Quantitative study of interactions between *Saccharomyces cerevisiae* and *Oenococcus oeni* strains

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Abstract This study examines the interactions that occur between *Saccharomyces cerevisiae* and *Oenococcus oeni* strains during the process of winemaking. Various yeast/bacteria pairs were studied by applying a sequential fermentation strategy which simulated the natural winemaking process. First, four yeast strains were tested in the presence of one bacterial strain leading to the inhibition of the bacterial component. The extent of inhibition varied widely from one pair to another and closely depended on the specific yeast strain chosen. Inhibition was correlated to weak bacterial growth rather than a reduction in the bacterial malolactic activity. Three of the four yeast strains were then grown with another bacteria strain. Contrary to the first results, this led to the bacterial stimulation, thus highlighting the importance of the bacteria strain. The biochemical profile of the four yeast fermented media exhibited slight variations in ethanol, SO_2 and fatty acids produced as well as assimilable consumed nitrogen. These parameters were not the only factors responsible for the malolactic fermentation inhibition observed with the first bacteria strain. The stimulation of the second has not been reported before in such conditions and remains unexplained.

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Introduction

The winemaking process can consist of two main steps where alcoholic fermentation (AF) led by *Saccharomyces cerevisiae*, is occasionally followed by malolactic fermentation (MLF) carried out by lactic acid bacteria (mainly *Oenococcus oeni*). This secondary fermentation which consists of the enzymatic decarboxylation of L-malic acid into L-lactic acid, is required during the vinification of most red wines and certain white and sparkling wine styles. In addition to deacidification, the MLF can increase microbiological stability and enhance wine flavour and aroma [3, 19, 21, 25]. Consequently, achieving a successful MLF is a key factor which has an impact on the quality and cost of wine. This step is often difficult to accomplish; however, due to the inadequate physico-chemical conditions of wine such as a high concentration of ethanol [6, 35], low pH [5, 35], temperature [5] and nutrient depletion [4, 27, 30], as well as some common inhibitory metabolites from yeasts such as SO_2 [7, 20, 28] and fatty acids [6, 12, 24]. Therefore, in order to control MLF, abundant knowledge of the interactions existing between the yeast *Saccharomyces cerevisiae* and the lactic acid bacterium *Oenococcus oeni* is required. In the literature, the most common kind of interaction described was the bacterial inhibition by yeasts whereas stimulation and neutralism were less frequent. These three kinds of interactions were evaluated in most cases by applying the classical method on Petri dishes inspired by antibiograms proposed by Lemaresquier [23] and subsequently improved by others [2, 34]. The results

obtained using this semi-quantitative method which evaluates only bacterial growth and not malolactic activity were not always extrapolated successfully to natural media due to several disadvantages of this method [2, 34]. Therefore the different interactions observed in the seven *Saccharomyces cerevisiae*/*Oenococcus oeni* pairs studied in this work, were quantified through measuring both growth and demalication as well as using similar conditions to those present during natural vinification. The strategy adopted was to use a sequential fermentation which simulated the natural winemaking process. In other words, MLF started when AF was achieved. The alcoholic fermentations took place in a synthetic grape juice of a similar composition to that of natural grape must. Four yeast fermented media were utilized with the first bacterial strain and then three of them were tested with another strain.

Materials and methods

Strains and storage conditions

Four strains of *Saccharomyces cerevisiae* (A, B, C and D) and two strains of *Oenococcus oeni* (X and Y) were used in this work. These strains were kindly provided by Lallemand Inc. (Toulouse, France). The stock cultures of *Saccharomyces cerevisiae* strains were kept at a temperature of 4°C in YEPD agar which is composed of glucose (20 g/l), yeast extract (10 g/l) (Oxoid, Hampshire, England), peptone (20 g/l) and agar (20 g/l).

Oenococcus oeni strains were kept frozen at −20°C in MRS broth (Biokar, Beauvais, France) containing 20% glycerol (v/v).

Growth media

Synthetic grape juice medium

The medium composition that simulated natural grape juice consisted of: glucose (100 g/l), fructose (100 g/l), yeast extract (Oxoid) (1 g/l), $(NH_4)_2SO_4$ (2 g/l), citric acid (0.3 g/l), L-malic acid (5 g/l), L-tartaric acid (5 g/l), $MgSO_4$ (0.4 g/l) and KH_2PO_4 (5 g/l). The pH was adjusted to 3.5 using a 10 N NaOH solution.

