Aitor BALMASEDA RUBINA

EFFECT OF OENOLOGICAL NON-*Saccharomyces* YEASTS ON MALOLACTIC FERMENTATION AND *Oenococcus oeni*

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Effect of oenological non-*Saccharomyces* yeasts on malolactic fermentation and *Oenococcus oeni*.

Balmaseda, A., Reguant, C., Bordons, A., Bautista-Gallego, J.

Dept Bioquímica i Biotecnologia, Facultat d'Enologia, Universitat Rovira i Virgili, 43007-TARRAGONA, SPAIN.

email: aitor.balmaseda@estudiants.urv.cat

RESUMEN

En los últimos años se ha incrementado notablemente el interés por el uso de levaduras no-*Saccharomyces* en la elaboración de vino. Normalmente estas levaduras se inoculan para iniciar la fermentación alcohólica (FAL) del mosto y posteriormente se inocula *Saccharomyces cerevisiae* para finalizar el proceso. Este tipo de inoculación secuencial de levaduras no-*Saccharomyces* se ha asociado con la mejora organoléptica de algunos vinos.

Son diversas las especies que se han descrito como beneficiosas, entre ellas cabe destacar *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, *Hanseniaspora uvarum*, *H. vineae* y *Starmerella bacillaris* (syn. *Candida zemplinina*) entre otras. Concretamente de las dos primeras existen ya algunos cultivos iniciadores comerciales en el mercado. A pesar del creciente interés por las no-*Saccharomyces*, apenas se han publicado trabajos científicos que evalúen el posible efecto de estas levaduras sobre la fermentación maloláctica (FML). Algunas de estas cepas de no-Saccharomyces producen otros cambios que podrían afectar directamente al desarrollo de la bacteria láctica *Oenococcus oeni*, principal responsable de la FML. El aumento de ácido succínico y/o la disminución de ácido L-málico descrito para algunas no-*Saccharomyces* podrían actuar como inhibidores de la FML. Por otra parte la disminución de etanol y SO_2 podrían tener un efecto positivo en la adaptación de *O. oeni* al vino. Por ello, el presente trabajo plantea el estudio del efecto del uso de levaduras no-*Saccharomyces* sobre *O. oeni* y la FML. Se evaluaron diferentes fermentaciones experimentales mixtas con cepas conocidas de *H. vineae*, *H. uvarum*, *M. pulcherrima* con *S. cerevisiae*, junto con una fermentación control de S. cerevisiae. Tras la caracterización química de los vinos obtenidos, se inocularon dos cepas de *O. oeni*: PSU-1 y 1PW13 y se estudió la evolución de la FML en los distintos vinos.

Los vinos obtenidos tras la FAL de las diferentes combinaciones de no-*Saccharomyces* estudiadas, no mostraron una composición química muy diversa respecto a los parámetros evaluados. Sin embargo, las cepas de *O. oeni* inoculadas pudieron llevar a cabo una FML deseada únicamente en los vinos obtenidos a partir de las combinaciones de *H. uvarum* y *M. pulcherrima* con *S. cerevisiae*. En los otros casos, la FML no concluyó. Además, en este estudio no se observó que el aumento en la concentración de ácido succínico tuviera un efecto inhibidor directo en el desarrollo de la FML, tal y como se describe en bibliografía.

Los resultados obtenidos permitirán determinar los efectos más relevantes del uso de no-*Saccharomyces* sobre *O. oeni* y podrán ser aplicados al criterio de selección de combinaciones levadura-bacteria más beneficiosas para el desarrollo de la FML.

Palabras clave: no-*Saccharomyces*, *Oenococcus oeni*, fermentación maloláctica, interacciones

ABSTRACT

In the last few years it has increased the interest on the use of non-*Saccharomyces* yeasts in winemaking. Generally, these yeasts are inoculated to start the alcoholic fermentation (AF) of must and later, *Saccharomyces cerevisiae* is inoculated to finish the process. This type of sequential inoculation with non-*Saccharomyces* yeasts it has been linked with the organoleptic improvement of some wines.

Some species have been reported as beneficial, such as *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, *Hanseniaspora uvarum*, *H. vineae* and *Starmerella bacillaris* (syn. *Candida zemplinina*), etc. In particular, *Torulaspora delbrueckii*, *Metschnikowia pulcherrima* are available as starter cultures in winemaking. Despite the increasing interest in non-*Saccharomyces* yeasts, there are not many works in which are evaluated the possible effects of these yeasts upon the malolactic fermentation (MLF). Some strains produce other changes that could directly affect to the lactic acid bacteria *Oenococcus oeni* growth, the main agent of MLF. The increase of succinic acid and/or the consumption of L-malic acid that has been reported in some non-*Saccharomyces* could act as MLF inhibitors. What is more, the ethanol content and SO_2 decrease, could positively affect the adaptation of *O. oeni* to wine. For this purpose, this present work considers the study of the use of non-*Saccharomyces* upon *O. oeni* and MLF. There were evaluated experimental mixed fermentations with the species *H. vineae*, *H. uvarum*, *M. pulcherrima* with *S. cerevisiae*, as well as a control fermentation with *S. cerevisiae*. After the chemical characterization of the obtained wines, two strains of *O. oeni* were inoculated; PSU-1 and 1PW13, and it was studied their behaviour in those wines.

The obtained wines after the AF by the different mixes of the studied non-*Saccharomyces* did not show very diverse chemical composition in regards to the evaluated parameters. However, the inoculated *O. oeni* strains, could only undergo the MLF in the wines obtained by the AF of *H. uvarum* and *M. pulcherrima* with *S. cerevisiae*. In the other cases, the MLF did not conclude. What is more, in this study it was not observed that the increase in succinic acid concentration had a direct inhibitory effect upon the MLF, as it is described in literature.

The results will allow determining the main effects of the use of non-*Saccharomyces* yeasts upon *O. oeni* and will be employed as yeast-bacteria selection criteria.

Key words: non-*Saccharomyces*, *Oenococcus oeni*, malolactic fermentation, interactions

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1. INTRODUCTION

1.1. NON-*Saccharomyces* YEASTS IN WINEMAKING

Wine is the result of the alcoholic fermentation driven out by oenological yeasts in a complex microbial environment (Beltran et al., 2002; Constantí et al., 1997). Although *Saccharomyces cerevisiae* is recognized as the main agent that carries out this complex process, there are more agents involved, since the grape must is a complex microbial environment. Other yeast species belonging to other genera, such as *Hanseniaspora/Kloeckera*, *Pichia*, *Candida* or *Metschinokowia* (Fleet et al., 1984) are implicated in the early stages of the alcoholic fermentation (AF). All these group of yeasts, belonging to another different genera apart from *Saccharomyces*, are known as non-*Saccharomyces* yeasts.

Even though the complex microbial environment, and the fact that some non-*Saccharomyces* yeasts can start the AF, *S. cerevisiae*, has the ability to overcome the growth of other competitor yeasts. That is why, *S. cerevisiae* dominates the fermentation since middle to the final fermentative stages (Fleet et al., 1984; Heard and Fleet, 1985). Beside its resistance to ethanol and SO_2, *S. cerevisiae* was considered candidate to carry out the technology of starter yeast culture in winemaking (Romano and Suzzi, 1993).

However, nowadays winemaking is changing because of the emerging interest of using non-*Saccharomyces* yeasts during the alcoholic fermentation to increase wine complexity and differentiation.

Current knowledge of non-*Saccharomyces* yeasts leaded to use them as starter cultures (Comitini et al., 2011; Viana et al., 2008). Despite their positive role, it has been studied that their use, as sole starter culture, can increase the appearance of acetic acid, ethanol and other undesirable compounds in the final wine. Even more, their main inconvenient, when used as only starter, is their low resistance to SO_2 (Jolly et al., 2006) and their poor fermentative activity (Contreras et al., 2014). On the other hand, it has been reported the positive role of non-*Saccharomyces* yeasts on wine fermentation and the final wine, when used as mixed culture with *S. cerevisiae*. This positive role of non-*Saccharomyces* ranges from a better fermentation performance to an improved wine quality and complexity (Comitini et al., 2011; Fleet, 2008; Jolly et al., 2014; Lema et al., 1996; Medina et al., 2013; Viana et al., 2008).

