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Cédric Brandam, Noura Fahimi, Patricia Taillandier. Mixed cultures of *Oenococcus oeni* strains: A mathematical model to test interaction on malolactic fermentation in winemaking. *LWT - Food Science and Technology*, 2016, 69, pp.211-216. <10.1016/j.lwt.2016.01.045>. <hal-01911992>

HAL Id: hal-01911992

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To cite this version:

Brandam, Cédric and Fahimi, Noura and Taillandier, Patricia Mixed cultures of *Oenococcus oeni* strains: A mathematical model to test interaction on malolactic fermentation in winemaking. (2016) LWT - Food Science and Technology, 69. 211-216. ISSN 0023-6438

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Mixed cultures of *Oenococcus oeni* strains: A mathematical model to test interaction on malolactic fermentation in winemaking

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A B S T R A C T

The control of malolactic fermentation (MLF) by *Oenococcus oeni* is an essential step in winemaking process. Although selected *O. oeni* strains are available for winemakers, the MLF is not always successful, sometimes because of indigenous presence of other strains. In this work, interaction during MLF between five strains of *O. oeni* was studied. Experiments were performed in MRS medium modified to be closer to wine conditions (pH 3.5, 10% ethanol). Interaction in mixed cultures of 10 pairs formed by the five strains were analysed from experiments performed in a Membrane Bioreactor (MBR). Pairs were classified in three different classes among growth interaction: 1) negative reciprocal interaction of both strains (6 pairs), 2) interaction that affect negatively one strain and positively the other (3 pairs), and 3) interaction with positive effect on one strain and no effect on the other (1 pair). Thanks to a mathematical model previously established during pure cultures to link growth and malic acid consumption, effect of mixed cultures on the specific activity of cells to consume malic acid has been equally evaluated. This capacity seemed not to be affected for 4 pairs whereas it seemed activated for 6 pairs.

Keywords:

Oenococcus oeni
Malolactic fermentation
Model
Mixed culture
Interaction

1. Introduction

In winemaking Malolactic Fermentation (MLF) is an important step that consists to convert L-malic acid into L-lactic acid after the alcoholic fermentation. Among lactic acid bacteria, *Oenococcus oeni* is the major specie responsible for MLF. The acidity decrease resulting of MLF is also accompanied by production of flavor and aroma in wine. In fact, the good monitoring of MLF is mandatory to ensure the good quality and the suit cost of wine. Actually, selected *O. oeni* strains are proposed by microorganism producers to inoculate musts after alcoholic fermentation but the good control of MLF remains difficult. One of the reasons could be that the majority of studies on MLF were done with pure cultures of *O. oeni*. However, in the real process, several indigenous strains are present in grape musts. Interactions that probably occur between lactic acid bacteria can impact the onset and/or the progress of the MLF. In the literature several studies have shown very different interaction effects between microorganisms: inhibition (Nissen & Arneborg, 2003), stimulation (De Souza Oliveira, Perego, Converti, & De Oliveira,

2009), competition (Bely, Stoeckle, Masneuf-Pomarède, & Dubourdieu, 2008; Holm, Nissen, Sommer, Nielsen, & Arneborg, 2001; King & Beelman, 1986; Lonvaud-Funel, Joyeux, & Dessens, 1988), amensalism (Carrau, Neirotti, & Gioia, 1993; Fernandez, Beaufort, Brandam, & Taillandier, 2014; Osborne & Edwards, 2007; Pommier, Strehaiano, & Déla, 2005; Taillandier, Gilis, & Strehaiano, 1995; Taillandier, Julien-Ortiz, Lai, & Brandam, 2014). All these studies concerned yeasts interactions. In our knowledge, only one interaction study was realized with lactic acid bacteria but it was to study its interaction with wine yeasts (Nehme, Mathieu, & Taillandier, 2010). In this work, *O. oeni* intra-specy interactions were investigated. The behaviour of 10 pairs of 5 strains of *O. oeni* in mixed cultures was analysed. A difficulty to study microorganisms belonging to the same species in mixed cultures is to distinguish each strain population. For that, a specific tool developed in our laboratory, the membrane bioreactor with double compartments, was used. It was conceived to study indirect interactions between microorganisms i.e. interactions due to metabolites excreted in the medium. To evaluate interaction between strains on the MLF, mixed cultures were compared to pure cultures. A mathematical model that we have been established in previous work (Fahimi, Brandam, & Taillandier, 2014) on pure cultures to represent the consumption of L-malic acid is used to help us to interpret mixed culture behaviour.