The amino acid composition of the yeast extract was as follows: valine (1%), tyrosine (4.95%), tryptophan (0.85%), threonine (2.73%), serine (3.42%), proline (0.88%), phenylalanine (3.78%), methionine (0.8%), lysine (5.4%), leucine (6.04%), isoleucine (4.81%), glycine (5.95%), glutamic acid (13.49%), cystine (0.76%), aspartic acid (7.07%), arginine (3.31%) and alanine (0.91%).

Synthetic wine medium

This medium composition simulated wine, yet it was lacking yeast metabolites with the exception of ethanol: glucose (0.5 g/l), fructose (0.5 g/l), yeast extract (Oxoid) (0.5 g/l), $(NH_4)_2SO_4$ (0.2 g/l), citric acid (0.3 g/l), L-malic acid (4 g/l), L-tartaric acid (5 g/l), $MgSO_4$ (0.2 g/l) and KH_2PO_4 (2 g/l), pH 3.5. After autoclaving, 80 g/l of ethanol [10% (v/v)] were added by sterile filtration through 0.2 μm membranes and the pH was readjusted to 3.5 using an 85% orthophosphoric acid solution.

Sequential fermentations

Seven sequential fermentations were studied using the following pairs:

- *Saccharomyces cerevisiae* A strain/*Oenococcus oeni* X strain
- *Saccharomyces cerevisiae* B strain/*Oenococcus oeni* X strain
- *Saccharomyces cerevisiae* C strain/*Oenococcus oeni* X strain
- *Saccharomyces cerevisiae* D strain/*Oenococcus oeni* X strain
- *Saccharomyces cerevisiae* B strain/*Oenococcus oeni* Y strain
- *Saccharomyces cerevisiae* C strain/*Oenococcus oeni* Y strain
- *Saccharomyces cerevisiae* D strain/*Oenococcus oeni* Y strain.

Alcoholic fermentation step

Saccharomyces cerevisiae strains were cultured at 22°C with an agitation of 150 rpm at an initial concentration of 3×10^6 cells/ml in 400 ml of the synthetic grape juice medium. Stock cultures of the four yeast strains were first reactivated in YEPD broth (composition described in "Strains and storage conditions", excluding agar) at a temperature of 22°C and an agitation of 150 rpm. After a 24 h incubation period, each pre-culture was used to inoculate an intermediate synthetic medium. The only difference present between the intermediate synthetic medium composition and the synthetic grape juice is the sugar concentration that is 50 g/l of glucose. This step was carried out using the same temperature and agitation for 24 h and provided the inocula.

The yeast growth was determined by direct cell counts under microscope using the Thoma haemacytometer. The biomass was also measured by weighing cells after dryness and was expressed in g/l.

Yeast fermented media used to inoculate malolactic bacteria

After the completion of AF as determined by the total or discontinuation of sugar consumption (remaining sugar concentration lower than 2 g/l), the yeast fermented media were subjected to various procedures before inoculating with malolactic bacteria. First, cells were removed by centrifugation (2,000 rpm for 20 min at 4°C). Then, malic acid concentration of the supernatant was measured and readjusted to 5 g/l. Subsequently the pH was adjusted to 3.5 using a 10 N NaOH solution. Finally, yeast fermented media were filtered in sterile conditions through 0.2 μm membrane filters and 150 ml of each medium was placed in an autoclaved Erlenmeyer flask (250 ml).

Malolactic fermentation step

Stock cultures of *Oenococcus oeni* X and Y strains were first reactivated in MRS broth (Biokar) containing 3% ethanol (v/v) at 22°C with an agitation of 150 rpm. After 24 h, these pre-cultures were inoculated into the intermediate synthetic media (composition described in "Alcoholic fermentation step") with 6% ethanol (v/v) added. 24 h later, the yeast fermented media were inoculated with the pre-cultures of *O. oeni* X and Y strains at an initial concentration of 2×10^6 cells/ml. These fermentations were followed until the cessation of malic acid consumption. Bacterial growth was determined by direct cell counts under a microscope using the Petit Salumbeni haemacytometer. Biomass was also determined by weighing cells after dryness and was expressed in g/l.

Bacterial control cultures

As a reference for growth and malic acid degradation kinetics, two control cultures of the X strain were performed using synthetic grape juice medium and synthetic wine medium while only the synthetic grape juice control culture was carried out for the Y strain. These cultures were conducted following the same steps described in "Malolactic fermentation step" and starting with an initial concentration of 2×10^6 cells/ml in 400 ml of both synthetic media (composition described in "Synthetic grape juice medium" and "Synthetic wine medium").