In particular, the most studied non-*Saccharomyces* species to modulate the organoleptic profile of wines are *Kloeckera apiculata, Torulaspora delbrueckii, Hanseniaspora guillermondi, H.*

uvarum, H. vineae, *Candida zemplinina*, etc (Fleet, 2008; Izquierdo Cañas et al., 2014; Jolly et al., 2006; Loira et al., 2014; Padilla et al., 2016; Strauss et al., 2001). According to produce more glyceric wines, *C. stellata* was used (Fleet, 2008).

It is because their potential use in mixed fermentations that our group participated in the European project "WildWine". In such project, a big amount of non-*Saccharomyces* and *Oenococcus oeni* strains were isolated and characterized from the wine-producing region of the D.O.Ca. Priorat (Wang et al., 2014, 2015). Those non-*Saccharomyces* were mainly species of *H. uvarum, H. vineae, T. delbrueckii, Metschnikowia pulcherrima*, etc.

1.2. MALOLACTIC FERMENTATION AND *Oenococcus oeni*

Malolactic fermentation (MLF) consists on a fairly simple reaction: a unique enzymatic decarboxylation of the L-malic acid to L-lactic acid (Liu, 2002). It is known as the second fermentation of wine, usually performed in red wines or high acidity white wines. This fermentation is carried out by lactic acid bacteria (LAB), in which the main species and the one that dominates in wine is *O. oeni* (Carreté et al., 2005; Liu, 2002).

This MLF is related to a quality improvement in wine since this biotransformation leads to a pH increase, because of the decarboxylation of L-lactic acid, which is a dicarboxylic acid to L-lactic acid, a monocarboxylic acid (Liu, 2002). During MLF, LAB consume L-malic acid and other nutrients, impoverishing wine. Therefore, in warm regions, where musts do not present high acidity, it is usually desirable to perform this MLF, due to the microbial stability that is achieved (Liu, 2002).

However, it should be noted that MLF is not favourable for all wines (Davis et al., 1985; Henick-Kling and Hee Park, 1994). Besides, some LAB can synthesise undesirable compounds that compromise the organoleptic characteristics of the product (Lonvaud-Funel, 1999), so MLF has to be a controlled process.

Ecological dynamics of LAB have been thoroughly studied for years. Generally, low population LAB density is detected in early stages of AF, coming from grape skins. The LAB diversity is maintained until the alcoholic content is not very high. When the ethanol concentration starts to increase, the bacterial population begin to decrease. At that point, contrary to the behaviour of other LAB species, *O. oeni* commence to grow actively (Lonvaud-Funel, 1999). Basically, *O. oeni* become dominant because of its high tolerance to ethanol, SO_2, low pH, and other stressful conditions (Henick-Kling and Hee Park, 1994; Kunkee, 1991; Wibowo et al., 1985).

Moreover, it has been reported that a minimum population of 10^6 cells/mL is needed to start the consumption of L-malic acid (Lonvaud-Funel, 1999). Under the stressful environmental conditions (Fleet et al., 1984) that present wine, the failure of MLF is usual. Trying to solve this problem, similarly to what happens in the AF, the starter culture technology was developed with LAB (Antalick et al., 2013; Henick-Kling and Hee Park, 1994). For this purpose *O. oeni* was selected as candidate due to its high adaptation to wine conditions. In addition to the selection of tolerant wine strains, there is an increasing consciousness of the potentially effects of the interaction between yeast strains used to perform AF and the ability of the LAB bacteria to carry out the MLF.

1.3. YEAST-BACTERIA INTERACTIONS

As introduced before, the performance of MLF by LAB is highly affected by the physic-chemical intrinsic properties of wine. Moreover, it is also influenced by the possible interaction between yeasts which have conducted the AF. Those interactions range from inhibitory, to neutral and stimulatory. There is not much literature about this topic, but it is agreed that the type and impact of the interactions is dependant by several factors like (1) yeast/bacteria strain combination, (2) the uptake and release of nutrients by yeasts and (3) the ability of yeasts to produce metabolites that affect somehow LAB (Alexandre et al., 2004; King and Beelman, 1986; Lonvaud-Funel et al., 1988).

1.3.1. *S. cerevisiae* interactions with *O. oeni* and the MLF

Since the purpose of this current study is to evaluate the unknown effect of the non-*Saccharomyces* yeasts upon *O. oeni* and the MLF, it is necessary to understand the known effect of *S. cerevisiae* to compare and discern the possible specific effects of the non-*Saccharomyces* yeasts.

Regarding to the inhibitory interactions, it has to be mentioned that the main inhibitory compound secreted by *S. cerevisiae* with a strong effect upon *O. oeni* and the MLF is the ethanol itself (Alexandre et al., 2004). Ethanol concentrations higher than 12% cause great difficulties in LAB growth. Apart from ethanol, SO_2, which is produced by *S. cerevisiae* based on must composition, is known as a main inhibitory compound (Lonvaud-Funel et al., 1988). Other compounds, such as medium chain fatty acids, mostly C10 and C12, can lead an inhibitory effect upon *O. oeni* playing synergically with ethanol and the low pH (Carreté, 2002; Nehme et al., 2008).

When it comes to yeast/bacteria strain combination, it has been reported that some *S. cerevisiae* strains can produce some antimicrobial proteins (Comitini et al., 2005; Dick et al., 1992). Moreover, it has been noticed that wine produced with criotolerant strains of *S. cerevisiae*, which are related with higher production of succinic acid and phenyl-ethanol, present much MLF performance problems (Caridi and Corte, 1997).

On the contrary, it has to be considerate the positive effects of *S. cerevisiae* upon *O. oeni* and MLF. Generally, MLF performance in the presence of yeast lees is positively influenced. This positive effect is attributed to the presence of mannoproteins that could help hijack toxic compounds of the medium (Diez et al., 2010; Guilloux-Benatier et al., 1995). Besides mannoproteins, other compounds are released from yeast lees result of the autolysis phenomena (Alexandre et al., 2004). Aminoacids, peptides, proteins or glucans are examples of other released compounds that can stimulate the growth of *O. oeni* in wine (Guilloux-Benatier et al., 1995; Martínez-Rodriguez et al., 2001).

1.3.2. Non-*Saccharomyces* possible interactions with *O. oeni* and the MLF

There is no much information about the possible interactions of non-*Saccharomyces* yeasts with *O. oeni*. In regards to yeast-bacteria interactions, in non-*Saccharomyces* mixed fermentations, conflicting results are found. Mendoza et al., 2011 showed that mixed fermentation of *S. cerevisiae* and *Kloeckera apiculata* had no effect in MLF performance and that the sequential inoculation of *O. oeni* had a product quality improvement. However, the same group detected the presence of some inhibitory compounds that affected *O. oeni* (Mendoza et al., 2010).

Since a mixed fermentation can directly impact in wine chemical composition, *O. oeni* can be affected by those changes. One of the desired chances in wine quality is the increase in glycerol content (Jolly et al., 2006). At the beginning, contrary to other LAB (Liu, 2002), *O. oeni* has no ability to assimilate this compound, but there is no information of the effect that glycerol increase can cause upon the MLF.

Naturally, one of the compounds that have higher impact upon *O. oeni* and MLF is the L-malic acid. It has been reported that the use of non-*Saccharomyces* can cause a decrease in L-malic acid concentration (Belda et al., 2014), which can negatively affect the MLF performance. Citric acid is another important organic acid in this field because it can be assimilated by *O. oeni* having a positive effect upon the MLF (Liu, 2002). There is no clear conclusion in citric acid concentration variation by non-*Saccharomyces* yeasts. Some authors reported a little increase in

its concentration (Giaramida et al., 2013); meanwhile others showed no changes in citric acid composition (Izquierdo Cañas et al., 2014).

As introduced before, *S. cerevisiae* strains can produce significant concentrations of succinic acid (Caridi and Corte, 1997). Succinic acid can inhibit the MLF since it acts as competitive inhibitor of L-malic acid by the malolactic enzyme (Lonvaud-Funel and Strasser de Saad, 1982). Similarly to that happens with citric acid, some groups reported an increase of succinic acid in mixed fermentations with non-*Saccharomyces* (Contreras et al., 2014), whereas other reported a decrease in its concentration (Magyar et al., 2014).

To sum up, the use of non-*Saccharomyces* in winemaking can alter the wine composition, and consequently affect *O. oeni* and MLF. As explained above, these effects can range from inhibitory, to neutral and stimulatory. Since the interactions between non-*Saccharomyces*, *S. cerevisiae* and *O. oeni* in winemaking are very complex, they should be studied. For this reason, the main objective of this work is to characterize the interaction between different non-*Saccharomyces* yeasts and *O. oeni* in sequential inoculations.

