EFFECT OF YEASTS/BACTERIA CO-INOCULATION ON MALOLACTIC FERMENTATION OF TEMPRANILLO WINES

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INTRODUCTION

Many scientific literature talks about the inoculation of lactic acid bacteria (LAB) to carry out malolactic fermentation (MLF) in wines. The best moment to inoculate LAB for MLF is under discussion [1]. Usually, the inoculation has been recommended at the end of alcoholic fermentation (AF) to prevent the synthesis of D-lactic and acetic acids. Early studies propose the possibility of inoculation of bacteria and yeasts in must simultaneously by co-inoculation [2]. Other authors advise an early inoculation before the end of AF [3]. Another possibility that several authors present for better stabilization of colour is to induce MLF after some time once ended the AF [4].

Traditionally, inoculation of LAB to carry out MLF is by sequential inoculation, after AF. Winemakers of Bordeaux recommend this inoculation strategy to prevent the *piqûre lactique* of wine [4]. It takes usually long times, because it occurs after AF and when LAB population is high enough [6]. Moreover, LAB also avoid much of toxicity of carboxylic acids synthesized by yeasts, as its concentration decreases after AF [25]. After AF the availability of nutrients for LAB also increases by yeast death and autolysis [26]. This strategy is commonly applied with malolactic bacteria of *Oenococcus oeni*, which is highly resistant to ethanol. But other approaches are possible, and the respective pros and cons are still controversial.

Many authors recommend the inoculation of LAB simultaneously to yeasts for MLF with good results [2, 5]. They have observed a better implantation of LAB in this stage than if bacteria are inoculated at the end of AF. Characteristics of grape must are more favourable than wine for growth of LAB: LAB have a chance to gradually adapt to conditions for carry out MLF, and become tolerant to ethanol along AF; also they have a large amount of nutrients at this stage [6-10]. Another positive aspect to induce bacterial growth in this stage is the combination of most of the sulphur dioxide by carbonyl compounds synthesized by yeasts [7].

Furthermore, interactions between LAB and yeasts have also been taken into account [9, 11-16]. LAB growth could be delayed by synthesis of toxic compounds by yeasts such as ethanol and sulphur dioxide [9]. The fast growth of yeasts can sometimes produce an antagonist effect for bacterial growth. Several authors attribute this inhibition of LAB growth to the presence of metabolites synthesized by yeasts such as decanoic acid [17, 18], and metabolization of substances for LAB nutrition by yeasts. However, several authors have demonstrated that the yeast growth along the AF was not affected by presence of LAB [19]. Nevertheless, some researchers consider a fast death rate of yeast in mixed cultures where there is fast growth of LAB [7]. Sometimes, it has also been seen that MLF occurring during AF can cause a stop in AF [20].

Several authors have also shown that with co-inoculation the MLF is completed more quickly [7] and also decreases the content of biogenic amines in wine [21]. Wines

generated by co-inoculation have a higher microbiological stability. The early carrying out MLF allows earlier addition of sulphur dioxide in wine and avoids to be wine unprotected for long time [7, 20, 22]. On the other hand, some researchers have described that the co-inoculation yields wines with lower final sugar contents, which increases microbiological stability in comparison to sequential inoculation of LAB [6, 7]. Because of that, co-inoculation avoids the possible growth of spoilage microorganisms, such as spoilage bacteria and *Brettanomyces* [20]. A risk of co-inoculation is the *piqûre lactique* of wine. Along LAB growth, they can metabolize sugars and synthesize D-lactic and acetic acids [7, 23]. Some authors have demonstrated that an early inoculation of LAB does not suppose the synthesis of high quantities of acetic acid [8]. Assays of co-inoculation and sequential inoculation did not provide differences in acetic acid concentrations [24].

The present study investigates the effect of the time of bacterial inoculation on vinification kinetics and important physico-chemical and microbiological parameters. For this, a traditional vinification, where LAB were inoculated after completion of AF, was compared to a simultaneous inoculation, where yeast and bacteria were inoculated concurrently. The vinifications were carried out in a pilot plant. A Tempranillo grape must was chosen as a typical example of a Spanish red wine vinification with MLF.