Analytical methods

Sugar consumption by yeasts

Sugar consumption was followed colorimetrically using the DNS method [26] and the results were expressed in g/l.

Malic acid degradation by bacteria and yeasts

L-malic acid concentration was determined using an enzymatic assay (Microdom, kit no 110 05 011 00, Taverny, France) and the results were expressed in g/l.

Ethanol production by yeasts

Ethanol concentration was measured using the HPLC method. The column used was an Aminex® HPX-87H Biorad presenting a cationic H^+ coverage thermostated at 40°C and the solvent was a 0.005 M sulphuric acid solution at a flow rate of 0.4 ml/min. The HPLC was coupled to a refractometer detector. The results were expressed in g/l.

Acetic acid production by yeasts

Acetic acid concentration was determined using an enzymatic assay (Boehringer Mannheim, kit no 10 148 261 035, Darmstadt, Germany) and the results were expressed in g/l.

SO_2 production by yeasts

SO_2 concentration was evaluated using the ripper iodimetric method [31]. The results were expressed in mg/l.

Nitrogen consumption by yeasts and bacteria

The assimilable nitrogen in the medium identified as ammoniacal nitrogen (NH_4^+) and α-amino nitrogen was measured using two enzymatic assays: one for the quantification of NH_4^+ (Microdom, kit no 110 05 037 00) and other for the quantification of α-amino nitrogen (Microdom, kit no 110 10 110 00). The results were expressed in mg/l.

Fatty acids produced by yeasts

The fatty acids were measured using gas chromatography. The results were contracted out in the Faculté D'Oenologie de l'Université Victor Segalen, Bordeaux 2 and were expressed in mg/l.

Results

Alcoholic fermentation

Alcoholic fermentations, carried out by the four strains of *S. cerevisiae*, lasted approximately for 5 days during which cell growth and sugar consumption were followed (kinetics not shown). At the end of the alcoholic fermentation A, B, C and D strains had attained a maximal biomass of 5.25 g/l

(334×10^6 cells/ml), 4.95 g/l (275×10^6 cells/ml), 6.6 g/l (300.6×10^6 cells/ml) and 7 g/l (398×10^6 cells/ml), respectively, and left 1.58, 1.16, 0.45 and 0.65 g/l of sugar, respectively. Table 1 shows some of the biochemical characteristics of the four yeast fermented media which reveal slight differences amongst them.

Malolactic fermentation using *O. oeni* X strain

In this part of the study, the synthetic wine medium was used as a control for *O. oeni* X strain in addition to the synthetic grape juice medium in order to evaluate the part of the inhibition due to ethanol by comparing the behaviour of this strain in both media. The greater inhibition of the X strain growth and demalication observed with the four yeast/bacteria pairs was therefore due in addition to ethanol to other inhibitory conditions of the yeast fermented media. We also noticed that the inhibition extent varied widely from a pair to another and strongly depended on the yeast strain chosen. In addition, the MLF was achieved only in the case of one pair using *S. cerevisiae* A strain whereas it was incomplete within the three other pairs. In the synthetic wine control culture, the demalication was also completed yet it presented a long lag phase of 215 h (Fig. 1).

As shown in Table 2, although the initial biomass of *O. oeni* X strain was almost the same in the different fermentations, the maximal biomasses reached were different and strongly reduced in comparison to the synthetic grape juice control. In fact, with the pairs A/X, B/X, C/X and D/X, the maximal biomasses reached were, respectively, 3.7, 6.4, 12.5 and 23.3 times lower than in the synthetic grape juice control. Between the four pairs tested, *O. oeni* X strain grew more efficiently in the A fermented medium. In the synthetic wine control, the maximal biomass reached was greater than in the fermented media but it was still 2.4 times lower than in the synthetic grape juice control. In addition, the malic acid consumption rates were slower than in the synthetic grape juice culture and were reduced to different levels compared to this control (Table 2). However, the MLF was completed only in the A fermented medium and in the synthetic wine medium which presented the same malic acid consumption rates. Demalication was still three times slower than in the synthetic grape juice control. Through our observations, we noticed that the inhibition extent of bacterial growth evolved in the same way as demalication did during the four sequential fermentations. As an example, the lowest biomass formed as well as the lowest amount of malic acid consumed were both observed in the D fermented medium. In addition, the