3. MATERIALS AND METHODS

3.1. MICROORGANISMS, CULTURE MEDIA, SOLUTIONS AND REAGENTS

All microorganisms, culture media, solutions and reagents used and cited in this present work are described in **Annex I: Microorganisms, culture media, solutions and reagents.**

3.2. STRAINS' CONSERVATION

In this work four yeasts were used: *S. cerevisiae* (Lalvin-QA23), *H. vineae* (CECT T02/5AF), *H. uvarum* (CECT 13130) and *M. pulcherrima* (CECT 13131); and two *O. oeni* strains: *O. oeni* PSU-1 and *O. oeni* 1PW13, (**Annex I: Table I**). These yeasts and *O. oeni* strains were maintained on YPD plates and MRS_{mf} plates, respectively at 4ºC for maximum of one month. After that period, the strains were subcultured on new plates.

3.3. GROWTH CURVES AND INOCULUM OBTAINMENT

Firstly, for each microorganism strain the characteristic growth curve was established. The growth conditions were the following: yeasts were grown at 27ºC and LAB were grown at 28ºC in a 10% of CO_2 atmosphere.

Before the preparation of the inoculum, a preinoculum was grown. The same procedure was followed for yeast and bacteria strains. From isolated culture plates, YPD or MRS_{mf} agar plates respectively, a single colony was picked and grown on 5 mL of liquid medium. In the case of yeast strains, they were grown in YPD liquid medium, whereas *O. oeni* strains were grown in MRS_{mf} liquid medium. Similarly to the previous, yeasts were grown at 27ºC and LAB were grown at 28ºC in a 10% of CO_2 atmosphere. After 24 hours, four days and seven days, yeast strains, *O. oeni* 1PW13 and *O. oeni* PSU-1, respectively, were passed to fresh medium in a proportion of 1%. Usually these inoculums had a final volume of 40 mL; so 400 µL of grown preinoculum was used.

Each microorganism's growth was followed by the measurement of the O.D. at 600 nm (Spectro Genesis 10UV, ThermoScientific) and plating after appropriate dilution with sterile 0,85% saline solution.

3.4. STUDY OF *O. oeni* CONDITIONATED GROWTH

To furthermore study the effect of the yeast metabolism on LAB growth, *O. oeni* strains were grown in MRS_{mf} medium were yeasts had already been grown for 24 hours.

Fresh MRS_{mf} medium (40 mL in Falcon tubes) was inoculated with each of one yeast (**Annex I: Table I**) suspension to achieve a final population of 10^6 CFU/mL. After 24 hours, the medium was centrifuged at 8500 rpm for 5 minutes and was treated with 1% of dimethyl dicarbonate to kill any remaining yeast. The treated medium was conserved at 4ºC for 24 hours to get the compound decomposed. *O. oeni* strains were only inoculated when it was confirmed that there was no viable yeast remaining. After that, samples were taken to determine the concentration of some chemicals (pH, acetic acid, L-lactic acid, L-malic acid, and citric acid). The resulting medium was inoculated with a final population density of 10^7 CFU/mL of each *O. oeni* strains (**Annex I: Table I**).

The results of these growths were compared with the growth curves obtained in **3.5. Inoculum obtainment** to determine the influence of yeast's metabolism in the growth of *O. oeni* in rich medium.

3.5. EXPERIMENTAL FERMENTATION

The experimental fermentations were performed in flasks containing 500 mL of sterile fermentation must with an adjusted sugar concentration of 200 ± 10 g/L. Fermentations were performed in the presence of air because the caps were not screwed tightly on the flasks. These

musts were inoculated with a non-*Saccharomyces* yeast (**Annex I: Table I)** to a final population density of 10^6 CFU/mL. After 24 hours of the inoculation of the non-*Saccharomyces* yeast, *S. cerevisiae* Lalvin-QA23 was inoculated in a population density of 10^6 CFU/mL. The results of these fermentations were compared with the ones obtained in fermentations with *S. cerevisiae* as sole inoculum.

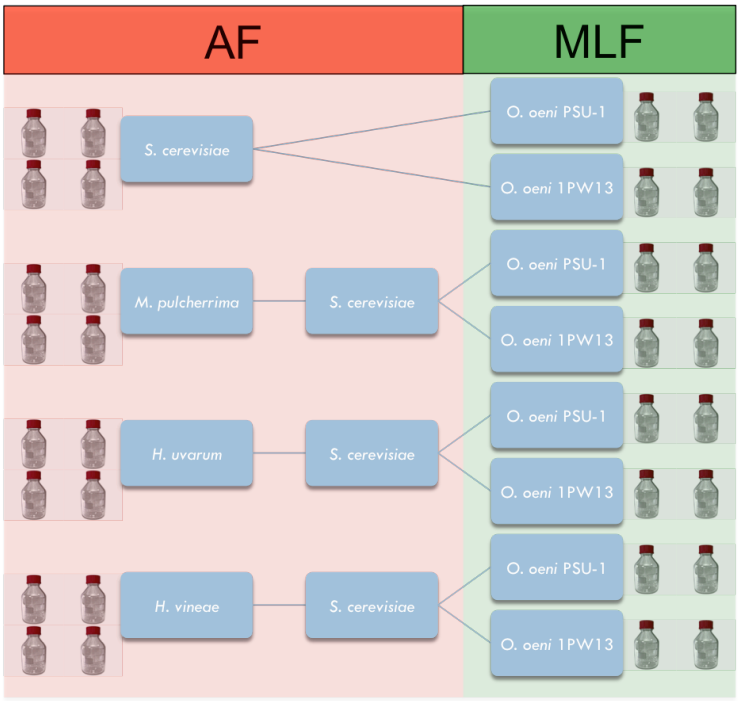

Figure 1. Simplified representation of the tested fermentation. Fermentations were carried out by duplicate for each non-*Saccharomyces* and *O. oeni* strain used.

When the AF was finished, the obtained wine was centrifuged at 8500 rpm for 5 minutes. Then, the wine was treated with 1% of dimethyl dicarbonate to kill any remaining yeast. The treated wine was conserved at 4ºC for 24 hours to get the compound decomposed.

The resulting wine was only inoculated with *O. oeni* when it was confirmed through YPD plates that there was no viable yeast remaining. After that, the wine was inoculated with a final population density of 10^8 CFU/mL. The MLF was considered to be finished when the concentration of L-malic acid was 0.00 g/L.

According to the used inocula, the excess was collected, centrifuged at 8500 rpm for 5 minutes and stored at -80ºC for future investigations.

3.5.1. Must characterization

As introduced before, fermentation musts were prepared using RCM with addition of sterile MilliQ purified water to achieve a final sugar concentration of 200 ± 10 g/L. After this, samples

of 1 mL were taken to measure some chemical compounds by Miura One chemical multianalyser (TDI SL, Gavà, Spain) (ISE S.r.l., Ref.: 13310001200). Furthermore, another 5 mL sample was taken for the measurement of the pH.

3.5.2. AF monitoring

Samples were taken everyday to follow sugar descent and yeast population evolution. Samples of sugar consisted on 1 mL of the fermenting must. After centrifuged at 8500 rpm for 5 minutes, the sample was diluted, if necessary, to be measured with the Miura One multianalyser.

According to the monitoring of yeast growth, samples of 100 µL were taken. These samples were diluted appropriately in sterile saline solution and plated on YPD medium and lysine agar medium plates and, finally, incubated at 28°C for 48 h. YPD rich medium provides total yeast counts while lysine agar medium only provides non-*Saccharomyces* cell counts since *S. cerevisiae* is not able to grow using lysine as a unique nitrogen source. After this period, the plates were counted.

Alcoholic fermentation was considered as finished when the sugar concentration was less than 1g/L. At this point, 120 mL of wine were taken to measure its alcoholic content, pH and some chemical parameters. The remaining wine was treated as introduced before.

3.5.3. Measurement of the alcohol content

The measurement of the alcohol content was driven by ebulliometry (Electronic ebulliometer uEBU6576, GabSystem). 100 mL of the finished wine (**3.6.2. AF monitoring**) were used for this purpose.

3.5.4. Wine characterization

When the AF was considered as finished, the wine was characterized in terms of sugar, pH, L-malic acid, L-lactic acid, acetic acid, succinic acid, citric acid and yeast population. Samples were taken and analysed with the Miura One multianalyser before and after the treatment with dimethyl dicarbonate.

