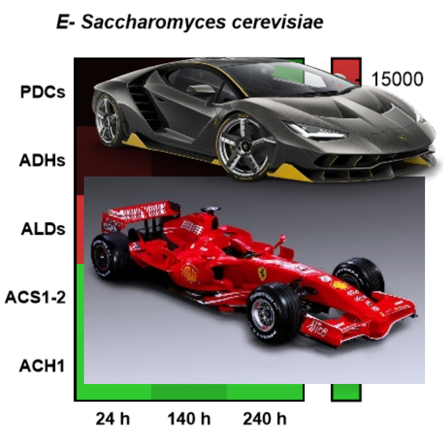


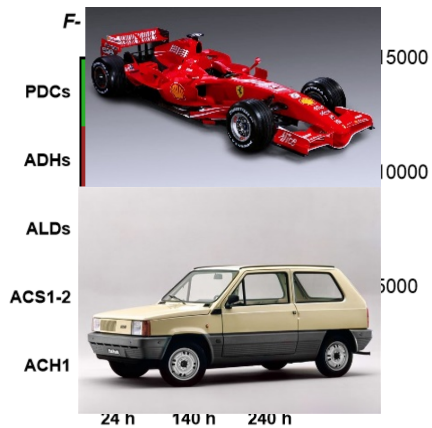


- Missing paralog genes
- *PGI1, TPI1, ENO2 = Candida glabrata*
- *PYK2 = Lachancea thermotolerans*
- *PDC1 = Kluyveromyces* sp

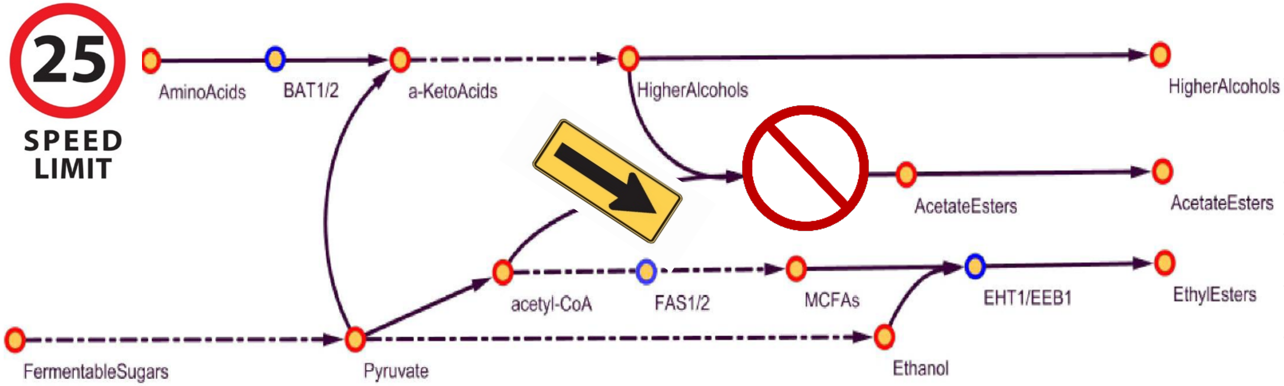


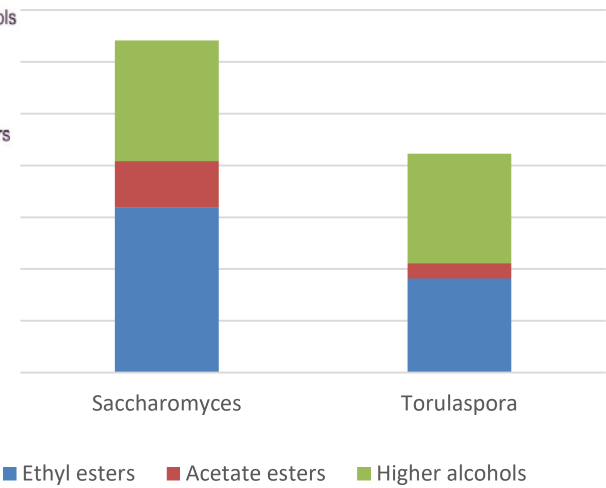




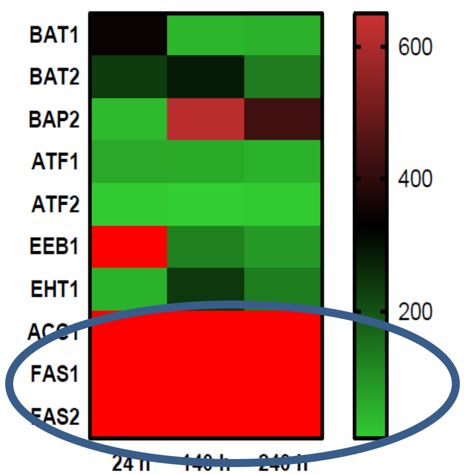


T. delbrueckii expresses
ADHs >8 times higher than *ALDs*

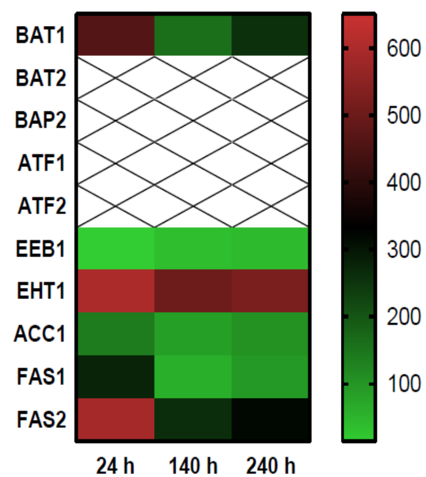




B- *Saccharomyces cerevisiae*



C- *Torulaspora Delbrueckii*



NO BAP2

NO ATF1-2

LOW FAS complex expression

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Int J Food Microbiol. 2017 Sep 18;257:183-191. doi: 10.1016/j.ijfoodmicro.2017.06.028. Epub 2017 Jun 27.

Influence of Torulaspora delbrueckii in varietal thiol (3-SH and 4-MSP) release in wine sequential fermentations.

Belda I[1], Ruiz J[1], Beisert B[2], Navascués E[3], Marquina D[1], Calderón F[4], Rauhut D[2], Benito S[4], Santos A[5].

Author information

Abstract

In last years, non-Saccharomyces yeasts have emerged as innovative tools to improve wine quality, being able to modify the concentration of sensory-impact compounds. Among them, varietal thiols released by yeasts, play a key role in the distinctive aroma of certain white wines. In this context, Torulaspora delbrueckii is in the spotlight because of its positive contribution to several wine quality parameters. This work studies the physiological properties of an industrial T. delbrueckii strain, for the production of wines with increased thiol concentrations. IRC7 gene, previously described in S. cerevisiae, has been identified in T. delbrueckii, establishing the genetics basis of its thiol-releasing capability. Fermentations involving T. delbrueckii showed improvements on several parameters (such as glycerol content, ethanol index, and major volatile compounds composition), but especially on thiols release. These results confirm the potential of T. delbrueckii on wine improvement, describing new metabolic features regarding the release of cysteinylated aroma precursors.

KEYWORDS: 4-MSP; Non-Saccharomyces; Torulaspora delbrueckii; Varietal thiols; Wine


WORK IN
PROGRESS

Thank you for your attention

Questions?

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