Table 1 Biochemical characteristics of the four yeast-fermented media

	A	B	C	D
Ethanol produced (g/l)	86 (±3)	81 (±2.5)	77 (±2)	86 (±2.7)
Acetic acid produced (g/l)	0.15 (±0.006)	0.15 (±0.006)	0.37 (±0.006)	0.19 (±0.01)
Malic acid consumed (g/l)	0.84 (±0.14)	0.63 (±0.11)	0.96 (±0.13)	0.94 (±0.1)
Initial ammoniacal nitrogen (mg/l)	380 (±4.54)	389 (±7)	379 (±4.52)	394 (±12.6)
Final ammoniacal nitrogen (mg/l)	0	0	0	0
Initial alpha-amino nitrogen (mg/l)	78 (±5.54)	94 (±6.11)	76 (±2.7)	82 (±1.13)
Final alpha-amino nitrogen (mg/l)	26.6 (±3.55)	10.2 (±2.34)	9.3 (±1.04)	13.4 (±0.62)
Free SO_2 (mg/l)	8.9 (±0.6)	6 (±0.4)	8.9 (±0.1)	8.96 (±0.8)
Molecular SO_2 (mg/l) at pH 3.5[a]	0.28	0.18	0.27	0.28
Total SO_2 (mg/l)	26.7 (±2)	13.44 (±1.2)	28.9 (±1.6)	20 (±1.4)
Total octanoic acid (mg/l)	16.7	5.7	24.8	0.7
Molecular octanoic acid (mg/l) at pH 3.5[b]	16	5.48	23.8	0.67
Total decanoic acid (mg/l)	1.9	0.5	2.9	<0.1
Molecular decanoic acid (mg/l) at pH 3.5[b]	1.8	0.48	2.8	<0.096
Total dodecanoic acid (mg/l)	0.2	<0.1	0.2	<0.1
Molecular dodecanoic acid (mg/l) at pH 3.5[b]	0.19	<0.095	0.19	<0.095

[a] $\text{Molecular}\,SO_2\ (\text{mg/l}) = \text{Free}\ SO_2\ (\text{mg/l})/\left(10^{(\text{pH}-\text{p}K\text{m})}+1\right)$

pH 3.5 corresponds to the initial pH at the beginning of the four MLF

$\text{p}K_\text{m}$ (SO_2) = 2 at 22°C and for an ethanol content between 9,7 and 11% (v/v) in the four yeast fermented media

[b] The molecular or undissociated form of the three organic acids was calculated following the Henderson–Hasselbach equation: pH = $\text{p}K_\text{A}$ + log [(ionic form)/(undissociated form)]. [Total organic acid] = [ionic form] + [undissociated form]. $\text{p}K_\text{A}$ of octanoic acid = 4.89. $\text{p}K_\text{A}$ of decanoic acid = 4.9. $\text{p}K_\text{A}$ of dodecanoic acid = 4.8

Each value is the mean of triplicate experiments ± SD

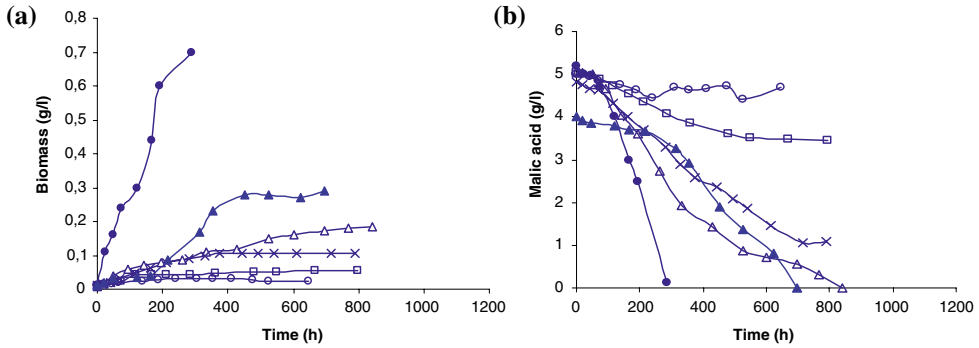

Fig. 1 Biomass evolution (**a**) and malic acid consumption (**b**) by *O. oeni* X strain in control cultures and the four sequential fermentations. *S. cerevisiae* A strain/*O. oeni* X strain (*open triangle*), *S. cerevisiae* B strain/*O. oeni* X strain (*times*), *S. cerevisiae* C strain/*O. oeni* X strain (*open square*), *S. cerevisiae* D strain/*O. oeni* X strain (*open circle*), Synthetic grape juice control culture (*filled circle*), Synthetic wine control culture (*filled triangle*). Each value is the mean of triplicate experiments ± SD