3.5.5. MLF monitoring

Similarly to AF monitoring (**3.6.2. AF monitoring**), samples were taken every 24 hours to follow L-malic acid descent and *O. oeni* population evolution. Samples of L-malic acid

consisted on 1 mL of the wine. After centrifuged at 8500 rpm for 5 minutes, the sample were analysed with the Miura One multianalyser.

According to the monitoring of *O. oeni* growth, samples of 100 µL were taken. These samples were diluted appropriately in sterile saline solution and plated on MRS_{mf}.

3.5.6. Final wine characterization

When MLF was considered as finished (0.00 g/L of L-malic acid, **3.6. Experimental Fermentation**), wine was characterized as previously explained in **3.6.4. Wine characterization**. Moreover, all remaining bacterial population was collected, centrifuged at 8500 rpm for 5 minutes and stored at -80ºC for future investigations.

3.6. STATISTICAL ANALYSIS

Each experiment was performed at least in two independent assays, analysing two technological replicates of each assay. It was calculated the average and standard deviation of all of them. For the statistical treatments and analysis of the results, the statistics program *IBM SPSS Statistics version 24* was used.

For every data the descriptive statistics and normality tests were performed. One way ANOVA was used to calculate the value of significance for the variation analysis, and included a *post-hoc* Tukey test when needed. It was always used a confidence interval of 95%, considering significant results values of $p \leq 0.05$.

4. RESULTS

4.1. *O. oeni* CONDITIONAL GROWTH

Under the studied conditions, the growth of the two *O. oeni* strains in MRS_{mf} medium conditional to yeasts metabolism showed different behaviour (**Figure 2**).

In regard to the growth of *O. oeni* PSU-1, globally there were no statistical differences between the population evolutions of the bacteria in the assayed media. In every studied condition, the starting population of $2.22 \cdot 10^6 \pm 6.26 \cdot 10^5$ CFU/mL, reached a similar final population in 96 hours. Nevertheless, the conditional growth curves showed a statistical different evolution ($p \leq 0.05$) at 48h and 72h regarding to the control.

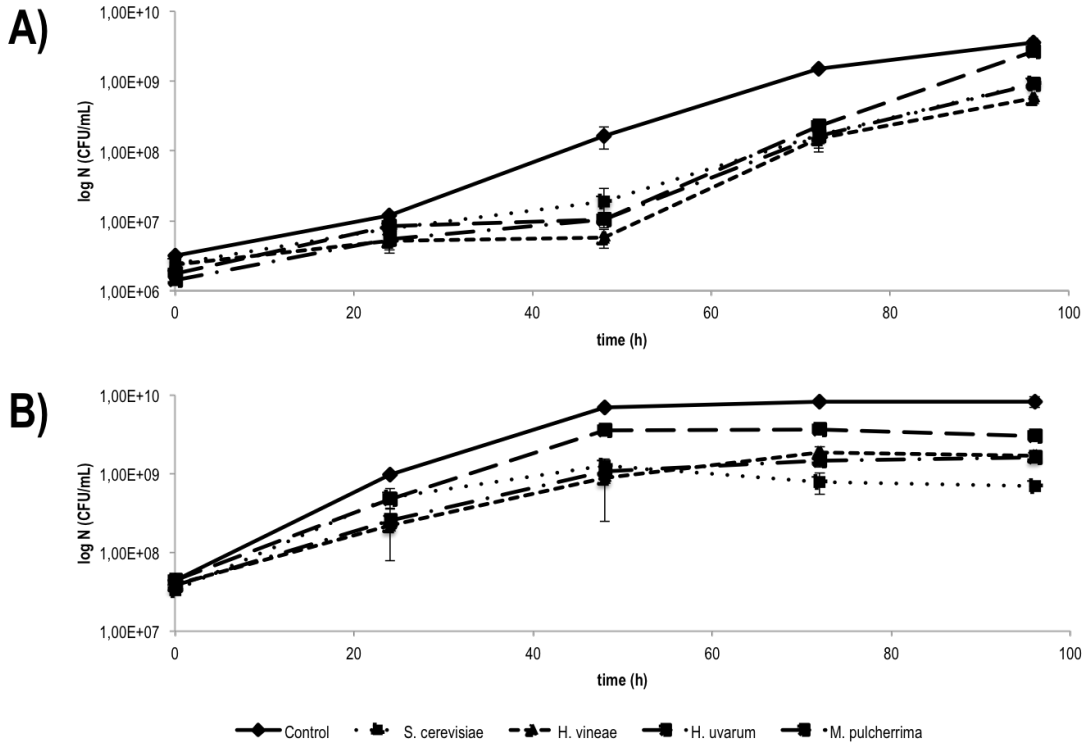

Figure 2. Evolution of *O. oeni* strains growth in MRSmf medium conditional to yeasts metabolism. A) Growth of *O. oeni* PSU-1. B) Growth of *O. oeni* 1PW13.

On the contrary, every *O. oeni* 1PW13 conditional growth exhibited the same tendency of the control during the 96 hours of the study. Besides, there are statistical differences ($p \leq 0.05$) between all the conditional growths in regards to the control at the final sampling (96 hours after inoculation).

4.2. EXPERIMENTAL FERMENTATIONS

4.2.1. Alcoholic fermentation

The four studied fermentations showed different behaviours according to the sugar consumption, *S. cerevisiae* imposition and time to reach a sugar concentration less than 1 g/L to consider AF as finished (**Figure 3)**.

To begin with, the control of the AF with *S. cerevisiae* Lalvin-QA23 as sole starter, finished in about 196 hours (**Figure 3A**). *S. cerevisiae* easily reached a population of $1.19 \cdot 10^8 \pm 1.05 \cdot 10^7$ CFU/mL in 24 hours. After that, yeast population began to decrease.

According to the mixed fermentation between *H. vineae* and *S. cerevisiae* presented a complicated competitive relationship (**Figure 3B**). It can be seen in the chart the erratic imposition of *S. cerevisiae* in the fermenting must. Viable *H. vineae* colonies were followed in

plates until 150 hours after their inoculation. Moreover, the sugar consumption described similar tendency in regards to the control (**Figure 3A**). Even though, this fermentation took more time in finishing, about 240 hours.

Analogous performances were noted when it came to the mixed fermentation between *H uvarum* and *M. pulcherrima* with *S. cerevisiae* (**Figure 3C** and **Figure 3D**, respectively). Viable colonies of these two non-*Saccharomyces* were only detected during the first 48 hours since they were inoculated; on the contrary, *S. cerevisiae* became dominant since then. Nevertheless, these two mixed fermentations showed different sugar consumption rate. The mixed fermentation between *H. uvarum* and *S. cerevisiae* took about 192 hours to conclude, whereas the fermentation between *M. pulcherrima* and *S. cerevisiae* needed much more time, about 280 hours.

Clearly the fermentation that showed the slower fermentation rate was the mix between *M. pulcherrima* and *S. cerevisiae*. In contrast, the mixed fermentation between *H. uvarum* and *S. cerevisiae* and the control fermentation needed the least time.