MATERIAL AND METHODS

Strains

Oenococcus oeni strain VP41 (Lallemand Inc. Montrêal, Canada) was used as the malolactic starter culture. *Saccharomyces cerevisiae* commercial strain VN (Lallemand) was used as starter to induce alcoholic fermentation.

Vinifications

Tempranillo grapes from a La Rioja (Spain) vineyard were harvest mechanically, destemmed and crushed, and 175 kg of grapes were pressed in a bladder press to yield 200 litres of must. The racked must had a pH of 4.1, and contained 120 g/L of glucose, 121 g/L of fructose, 2.2 g/L of malic acid and 0.24 g/L of citric acid.

Two different vinifications were conducted in tanks with co-inoculation and sequential inoculation with malolactic starter VP41, respectively to induct MLF. The musts were sulphited by adding 30 mg/L SO_2 and adding 3 g/HL of Lallzyme EX™ (Lallemand) prior to yeast inoculation. Yeast inoculations were done according to the manufacturer’s instructions. To induce simultaneous AF/MLF (co-inoculation), VP41 was inoculated 12 h after inoculation of yeast starter. To induce sequential AF/MLF (sequential inoculation), VP41 was inoculated at the end of alcoholic fermentation. A commercial freeze-dried preparation of the starter VP41 was used according to the manufacturer´s instructions. Commercial MLF nutrient (OPTI'MALO PLUS, Lallemand) was added according to the manufacturer’s instructions too.

AF was monitored by ethanol production and sugar depletion. MLF was monitored by L-malic acid degradation and L-lactic acid production. AF and MLF were considered complete when residual sugars were less than 1 g/L and L-malic was less than 0.15 g/L. The vinifications were conducted with temperature control, and the temperature ranged between 20-22℃. Final wines were stabilized by SO_2 addition and stored in bottles.

Analysis of must and wine

A sample of must was analyzed at the time of crushing, and fermentation samples were collected during vinifications until the end of AF and MLF.

pH of samples were measured with a pHmeter pH 330i (WTW) coupled to a SenTix 81 (WTW) electrode.

Glucose, fructose, malic acid, lactic acid, citric acid, glycerol and ethanol were analyzed by high-pressure liquid chromatography (HPLC) [36]. Before analysis, the samples were diluted 1:10 and sterilized by filtration (0.2 µm). The HPLC system (Agilent 1200) was equipped with high performance automatic injector (Agilent 1200), refractive index detector (Agilent 1200) and thermostated compartment (Agilent 1200). Columns were maintained at 70℃ by a temperature control unit. Injection volumes were 5 µL and run time for completion was 40 minutes. Mobile phase consisted of a solution of phosphoric acid 85% (Panreac) (0.75 mL/L) in Millipore Milli-Q water. The phosphoric acid solution was used as the solvent with 0.7 mL/min flow rate. The separation of different compounds was carried out by a precolumn (BIORAD) bound with 2 ion exclusion columns of 30 nm x 7.8 mm AMINEX HPX-87-H (BIORAD).

Microbiological analysis

O. oeni counts in samples were enumerated on MLO plates [27] which was added 1.5% Actistab (Gist-Brocades) to inhibit yeast growth. The plates were incubated at 28℃. After 6 days, colony counts were carried out and reported as colony-forming units per mL (cfu/mL). LAB were monitored until the completion of AF and MLF.

Implantation analysis of LAB

Must and wine samples were diluted adequately and plated on MLO to get isolated colonies of LAB. To analyze the implantation of malolactic starter, we performed a similarity analysis of RAPD fingerprintings of autochthonous isolates and the inoculated strain VP41. For this, we typified isolates by random amplification of polymorphic DNA (RAPD) [28]. Template DNA was prepared from cell suspensions in water [29]. DNA amplification was carried out in 50 µL. PCR mixture containing 200 µM dNTPs, 1 µM primer M13 [30], 10 mM Tris HCl (pH 8.8), 50 mM KCl, 0.1% Triton X-100, 2 mM $MgCl_2$, 1.5 U Dynazyme II DNA Polymerase (Finnzymes) and 1 µL template DNA prepared as described above. PCR was performed in a Techne thermal cycler. The amplification conditions used were described by Zapparoli, 2000 [31]. RAPD products were resolved by electrophoresis in 1.2% (w/v) SeaKem LE agarose (FMC) in 0.5 X TBE gels and 1 Kb plus ladder was used as a molecular size standard. Agarose gels were stained with ethidium bromide (0.5 µg/mL) and images digitized with a GelPrinter Plus system (TDI). Digitized images of RAPDs were analyzed by BioNumerics software 6.6 (Applied Maths), which carried out conversion, normalization and further analysis of the patterns. RAPD patterns were analysed using Pearson´s product moment correlation coefficient and the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) clustering method.