Table 2 MLF characteristics of the four sequential fermentations using *O. oeni* X strain and their corresponding control cultures

	Synthetic grape juice control	Synthetic wine control	Couple A/X	Couple B/X	Couple C/X	Couple D/X
Initial biomass (g/l)	0.013	0.013	0.01	0.01	0.008	0.011
Maximal biomass (g/l)	0.7	0.29	0.19	0.11	0.056	0.03
Initial malic acid concentration (g/l)	5.2	4	5.05	4.82	5.05	5
Final malic acid concentration (g/l)	0.12	0	0	1.1	3.5	4.63
Malic acid consumption rate (g/l/h)[a]	18×10^{-3}	6×10^{-3}	6×10^{-3}	5.2×10^{-3}	2.8×10^{-3}	2×10^{-3}
Inhibition percentage of demalication rate (%)[b]	–	67	67	71	84	89
Specific average demalication rate (g/g/h)[c]	26×10^{-3}	21×10^{-3}	32×10^{-3}	47×10^{-3}	56×10^{-3}	74×10^{-3}
Duration of MLF (h)[d]	288	695	840	715	548	187

[a] Malic acid consumption rate is defined as the malic acid concentration consumed (g/l) divided by the duration of MLF (h)

[b] The inhibition percentage of demalication rate is defined as the reduction of the demalication rate within a couple in comparison to the control (synthetic grape juice control culture) and is calculated as follows: [1 − (demalication rate within a couple/control demalication rate)] × 100

[c] Specific average demalication rate (g/g/h) = [malic acid consumed (g/l)/duration of MLF (h)]/biomass (g/l) present at this moment

[d] Duration of MLF takes into consideration the time until the cessation of malic acid consumption

values of the specific average demalication rates increased whenever the maximal biomass diminished. Therefore, the limitation of the total malic acid consumed throughout the sequential fermentations was related, in a certain extent, to a reduction of the formed biomass but not to a decrease in the malolactic activity.

Malolactic fermentation using *O. oeni* Y strain

Using *O. oeni* Y strain, three yeast/bacteria pairs were tested:

- *S. cerevisiae* B strain/ *O. oeni* Y strain
- *S. cerevisiae* C strain/ *O. oeni* Y strain
- *S. cerevisiae* D strain/ *O. oeni* Y strain.

In this part of the study, *S. cerevisiae* B, C and D strains were selected in order to evaluate the sensitivity of *O. oeni* Y strain towards them since they proved to inhibit the X strain strongly and to prevent it from achieving a complete MLF ("Malolactic fermentation using *O.oeni* X strain").

Growth and malic acid degradation kinetics of the Y strain in the synthetic grape juice medium and the yeast fermented media are presented in Fig. 2.

O. oeni Y strain showed a long lag phase of around 285 h in both synthetic grape juice control and in B fermented medium. The lag phase was also extended in the D fermented medium as it lasted for 216 h; whereas in the C fermented medium, it required only 70 h for the exponential growth phase to start (Fig. 2a). The growth of the bacteria strain was obviously stimulated in the B fermented medium in comparison to the control; whereas it was inhibited in the C fermented medium and was not affected in the D fermented one. In regards to the malic acid consumption, although it was complete in the control culture,

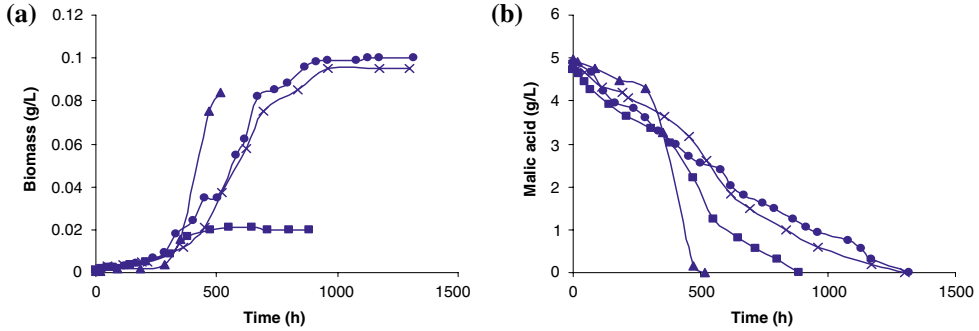

Fig. 2 Biomass evolution (**a**) and malic acid consumption (**b**) by *O. oeni* Y strain in control culture and the three sequential fermentations. *S. cerevisiae* B strain/*O. oeni* Y strain (*filled triangle*), *S. cerevisiae* C strain/*O. oeni* Y strain (*filled square*), *S. cerevisiae* D strain/*O. oeni* Y strain (*times*), Synthetic grape juice control culture (*filled circle*). Each value is the mean of triplicate experiments ± SD