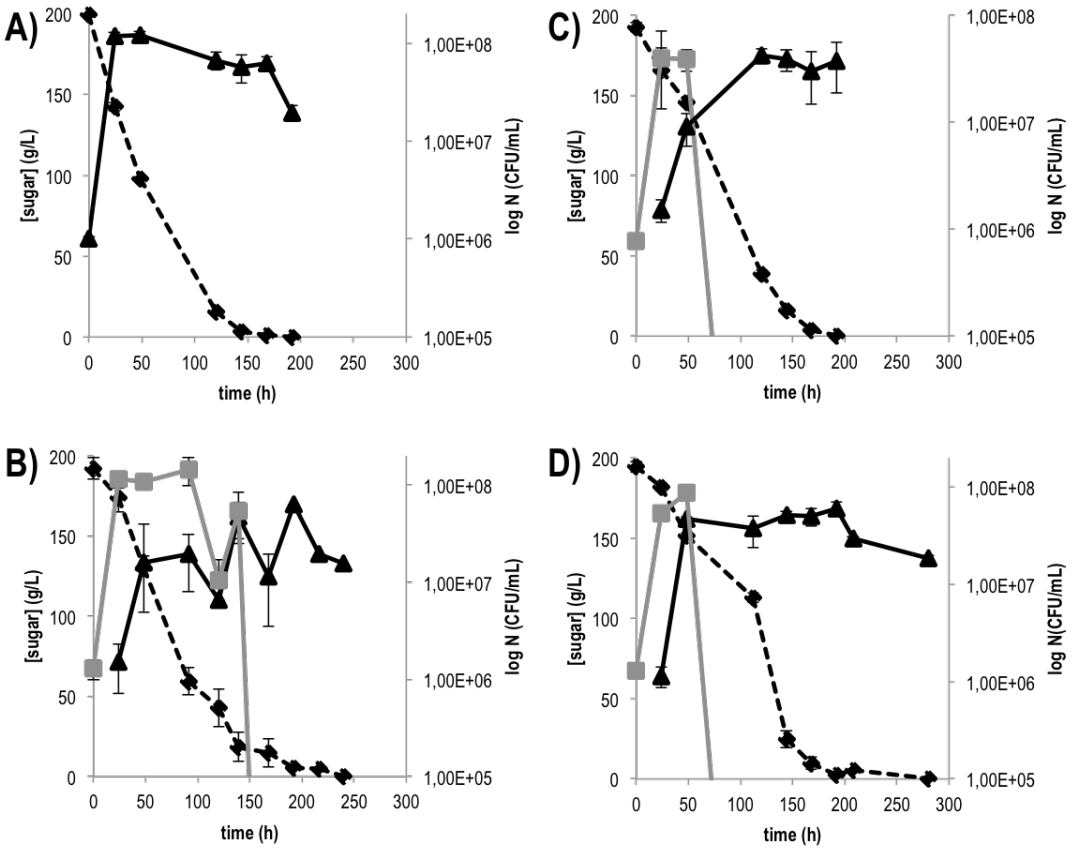

Figure 3. Evolution of alcoholic fermentations. (●) sugar concentration decrease, (▲) *S. cerevisiae* population, (■) non-*Saccharomyces* population. A) *S. cerevisiae* fermentation. B) Mixed fermentation of *H. vineae* and *S. cerevisiae*. C) Mixed fermentation of *H. uvarum* and *S. cerevisiae*. D) Mixed fermentation of *M. pulcherrima* and *S. cerevisiae*.

Those distinct alcoholic fermentation responses were also noticed in the chemical composition of the obtained wines (**Table 1**). Concerning to the alcoholic content and the pH of wines, there were no statistical differences regarding to the inoculated fermenting yeasts. What is more, all the obtained wines increased their volatile acidity in respect to the initial grape must. Mixed fermentation with *M. pulcherrima* did not really increase the concentration of this compound in the final wine (0.06 ± 0.04 g/L). Linked with this, statistical differences ($p \leq 0.05$) in the production of acetic acid were observed when it came to the mixed fermentation of *H. uvarum* with *S. cerevisiae* (0.56 ± 0.02 g/L) and the one of *M. pulcherrima* and *S. cerevisiae* (0.06 ± 0.04 g/L).

In addition, all the inocula tested consumed L-malic acid (**Table 1**). These consumptions of the mixed fermentations were statistically different ($p \leq 0.05$) from the *S. cerevisiae* control fermentation (0.17 ± 0.01 g/L). Apart from that, another significant differences were obtained in the L-malic acid consumption in the mixed fermentation of *H. vineae- S. cerevisiae* (0.45 ± 0.11 g/L) and *M. pulcherrima- S. cerevisiae* (0.36 ± 0.05 g/L).

Table 1. Chemical composition changes of the obtained wines after AF. Shown data about compounds production or consumption refers to the difference between the wine and the initial grape must.

Yeast mix	Alcoholic content (% vol/vol)	pH	Acetic acid production (g/L)	L-malic acid consumption (g/L)	Citric acid production (g/L)	Succinic acid production (g/L)
S. cerevisiae	13.1 ± 0.30	3.76 ± 0.14	0.15 ± 0.02^{ab}	0.17 ± 0.01^{a}	0.103 ± 0.01^{ab}	0.106 ± 0.06^{a}
H. vineae + *S. cerevisiae*	13.0 ± 0.14	3.62 ± 0.03	0.27 ± 0.06^{ab}	0.45 ± 0.11^{d}	0.190 ± 0.03^{b}	0.019 ± 0.04^{ab}
H. uvarum + *S. cerevisiae*	12.6 ± 0.61	3.76 ± 0.14	0.56 ± 0.02^{b}	0.35 ± 0.07^{cd}	0.053 ± 0.01^{a}	0.215± 0.03^{c}
M. pulcherrima + *S. cerevisiae*	12.3 ± 0.32	3.61 ± 0.01	0.06 ± 0.04^{a}	0.36 ± 0.05^{bc}	0.080 ± 0.03^{ab}	0.271± 0.04^{c}

Note: Column values followed by different superscript letters are significantly different at $p < 0.05$, according to a Tukey *post-hoc* comparison test.

As observed with the acetic acid, citric acid increased in wine in relation to the initial grape must (**Table 1**). This organic acid showed a homogeneous little increase in all the trials, being statistically different when it came to the fermentations of *H. vineae- S. cerevisiae* (0.190 ± 0.03 g/L) and *H. uvarum- S. cerevisiae* (0.053 ± 0.01 g/L).

Finally, succinic acid increased in all tested fermentation (**Table 1**). Under the studied conditions, the production of succinic acid of *S. cerevisiae* fermentation (0.106 ± 0.06 g/L) was statistically different when it came to the mixed fermentations with *H. uvarum* (0.215± 0.03

g/L) and *M. pulcherrima* (0.271± 0.04 g/L). Besides, more statistical differences were detected in the production of this organic acid between the mixes with *H. uvarum* and *M. pulcherrima*.

4.2.2. Malolactic fermentation

MLF in wines obtained by AF of the tested mixed non-*Saccharomyces* (**Figure 1**) and *S. cerevisiae* Lalvin-QA23 exhibited very unlike performances. Under the studied conditions, only 3 of the tried 8 MLF could be considered as finished ([L-malic acid]= 0.00 g/L).

Generally, *O. oeni* was not able to finish the MLF (data not shown). Indeed, the 10^8 CFU/mL population inoculated was lost in 24-48 hours. In most of the cases the bacteria usually could start consuming L-malic acid. Nevertheless, the MLF could not be finished because of the rapid viability loss. This behaviour was observed in both of the *O. oeni* strains studied: PSU-1 and 1PW13.

According to the evolution of the MLF that could finish, the three of them concluded in 24-48 hours since *O. oeni* was inoculated (**Figure 4**).

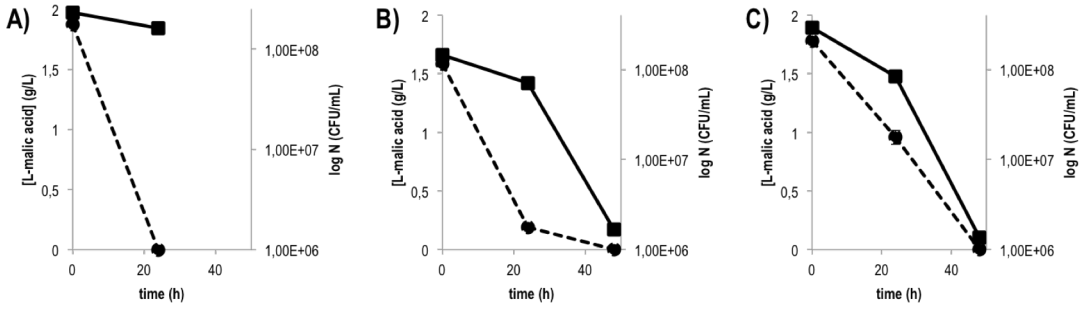

Figure 4. Evolution of finished malolactic fermentations. (●) L-malic acid concentration decrease, (■) *O. oeni* population. A) MLF of *O. oeni* PSU-1 in wine fermented by *H. uvarum* and *S. cerevisiae*. B) MLF of *O. oeni* PSU-1 in wine fermented by *M. pulcherrima* and *S. cerevisiae*. C) MLF of *O. oeni* 1PW13 in wine fermented by *M. pulcherrima* and *S. cerevisiae*.

The most rapid MLF performance was noticed with *O. oeni* PSU-1 in the wine obtained by the fermentation of the *H. uvarum* and *S. cerevisiae* (**Figure 4A**). L-malic acid concentration reached 0.00 g/L in less than 24 hours. Regarding to the bacterial population, the inoculated population was maintained on 10^8 CFU/mL in those 24 hours.