RESULTS

Population dynamics of VP41

Figure 1 shows the population dynamics of strain VP41 in co-inoculation and sequential inoculation. In co-inoculation, LAB were inoculated on first day of vinification, and in sequential inoculation they were inoculated on the 8th of vinification, after AF. LAB could grow well under both conditions, whether they were inoculated in must as if inoculated after AF. We have observed that if the bacteria are inoculated at the end of AF, LAB grow more rapidly to its maximum population than if they are inoculated at the start of AF. In co-inoculation, LAB population is almost constant during AF and decreases at the end of it. By contrast, if LAB are inoculated at the end of AF, they grow for approximately 10 days and then gradually decrease their population.

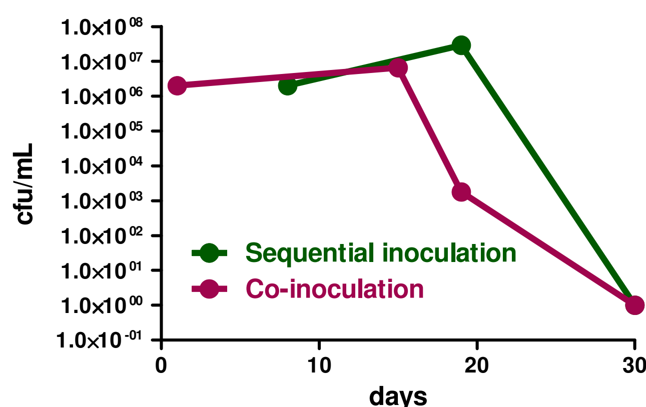

Figure 1. Population dynamics of strain VP41 in co-inoculation and sequential inoculation during vinifications.

Implantation of malolactic starter VP41 was 100% throughout the vinifications of both coinoculation and sequential inoculation. As shown in Figure 2, the isolates along vinification of tanks 3 (sequential inoculation) and 4 (coinoculation) were grouped into a single cluster with the fingerprinting of the inoculated strain VP41 with percentages of similarity of 87.0 and 83.9, respectively.