yet it was faster in the B fermented medium. Nevertheless, it presented a lag phase of 282 h which was not detected in the control culture (Fig. 2b). In this case, both bacterial growth and demalication started together after the completion of long lag phase. In the C fermented medium, demalication was stimulated after 378 h but was not as fast as observed in the B fermented medium. Finally, the D fermented medium showed total neutrality towards the demalication activity of the Y strain.

When examining their corresponding productivities, the B fermented medium showed a stimulatory effect on Y growth which was 2.5 times faster than in the control one. In contrary, in the C fermented medium, bacterial growth was inhibited and was 3.3 times slower than in the control (Table 3). Malic acid consumption was stimulated in both B and C fermented media and it was, respectively, 2.6 and 1.4 times faster than in the control when comparing the demalication rates. Comparing the specific average demalication rates in the B and C fermented media to the control one highlights the stimulation of malic acid consumption in both couples. And although the bacterial growth was inhibited in the C fermented medium, yet the specific average demalication rate was the highest, proving that these two activities were totally dissociated in this case. Finally, the maximal biomass reached, the biomass productivity and the demalication rate obtained in the D fermented medium and the control were practically the same revealing a total neutrality of the D strain towards the Y strain.

Discussion

The different pairs examined in this study showed various degrees of inhibition and stimulation depending on the yeast and bacterial strains chosen. The originality of the results obtained was to uncover the fact that the same yeast strain was able to, respectively, inhibit and stimulate two

Table 3 MLF characteristics of the three sequential fermentations using *O. oeni* Y strain and their corresponding control culture

	Synthetic grape juice control	Couple B/Y	Couple C/Y	Couple D/Y
Initial biomass (g/l)	0.0007	0.0006	0.0015	0.001
Maximal biomass (g/l)	0.1	0.084	0.02	0.095
Biomass productivity (g/l/h) [a]	1.34×10^{-4}	3.4×10^{-4}	0.4×10^{-4}	1.21×10^{-4}
Initial malic acid concentration (g/l)	4.88	4.96	4.72	4.84
Final malic acid concentration (g/l)	0	0	0	0
Malic acid consumption rate (g/l/h)	3.7×10^{-3}	9.6×10^{-3}	5.3×10^{-3}	3.7×10^{-3}
Activation percentage of demalication rate (%)[b]	–	160	43	–
Specific average demalication rate (g/g/h)	0.04	0.11	0.27	0.04
Duration of MLF (h)	1,319	518	886	1,300

[a] Biomass productivity is defined as the maximal biomass formed (g/l) divided by the time (h) at the end of the growth phase without taking into consideration neither the lag phase nor the stationary phase

[b] Activation percentage of demalication rate (%) is defined as the increase of the demalication rate within a couple in comparison to the control (synthetic grape juice control culture) and is calculated as follows: [(demalication rate within a couple/control demalication rate) − 1] × 100

different bacteria strains in conditions similar to winemaking: sequential cultures in liquid media. Moreover, *S. cerevisiae* D strain which was the most inhibiting one towards *O. oeni* X strain showed a neutral effect on the growth and demalication of *O. oeni* Y strain compared to synthetic grape juice control, despite the presence of ethanol and sulphur dioxide. To further comprehend the causes of growth and demalication inhibition observed when *O. oeni* X strain was utilized, yeast fermented media were subjected to biochemical analyses in order to search for possible inhibitory molecules. Ethanol, SO_2 and fatty acids produced by the four yeast strains in addition to the assimilable nitrogen consumed were determined. These parameters constitute the main inhibitors of lactic acid bacteria growth and demalication during winemaking.