Additionally, those wines obtained by the fermentation of the mixed inocula of *M. pulcherrima* and *S. cerevisiae* finished the MLF. Both of the strains used concluded the fermentation in about 48 hours (**Figure 4B** and **Figure 4C**). Contrary to what was observed in the previously explained MLF, in this case, the inoculated population descended during the fermentation performance. However, this viability loss occurred after the first 24 hours.

In order to understand the metabolic profile of *O. oeni* in wine, some chemical compounds were analysed (**Table 2**). Apart from the total consumption of L-malic acid (**Figure 4**), citric acid was also consumed.

Similarly to what was obtained with L-malic acid, citric acid was nearly totally consumed. Only few remaining traces were detected (0.05 ± 0.04 g/L). There were no statistical differences in the consumption of citric acid by the tested inocula, but *O. oeni* PSU-1 presented in the two tested wines higher consumption of this organic acid, 0.270 ± 0.025 g/L and 0.236 ± 0.075 g/L in the wines fermented by *H. uvarum- S. cerevisiae* and *M. pulcherrima- S. cerevisiae*, respectively.

Table 2. Chemical composition changes of the obtained wines after MLF. Shown data about compounds production or consumption refers to the difference between the final wine and the wine after AF.

Yeast mix	*O. oeni* strain	Acetic acid production (g/L)	L-lactic acid production (g/L)	Citric acid consumption (g/L)	Deacidification (pH units)
H. uvarum + *S. cerevisiae*	*O. oeni* PSU-1	0.185 ± 0.025	1.12 ± 0.02	0.270 ± 0.025	0.294 ± 0.037^{a}
M. pulcherrima + *S. cerevisiae*	*O. oeni* PSU-1	0.130 ± 0.035	1.09 ± 0.04	0.236 ± 0.075	0.498 ± 0.006^{b}
	O. oeni 1PW13	0.173 ± 0.002	1.04 ± 0.05	0.140 ± 0.030	0.429 ± 0.041^{ab}

Note: Column values followed by different superscript letters are significantly different at p <0.05, according to a Tukey *post-hoc* comparison test.

As expected, acetic acid concentration increased (**Table 2**). The fermentative combination of *H. uvarum- S. cerevisiae- O. oeni* PSU-1 presented the higher acetic acid production of the tested mixed starters (0.185 ± 0.025 g/L). Regarding to L-lactic acid production, the obtained results presented a homogeneous production of this acid around 1 g/L in each of the finished wines (**Table 2**), showing no contrast in the inoculated yeasts or *O. oeni* strain. The only statistical difference revealed in the chemical composition changes of the obtained wines after MLF was in the pH deacidification (**Table 2**). It was noticed a significant higher deacidification in the mixed of *H. uvarum- S. cerevisiae- O. oeni* PSU-1 (0.294 ± 0.037 pH units) in relation to the mixed of *M. pulcherrima- S. cerevisiae- O. oeni* PSU-1 (0.498 ± 0.006 pH units).

Finally, it has to be mentioned the similar evolution of the obtained wines (**Table 3**). Since must until the finished wine, the three wines that could undergo MLF presented similar chemical composition. The only observed significant difference was found in the pH of the wine fermented by *H. uvarum- S. cerevisiae- O. oeni* PSU-1 in relation to *M. pulcherrima- S. cerevisiae- O. oeni* PSU-1.

Table 3. Chemical characterization of finished wines since grape must to the obtained wines after MLF

	Alcoholic content (%vol/vol)			pH			L-malic acid (g/L)			Acetic acid (g/L)			Citric acid (g/L)			L-lactic acid (g/L)			Succinic acid (g/L)		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
Initial must	-	-	-	3.960 ± 0,002	3.903 ± 0.001	3.930 ± 0.006	2.26 ± 0.11	2.15 ± 0.03	2.15 ± 0.06	0.05 ± 0.00	0.035 ± 0,005	0.06 ± 0.00	0.225 ± 0.005	0.27 ± 0.023	0.07 ± 0.03	0.08 ± 0.00	0.0575 ± 0.042	0.028 ± 0,015	-	-	-
Wine (after AF)	12 ± 0.00	12.1 ± 0.1	12.4 ± 0.4	3.621 ± 0,008	3.616 ± 0.015	3.616 ± 0.016	1.735 ± 0.19	1.795 ± 0.05	1.795 ± 0.06	0.63 ± 0.01	0.127± 0.04	0.083 ± 0.004	0.265 ± 0.025	0.35 ± 0.012	0.25 ± 0.04	0.07 ± 0.01	0.055 ± 0.008	0.043 ± 0.013	0.194 ± 0.032	0.24 ± 0.045	0.30 ± 0.02
Wine (after MLF)				3.951 ± 0.016	4.115 ± 0.022	4.045 ± 0.056	0.00	0.00	0.00	0.785 ± 0.005	0.27 ± 0.07	0.265 ± 0.011	0.075 ± 0.035	0.08 ± 0.03	0.06 ± 0.03	1.15 ± 0.005	1.11 ± 0.025	1.055 ± 0.05			

Note: A): *H. uvarum – S. cerevisiae- O. oeni* PSU-1 fermented wine, B) *M. pulcherrima – S. cerevisiae- O. oeni* PSU-1 fermented wine; C) *M. pulcherrima – S. cerevisiae- O. oeni* 1PW13 fermented wine.

4. DISCUSSION

As introduced before, there has been an increasing interest in using non-*Saccharomyces* yeasts in combination with *S. cerevisiae* as wine starters (Ciani and Maccarelli, 1998; Padilla et al., 2016). *S. cerevisiae* is usually needed because the often lack of the non-*Saccharomyces* yeasts to finish the AF (Contreras et al., 2014). These non-*Saccharomyces* yeasts are appreciated in winemaking due to their unusual metabolic activities that directly impact the wines they ferment. It has been reported changes in wines that undergo AF with these two yeast groups (Fleet, 2008). Generally, these impacts positively improve the organoleptic characteristics of wines. Different non-*Saccharomyces* yeast species have been reported as beneficial in this context, such as, *H. vineae*, *H. uvarum*, *M. pulcherrima*, *T. delbrueckii* and *C. zemplinina* (Fleet, 2008; Izquierdo Cañas et al., 2014; Jolly et al., 2006; Loira et al., 2014; Padilla et al., 2016).

Besides, the use of non-*Saccharomyces* as culture starter can also affect other chemical compounds composition not directly linked with the organoleptic profile of wines. These chemical changes may influence the following fermentation, MLF. Since *O. oeni* is the most frequently used starter for MLF, it is of outstanding interest set in context the effect of those changes upon *O. oeni*.

The main subject of the present work was to evaluate non-*Saccharomyces* and *O. oeni* interactions in an oenological context. Still, to first study the effect of yeasts metabolism upon *O. oeni*, it was performed conditional growth analysis. The aim of these analyses was to evaluate the effect of the metabolism of yeast upon *O. oeni* growth in an ideal medium for *O. oeni*; MRS_{mf} liquid medium (**Figure 2**). 24 hours of active metabolic activity by yeast supposed a decrease on the population density in all sampling points, either with *O. oeni* PSU-1 and *O. oeni* 1PW13. There is no literature to compare these results, but in this work it was noticed no positive interaction between the yeasts and *O. oeni* strains tested.

Globally, the viability evolution of *O. oeni* PSU-1 (**Figure 4A**) did not show statistically different data in relation to the control of the bacteria growing in fresh MRS_{mf} medium. Even though, it has to be mentioned that significant differences were observed at 48 and 72 hours growing values between the control and all the conditional media. According to the viability evolution of *O. oeni* 1PW13 (**Figure 4B**), globally there were statistical different evolutions in the growth of the control in relation to all conditional media. Besides, these differences were also noticed in the 96 hours sampling.

Probably, the detected viability loss is mainly because of nutrient consumption by yeasts. Other chemical compounds were studied (data not shown), such as pH, but they did not appear to be significant changes. It seems that 24 hours are sufficient time to quite exhaust the medium by all yeasts, but not enough to distinguish different behaviour in regard to the inoculated yeast. So, this kind of trial is not a good method to test yeast-bacteria interactions.

To begin with the study of the non-*Saccharomyces* and *O. oeni* interactions in wine, different fermentations were carried out. It was followed the AF and MLF of different musts using distinct non-*Saccharomyces* inocula and *O. oeni* strains (**Annex I: Table I**). Moreover, changes of some chemical compounds described as potentially influential upon MLF and *O. oeni* were determined In these assays, six mixed fermentations were studied (**Figure 1**), as result of the combination of three non-*Saccharomyces* yeasts and two *O. oeni* strains. Besides, to fully understand the role of non-*Saccharomyces* in these cited effects, a control fermentation was studied with *S. cerevisiae* Lalvin-QA23 as sole starter.