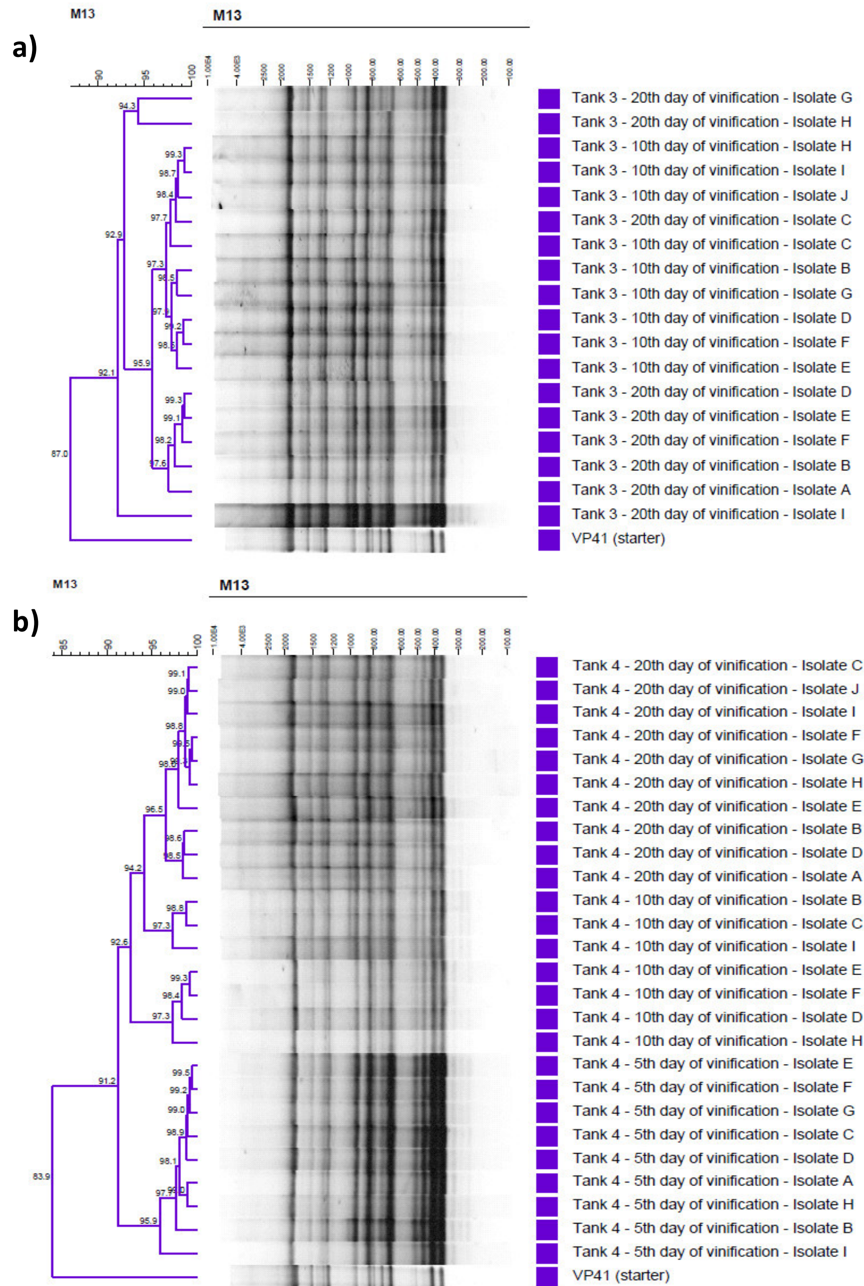

Figure 2. Dendrogram obtained for implantation analysis of VP41 in sequential (a) and co-inoculation (b) vinifications by BioNumerics 6.6 software. We used the isolates along vinifications and inoculated starter and using Pearson´s product moment correlation coefficient and UPGA as clustering method.

Evolution of vinifications

The physico-chemical parameters of the final wines are shown in Table 1.

Table 1. Composition of initial must and wines after alcoholic fermentation (AF) and malolactic fermentation (MLF) produced by induction of simultaneous or sequential inoculation of malolactic starter VP41.

	Must	Sequential inoculation	Coinoculation
pH	4.134	4.156	4.090
Glucose (g/L)	119.89	0	0.10
Fructose (g/L)	121.57	0.32	0.29
Malic acid (g/L)	2.24	0.14	0.15
L-lactic (g/L)	0	1.40	1.39
D-lactic (g/L)	0	0.22	0.37
Ethanol (% v/v)	0	14.87	13.37
Glycerol (g/L)	0	12.73	12.03

pH during vinifications remained higher by applying the strategy of coinoculation (Figure 3). The pH drop happened during AF was offset by the degradation of malic acid and lactic acid synthesis, showing a lower acidity during vinification and facilitating in this way the bacterial metabolism and survival.

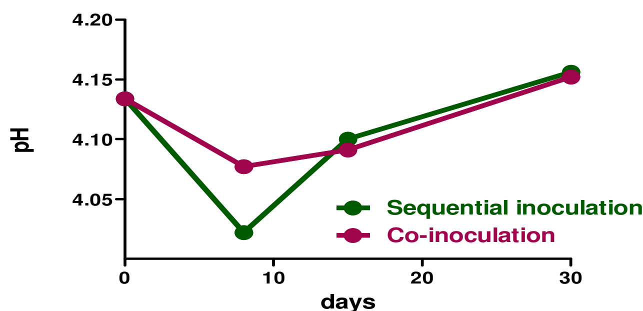

Figure 3. Kinetics of pH during vinifications carried out with the malolactic starter VP41 by co-inoculation and sequential inoculation.