Ethanol produced by yeasts during alcoholic fermentation affected the growing ability rather than the malolactic activity of lactic acid bacteria. Indeed, according to Capucho and San Romao [6], 8% (v/v) ethanol has been reported to strongly inhibit the cell growth. Nevertheless, malic acid was still degraded up to 90%. This finding supports our results obtained in the synthetic wine medium. Although the biomass was 2.4 times less in comparison to the synthetic grape juice control, the malic acid was totally consumed in the presence of 10% ethanol (v/v). In addition, the ethanol appeared to be responsible for diminishing the demalication rate by 67% (Table 2). The ethanol content of the four yeast fermented media was almost equivalent to that of the synthetic wine control and varied from 9.7 to 11% (v/v) (77–86 g/l). However, the biomasses of bacteria which reached were not as high and the demalication rates were not as fast except for the A fermented medium (Table 2). The outcome showed that ethanol had contributed to the MLF inhibition through consequent reduction in the demalication rate and the maximal biomass produced (Table 2). However, it was not the only component responsible for incomplete demalication observed in B, C and D fermented media. Therefore, combined with other inhibitory factors, ethanol has led to unsuccessful MLF in these media [5, 6, 34].

Sulphur dioxide (SO_2), which constitutes one of these inhibitory factors, has been investigated by many authors [7, 20]. The ability of *S. cerevisiae* to produce SO_2 is dependant upon various factors including the strain involved and the medium composition [14, 28, 33]. Most strains produce less than 30 mg/l SO_2 which is our case (Table 1) although, some have been reported to produce more than 100 mg/l [14]. Free SO_2 at a concentration of more than 15 mg/l at a pH of 3.5 can considerably reduce the number of viable cells and inhibit *O. oeni* growth [17]. In addition, the molecular SO_2 which is the toxic form [33] was found to inhibit the bacterial growth at concentrations above 0.5 mg/l. According to Delfini and Morsiani [10], levels of inhibition ranged from 0.5 to 0.84 mg/l of molecular SO_2 depending on the bacterial strain. As shown in Table 1, our concentrations were lower than the ones reported in the literature. Moreover, during MLF, the pH of A, B, C and D fermented media increased from 3.5 to 3.9, 3.86, 3.6 and 3.55, respectively, leading to a decrease in the amounts of molecular SO_2 to 0.11, 0.08, 0.21 and 0.25 mg/l at the end of MLF. Nevertheless, this SO_2 may have contributed to inhibition by reducing the maximal biomass and the malic acid activity (Table 2) in association with other factors [7]. In addition, combined SO_2 at a concentration of 20 mg/l was shown to reduce malolactic activity by 13% [22]. In our study, the C fermented medium contained 20 mg/l of combined SO_2, and the demalication was reduced by 84% (Table 2). This proved that SO_2 was not the only contributing factor to inhibition of X strain.

Medium chain fatty acids, such as decanoic acid, can also inhibit malolactic bacteria [12, 13, 24]. In addition to limiting the bacterial growth, medium chain fatty acids can considerably reduce the ability of malolactic bacteria to catabolise malic acid, although these effects are highly dependant upon the type and concentration of the fatty acid present as well as the medium pH [6, 7]. The octanoic, decanoic and dodecanoic acids have close pK_A of 4.89, 4.9 and 4.8, respectively, meaning that the major form of these acids in our media (pH 3.5) was the undissociated form (Table 1) which is the toxic form [6]. Despite the augmentation of the pH values during MLF, the undissociated form remained as a predominant one as the pH was still lower than the three pK_A. Edwards and Beelman [12] showed that the addition of 5–10 mg/l of decanoic acid to grape juice inhibited bacterial growth and MLF, whereas 30 mg/l were lethal. Moreover Lonvaud-Funel et al. [24] found that the addition of either 23 μM (4 mg/l) decanoic acid or 2.5 μM (0.5 mg/l) dodecanoic acid caused the inhibition of MLF in wine. Furthermore, these authors found that the combined addition of hexanoic, octanoic and decanoic acids resulted in a greater inhibition than the addition of each one individually. In our case, although decanoic and dodecanoic acids' concentrations were lower than the ones found in the literature (Table 1) and reached a maximum of 2.9 and 0.2 mg/l, respectively, in the C fermented medium, we may suggest that their combination with octanoic acid contributed to a certain extent in the inhibition of bacterial growth and malic acid consumption.