AF was followed in terms of sugar consumption and yeast population evolution (**Figure 3**). The value of [sugar] < 1 g/L to consider AF finished was not arbitrarily chosen. Since *O. oeni* is able to consume sugars (Liu, 2002), it was important to reduce their concentration to reflect *O. oeni* metabolic activity in an real exhausted wine.

The control AF of *S. cerevisiae* (**Figure 3A**) finished in about 192 hours showing a rapid sugar consumption. It was expected to achieve delays in AF finishing time in every mixed fermentation as a result of the competition between the yeasts. *S. cerevisiae* showed a rapid imposition in the fermenting must when it was inoculated with *H. uvarum* and *M. pulcherrima* (**Figure 3C**, **3D**). In 24 hours after the inoculation, *S. cerevisiae* became dominant and no viable *H. uvarum* and *M. pulcherrima* were detected. The mixed fermentation of *H. vineae* and *S. cerevisiae* finished in 240 hours, showing a very clear competitive behaviour between these two yeasts (**Figure 3B**). In this fermenting must, *S. cerevisiae* had difficulties to become dominant and overcome the growth *H. vineae*. It was not until 126 hours after *S. cerevisiae* inoculation, when *H. vineae* was not detectable in plates. Since non-*Saccharomyces* population was followed by counting CFU in Lys medium, it cannot be clearly demonstrated the sudden viability loss. Nevertheless, it is accepted the quick imposition of *S. cerevisiae* since middle fermentative stages (Fleet, 2008; Wang et al., 2016).

Moreover all the tested fermentation concluded with no different pH (**Table 1**). Larger pH differences would have shown different MLF performance since *O. oeni* is very dependant to wine pH (Wibowo et al., 1985).

On top of that, the chemical composition of the different obtained wines presented particularities (**Table 2**). Regarding to the alcoholic content, there were no significant differences in mixed fermentation in relation to the control fermentation. Nowadays the decrease of alcoholic content is a current challenge. Few works have been published about the use of non-*Saccharomyces* yeast to reduce the alcoholic content (Contreras et al., 2014; Quirós et al., 2014). Although in this work, non-*Saccharomyces* yeasts were inoculated, they were the only fermenting yeast for barely 24 hours. As well as the vigorous fermentative profile of *S. cerevisiae*, there were not observed reductions in ethanol concentration in the mixed fermentations.

Volatile acidity in wine is directly linked with acetic acid concentration in wine. In general, mixed fermentation with non-*Saccharomyces* yeasts present higher concentrations of acetic acid in regards to *S. cerevisiae* fermentation (Jolly et al., 2006; Rojas et al., 2003). In this study, the two mixed fermentation of the genera *Hanseniaspora* showed a higher concentration tendency that became statistically significant ($p \leq 0.05$) with *H. uvarum* (0.56 ± 0.02 g/L). Surprisingly, the use of *M. pulcherrima* decreased the volatile acidity of the obtained wine (0.06 ± 0.04 g/L) in relation to the control (0.17 ± 0.01 g/L).

The results of the L-malic acid consumption agreed with the ones published (Belda et al., 2014; Su et al., 2014). All the mixed fermentation with non-*Saccharomyces* yeasts showed a significant higher L-malic acid consumption ($p \leq 0.05$). Indeed, the mixed fermentation with *H. vineae* showed the highest rate (0.45 ± 0.11 g/L). However, the initial must contained around 2.12 ± 0.09 g/L of L-malic acid, so even after AF, there was always sufficient amount of this acid to perform a successful MLF.

According to the citric acid production, the tested mixed fermentation did not show statistically different results in relation to the *S. cerevisiae* control fermentation. This result fixes with other already published works that reported the same citric acid production (0.25 g/L) in *S. cerevisiae* fermentation and in a mixed fermentation with *Wickerhamomyces anomalus* (Izquierdo Cañas et al., 2014). For the moment the only mixed fermentation that clearly increased citric acid concentration is with *C. zemplinina* (Giaramida et al., 2013).

One of the organic acids that have been related with a negative effect upon MLF is succinic acid. This organic acid is described as a competitive inhibitor of the malolactic enzyme, leading

larger extension of the MLF length (Caridi and Corte, 1997; Lonvaud-Funel and Strasser de Saad, 1982). There have been reported different behaviours related to the production of succinic acid by non-*Saccharomyces* yeasts. In the present work it was observed that mixed fermentation with *H. uvarum* and *M. pulcherrima* (0.215 ± 0.03 g/L, 0.271 ± 0.04 g/L, respectively) supposed a significant increase in succinic acid production ($p \leq 0.05$) in relation to the control (0.106 ± 0.06 g/L). This result agree with previously published work about *M. pulcherrima* (Contreras et al., 2014). On the contrary, it was noticed a significant decrease in the production of this acid when *H. vineae* was inoculated (0.019 ± 0.04 g/L).

As result of the carried out fermentations trials, the obtained wines were inoculated with two *O. oeni* strains (**Annex I: Table I**). As introduced before (**4.2.2. Malolactic fermentations**), only three of possible eight MLF could be considered as finished.

Generally, *O. oeni* exhibited a strain specific adaptation to wine. *O. oeni* PSU-1, when not finishing MLF, suddenly lost its viability once it was inoculated (data not shown). Wines fermented by *S. cerevisiae* and the mixed fermentation with *H. vineae* appeared to be extremely hostile medium to the growth of *O. oeni*. On the contrary, wines obtained from the mixes of *H. uvarum* and *M. pulcherrima* looked less unfriendly to the bacteria, leading MLF performance (**Figure 4**). *O. oeni* PSU-1 when inoculated in *H. uvarum- S. cerevisiae* wine, concluded MLF in 24 hours (**Figure 4A**). During this time the population remained stable, higher than 10^8 CFU/mL. Besides, *O. oeni* PSU-1 in *M. pulcherrima- S. cerevisiae* wine needed 48 hours to conclude (**Figure 4B**). There was a rapid consumption of L-malic acid in the course of the first 24 hours that reached 0.00 g/L in less than 48 hours. Interestingly, *O. oeni* population continued steady at $7.05 \cdot 10^7 \pm 5 \cdot 10^5$ CFU/mL, as observed previously. It was not until the first 24 hours were finished, when an abrupt viability fall of two orders occurred ($1.67 \cdot 10^6 \pm 2.1 \cdot 10^5$ CFU/mL).

O. oeni 1PW13 strain showed higher resistance to the obtained wines. At the inoculation time, *O. oeni* preserved its viability, being counted in plates the same bacterial population of $\approx 10^8$ CFU/mL regarding to the used inoculum. Besides, *O. oeni* 1PW13 was only able to conclude MLF when it was inoculated in *M. pulcherrima- S. cerevisiae* wine (**Figure 4C**). The performance of this MLF was similar to the one of *O. oeni* PSU-1 in *H. uvarum- S. cerevisiae* wine (**Figure 4B**). The only difference between this two MLF was the linear consumption of the malic acid during the 48 hours.

These three obtained wines had a L-malic acid concentration of 0.00 g/L, as consequence of the concluded MLF (**Table 4**). As result of MLF, L-malic acid was decarboxilated to L-lactic acid (**Table 2**), causing a pH increase. This pH increase was more notable when *O. oeni* 1PW13 performed the MLF. Regarding to the relation of these two acid by the MLF, L-lactic acid has a direct stoichiometric connection (Liu, 2002). So, since it was formed less L-lactic acid than consumed L-malic acid, it could be reported that *O. oeni* is assimilating L-malic acid, not only as energy source, but as metabolic intermediary.

Citric acid was also consumed in these three fermentations (**Figure 3**). This metabolic activity is found in some *O. oeni* strains as a response to acidity or ethanol stress (Olguín et al., 2009). What is more, it has to be remembered that the only energy sources of wine for *O. oeni* are sugar traces, L-malic acid, citric acid and few macromolecules. That is why, as well as L-malic acid, there is no citric acid in wine after MLF (**Table 3**). As result of the consumption of citric acid, *O. oeni* increased volatile acidity (Liu, 2002; Lonvaud-Funel, 1999). Anyway, since citric acid concentration in the obtained wines was not very high, acetic acid did not much increase (**Table 2**).