The inoculation strategy of malolactic starter did not affect the development of AF. The residual sugars in both final wines were lower than 0.4 g/L. Regarding MLF (Figure 4). The overall vinification process was shorter in co-inoculation vats. However, we observed in co-inoculated wines a slightly higher amount of D-lactic, and slightly lower pH of the final wine made by this strategy.

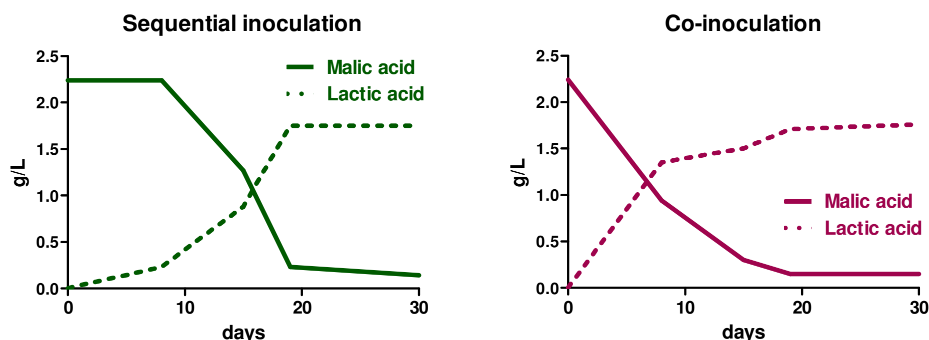

Figure 4. Kinetics of MLF during vinifications carried out with the malolactic starter VP41 by co-inoculation and sequential inoculation.

Fermentation parameters such as ethanol or glycerol showed no significant variation between the two inoculation strategies.

Furthermore, the co-inoculated wine was the best valued for its higher aromatic intensity, with more varietal and fruity notes. On taste, it presented a smoother and more persistent mouthfeel.

DISCUSSION

The practice of yeast-bacteria co-inoculation is often criticized because of the possible interference of bacteria in the course of AF, and because of the risk of volatile acid production and an excessive increase in D-lactic production, causing a reduction in wine quality [32]. In this work, the simultaneous inoculation of yeast and bacteria into must was compared to a traditional vinification protocol, where MLF was induced by inoculation of malolactic starter VP41 after completion of AF.

Inoculated bacteria VP41 of *O. oeni* as malolactic culture was capable of carrying out the process of MLF using two strategies. The implantation of the inoculated bacteria was 100% in both cases. Co-inoculation of bacteria VP41 into must did not affect the development of AF, as the wines were dry at the end of vinification.

MLF took place more quickly in wines made by co-inoculation. In this case, the performance of the bacteria when they are inoculated into grape must along with the yeast (co-inoculation) is enhanced by allowing for a period of bacterial adaptation to the gradual ethanol concentration synthesized during AF. By sequential inoculation, the acclimatization period drastically affects bacterial malic acid consumption before alcohol toxicity becomes problematic for the bacteria.

Co-inoculation is interesting for the more favourable conditions of must for bacteria. Furthermore, the MLF is performed simultaneously to the AF and thereby prevents the wine unprotected of SO_2 during long periods of time, with the risk that spoilage microorganisms are developed as acetic bacteria, other LAB, *Brettanomyces*…[33].

In final wines made with both strategies, there were not found significant differences in the physico-chemical characteristics. Although acetic acid is a final product of sugar metabolism by heterolactic bacteria, this acid did not increased significantly in wines produced by co-inoculation [18, 34, 35].

In contrast to earlier studies, the current work has found no evidence of a negative impact of simultaneous AF/MLF on fermentation success and kinetics, or on final wine parameters. Instead, several aspects suggest a microbiological and technological advantage of applying this fermentation protocol in Tempranillo wines. Specifically, co-inoculation may be advantageous for the reduction in time of the winemaking process, and the increase of the aromatic quality of wines. In addition, AF is not affected by the inoculation of bacteria and MLF is done without difficulty, without alterations of physico-chemical parameters of the final wine; even more, co-inoculated wine was preferred and more valued for its higher aromatic intensity, with more varietal and fruity notes, and it presented a smoother and more persistent mouthfeel.

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