Inhibition of malolactic bacteria has also been reported to result from a nutrient depletion in the yeast fermented media [4, 27, 30]. In fact, after the completion of AF, the wine may be lacking some nutrients such as vitamins and amino acids which are essential for malolactic bacteria metabolism and survival. In our case, the remaining nitrogen in the yeast fermented media was measured. As

we notice in Table 1, ammoniacal nitrogen was entirely consumed by yeasts. But since our analyses on the synthetic grape juice control of *O. oeni* X strain have shown no consumption of ammoniacal nitrogen we can exclude this factor from inhibitory reasons (data not shown). Nitrogen from α-amino acids is essential for bacterial metabolism and survival, but this strain, in synthetic grape juice control culture, only consumed 8.6 mg/l of the 81.2 mg/l α-amino nitrogen initially present in the medium. This amount seemed to be sufficient for this strain to completely achieve MLF in 12 days, consuming 5.08 g/l of malic acid and reaching a biomass of 0.7 g/l. Through comparing the concentrations of α-amino nitrogen remaining in the yeast fermented media (Table 1) and taking into consideration the yeast extract used (oxoid) which contained all essential amino acids required for bacterial growth, we can suggest that from a quantitative point of view there was no lack in the nitrogen requirements of the X strain. Besides, Remize et al. [32] have shown that the levels of nitrogen from essential amino acids as low as 0.7 mg N/l in the case of tyrosine were sufficient to allow the growth and activity of *O. oeni*. Yeasts, however may have depleted some essential amino acids to concentrations that were not sufficient for bacterial growth and metabolism [4, 32]. Therefore, from a qualitative point of view, we cannot exclude this factor from being considered as one of the inhibitory reasons. In addition the yeast fermented media may have lacked some essential vitamins or trace elements that we did not measure.

As a summary, the synergistic inhibitory effects of ethanol, SO_2, fatty acids and reduced nutrient content may partly explain the growth and demalication inhibition but do not clarify it entirely. For example, the yeast strain D which was the most inhibiting one was not the yeast which produced the biggest amount of ethanol, SO_2 or fatty acids nor the one which consumed the biggest amount of nitrogen compared to the other strains. In addition, the synthetic wine control culture confirmed the fact that a complete MLF can be achieved even in the presence of ethanol and a reduced nutrient composition. Therefore, incomplete MLF in B, C and D fermented media was probably due to other yeast metabolites. These metabolites can possibly be of protein nature. Few studies on such metabolites have been reported till now. Dick et al. [11] isolated two different cationic proteins. Recently, Comitini et al. [8] as well as Osborne and Edwards [29] have partially characterized two different yeast compounds of protein nature active against *O. oeni.*

While *O. oeni* X strain was more or less inhibited depending on the yeast fermented medium, *O. oeni* Y strain showed a stronger resistance towards the three yeast fermented media tested as its demalication rate was approximately twice faster than the X one in the three of them. Moreover, the Y strain was stimulated by the B and C fermented media compared to the synthetic grape juice control (Fig. 2). In fact, with B fermented medium, both growth and demalication were faster than in the control culture. But in C fermented medium, only the demalication was stimulated whereas the growth was inhibited (Table 3). This confirms the fact that these two activities are not always correlated [6]. The D fermented medium, which was the most constraining towards the X strain, had a neutral effect on the behaviour of the Y strain stressing once again on the capacity of Y strain to perform MLF under hard inhibitory conditions.

Some researchers tried to clarify the resistance of certain *O. oeni* strains to the harsh environmental conditions of wine by investigating the presence of stress-responsive genes whose expressions increased as a reaction to the different stressing factors such as ethanol, SO_2, fatty acids, temperature, acidity, etc. Various regulatory mechanisms that respond to environmental stress have been identified in Gram positive bacteria including the synthesis of so-called heat shock proteins such as the Hsp 18 protein [9, 18]. Similar mechanisms responding to some stressing factors might have presented an enhanced expression within the Y strain.

Besides the resistance of Y strain to the inhibitory conditions described earlier, this strain was stimulated by B and C fermented media. It is generally recognized that substances released by yeasts, particularly nitrogenous compounds such as amino acids [15] and mannoproteins [16], can play a major role in influencing the bacterial growth and malolactic activity in wine. Furthermore, other yeast-derived components may stimulate bacterial growth and demalication. They include: vitamins, nucleotides and lipids (such as long chain fatty acids) which have been poorly studied in relation to their potential stimulatory effects on bacterial growth in wine [1].

Finally, the study reported in this paper shows that in order to perform a complete and successful MLF, winemakers should carefully choose the yeast and bacteria strains within a couple. A single bacteria strain can be variably inhibited depending on the yeast responsible for the alcoholic fermentation. The same yeast can exhibit opposite effects towards two different strains of *O. oeni*. Further analyses are required in future studies in order to investigate other inhibitory yeast metabolites such as proteins or peptides. It would be also interesting to search for some stress-responsive genes within the resistant bacterium and to try to identify which yeast molecules are possibly stimulatory ones.

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