Interactions between yeasts and LAB in wine are very complex mechanisms (King and Beelman, 1986; Lonvaud-Funel et al., 1988). The present work tried to study and quantify, if possible, those effects regarding to the different possible interactions produced in wines fermented by *S. cerevisiae* or a mix between non-*Saccharomyces* and *S. cerevisiae.* The distinct starter cultures used to undergo the AF leaded to chemically different wines (**Table 1**). So, these wines were perfect tests to evaluate different *O. oeni* responses. However, the obtained wines appeared to be very hostile medium for the growth or even survival of the bacterial, even the control fermentation with *S. cerevisiae* Lalvin-QA23.

It was inoculated a population density of $\approx 10^8$ CFU/mL which is sufficient to conclude MLF (Lonvaud-Funel, 1999). Nevertheless, only three wines could undergo MLF (**Figure 4**, **Table 4**). These wines had similarities regarding to the alcohol content, pH, L-malic acid and citric acid concentration (**Table 3**). Surprisingly, wines with higher concentration of succinic acid were the ones that could undergo MLF, in contrast to what is reported (Caridi and Corte, 1997). Moreover, the mixed fermentations of the non-*Saccharomyces* producing less citric acid were the only ones that finished MLF.

5. CONCLUSIONS AND PERSPECTIVES

The drawn conclusions of this study of the effect of non-*Saccharomyces* yeasts upon MLF and *O. oeni* are:

- The study of the conditional growth of *O. oeni* to yeasts in MRS_{mf} liquid medium showed similar trends respect the control curve. Only in the case of *O. oeni* 1PW13 significant differences were detected.
- 24 hours of non-*Saccharomyces* yeast metabolic activity are not enough to obtain clear chemical composition changes in wine after AF.
- Succinic acid seems to have no direct negative influence upon MLF.
- The set experimental conditions produced hostile wines for *O. oeni*.
- In this study, *M. pulcherrima* and *H. uvarum* presented the best positive effect upon MLF and *O. oeni* that counteract wine hostility.

To sum up, this work has presented few indications of the potential positive interactions between non-*Saccharomyces* yeasts and *O. oeni*. However, the tested conditions did not allow a fluent MLF performance, hindering the possibility to obtain robust conclusions of the interactions. Moreover, very few studies are currently published and, again, their results appear to be contradicted. That is why I propose to quite change the fermenting media and conditions. As well as to enlarge the tested non-*Saccharomyces*, *S. cerevisiae* strains and *O. oeni* strains to fully study this field. I strongly believe that this field of outstanding interest for science and winemaking will have rapid application. So, much more effort and dedication in it will worth it.

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Annex I:

Microorganisms, culture media, solutions and reagents

I. MICROORGANISMS

In the present work three non-*Saccharomyces* yeasts and a single *Saccharomyces cerevisiae* strain were used (**Annex I: Table I**). Moreover, regarding to *O. oeni*, two strains were used (**Annex I: Table I)**.

Table I. Relation of the microorganisms used in this work

	Species	Strain	Origen
Yeasts	*Saccharomyces cerevisiae*	Lalvin-QA23	Lallemand
	Hanseniaspora uvarum	CECT 13130	D.O.Q. Priorat (WildWine Proyect)
	Hanseniaspora vineae	CECT T02/5AF	Universidad de la República, Montevideo, Uruguay
	Metschnikowia pulcherrima	CECT 13131	D.O.Q. Priorat (WildWine Proyect)
Lactic acid bacteria	*Oenococcus oeni*	PSU-1	Pennsylvania State University, USA
	Oenococcus oeni	1PW13	D.O.Q. Priorat (WildWine Proyect)

I. CULTURE MEDIA

II.I. Yeast-Peptone-Dextrose (YPD) liquid medium

Glucose (Panreac, Ref. 131341.1211)	20 g
Peptone (Panreac, Ref. 403695.1210)	20 g
Yeast extract (Panreac, Ref. 403687.1210)	10 g
Distilled water	1000 mL

After the hydration of the medium, the pH was adjusted to 5 with HCl. The medium was sterilized at 121ºC for 20 minutes on the autoclave. Stored at room temperature.

II.II. Yeast-Peptone-Dextrose (YPD) broth medium

Glucose (Panreac, Ref. 131341.1211)	20 g
Peptone (Panreac, Ref. 403695.1210)	20 g
Yeast extract (Panreac, Ref. 403687.1210)	10 g
Agar (Panreac, Ref. 402303.1210)	17 g
Distilled water	1000 mL

This medium was prepared similarly to YPD liquid medium. Agar was only added when the medium was hydrated and the pH was adjusted to 5. The medium was sterilized at 121ºC for 20 minutes on the autoclave. Then, the medium was plated in Petri dishes. After the gelation of the agar, the YPD broth plates were stored at room temperature.

II.III. Man, Rogosa and Sharpe malic and fructose (MRS_{mf}) liquid medium

MRS broth (Difco, Ref. 288130)	55 g
DL-Malic acid (Panreac, Ref. 142051.1211)	4 g
D-Fructose (Panreac, Ref. 142728.1211)	5 g
Distilled water	1000 mL

After the hydration of the medium, the pH was adjusted to 5 with NaOH. The medium was sterilized at 121ºC for 20 minutes on the autoclave. Stored at room temperature.

II.IV. Man, Rogosa and Sharpe malic and fructose (MRS_{mf}) agar medium

MRS broth (Difco, Ref. 288130)	55 g
DL-Malic acid (Panreac, Ref. 142051.1211)	4 g
D-Fructose (Panreac, Ref. 142728.1211)	5 g
Agar (Panreac, Ref. 402303.1210)	20 g
Distilled water	1000 mL

This medium was prepared similarly to MRS_{mf} liquid medium. Agar was only added when the medium was hydrated and the pH was adjusted to 5. The medium was sterilized at 121ºC for 20 minutes on the autoclave. Then, the medium was plated in Petri dishes. After the gelation of the agar, the MRS_{mf} broth plates were stored at room temperature.

II.V. Lysine (Lys) broth medium

Lysine medium (Oxoid, Ref. CM0191)	66 g
Potassium L-lactate solution (Sigma, Ref. 60389)	5 mL
Lactic acid (Sigma, Ref. L6661)	2 mL
Distilled water	1000 mL

The medium was prepared following the manufacturer instructions. The Lys medium was suspended in distilled water containing 1 mL of potassium L-lactate solution and boiled to

dissolve completely. Then, the medium was cooled to approximately 50ºC and 2 mL of lactic acid were added to adjust to pH 4.8 ± 0.2. Finally, the medium was dispensed into petri dishes.

II.VI. Fermentation must

Rectified concentrated grape must (Most Concentrar Blanc 65 Brix, Concentratrats Pallejà S.L.)

Sterile MilliQ purified water

The fermentation must used in this work was prepared using rectified concentrated grape must (RCM) with addition of sterile MilliQ purified water. The proportion of both reactive depended on the sugar concentration of the RCM to adjust a volume of 500 mL to [sugar]= 200 ± 10 g/L, analysed with the Miura One multianalyser.

III. SOLUTIONS

III.I. Saline solution

NaCl	0.85 g
Distilled water	100 mL

The solution was sterilized at 121ºC for 20 minutes on the autoclave and stored at room temperature.

III.II. Dimethyl dicarbonate

Sigma, Ref. D5520

Stored at 4ºC.

IV. MIURA ONE, CHEMICAL MULTIANALYSER

Miura One (ISE S.r.l., Ref.: 13310001200) (TDI SL, Gavà, Spain)

Miura one Automatic multiparametric analyser was used to analyse some chemical compounds of the musts and wines.

IV.I. Reagents and standards

IV.I.I. Acetic acid enzymatic detection kit

TDI, Ref.: 2401

Stored at 4ºC.

IV.I.II. L-lactic acid enzymatic detection kit

TDI, Ref.: 2403

Stored at 4ºC.

IV.I.III. Citric acid enzymatic detection kit

TDI, Ref.: 2406

Stored at 4ºC.

IV.I.IV. L-malic acid enzymatic detection kit

TDI, Ref.: 2402

Stored at 4ºC.

IV.I.V. Glucose/Fructose enzymatic detection kit

TDI, Ref.: 2404

Stored at 4ºC.

IV.I. VI. Multiparametric standard Enocal LD

TDI, Ref.: 2100D

Stored at 20ºC.

V. OTHER REAGENTS

V.I. Succinic acid detection manual Kit

Megazyme, Ref.: K-SUCC

Stored at 4ºC.