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2. Materials and methods

2.1. Strains and storage conditions

Five strains of *Enococcus œni* named A, B, C, D, and E were studied in this work. These strains belong to the DIVOENI ANR collection at the faculty of œnology, Bordeaux, France (n°ANR-07 BDIV 011-01). The strains were kept frozen at -20°C in MRS broth (Biokar diagnostic, Beauvais, France) containing 20% glycerol (v/v).

2.2. Cultures conditions

2.2.1. Reactivation

One hundred μL of the frozen strains A, B, C, D, and E were reactivated 65 h in 10 ml of MRS broth supplemented with L-malic acid (4 g/L) at 28°C , pH 5.2 without agitation in Erlenmeyer flasks.

2.2.2. Inoculum

The modified MRS_m (MRS broth + 4 g/L of L-malic acid + 2 g/L of D-fructose) was used with adjusting the pH at 4.8 using a 85% orthophosphoric acid solution. After autoclaving, 5% (v/v) of ethanol were added and then the medium was inoculated at 1% (v/v) using reactivated cultures. The cultures were incubated at 28°C in Erlenmeyer flasks without agitation.

2.2.3. Malolactic fermentation conditions (MLF)

A tool designed specially to study the indirect interactions between two microorganisms was used: a lab-made, two-compartment, membrane bioreactor (MBR) (See Fig. 1). The complete system has been described in detail (Albasi, Tataridis, Salgado Manjarrez, & Taillandier, 2001). It is composed of two jars interconnected by a hollow fibre membrane module immersed in one of the jars. The membrane fibre diameter of $0.1\ \mu\text{m}$ allows the medium, but not the microorganisms, to pass through the fibres. Each strain is inoculated into only one compartment, which can be sampled and analysed separately. By applying pressure into the headspace of each of the vessels alternately, the medium is made to flow and mix. Compressed, filter-sterilised nitrogen is used to apply the pressure and a system of valves controls its admission and expulsion according to the liquid levels. The time and quantity of liquid inversion is calculated to ensure perfect homogenisation between the two jars. Hence, the microorganisms grow as if they were in the same liquid medium but they are physically separated, thus allowing the dynamics of each population to be easily followed by microscopic counting. So, this specific system can be used to study indirect interactions without needing a sophisticated method to follow each dynamic population based for example on molecular biology. It is not suitable for direct interactions since the strains are cultivated separately.

For these cultures, the pH of the modified MRS_m was adjusted to 3.5 and 10% (v/v) of ethanol was added. The fermentation was carried out at 20°C . Strains were grown in anaerobiosis conditions under 0.45 bar of nitrogen atmosphere pressure in the membrane bioreactor of 4 L, and with an agitation of 100 rpm. For pure culture, fermentations were performed exactly in the same conditions as mixed culture but without the membrane in reactor. Three culture replicates have been done for each strain in pure culture.

Ten mixed cultures were studied in this work using the following crossings between strains: A/B, A/C, A/D, A/E, B/C, B/D, B/E, C/D, C/E and D/E. The experiments of A/C, A/D and B/D pairs were repeated 3 times, the other pairs were repeated 2 times. All the presented results (growth and L-malic acid concentrations) had no more 8% of variation between the 2 or 3 repetitions. The inoculum was adjusted in order to start the MLF with 2×10^6 CFU/mL in all cases: 2×10^6 CFU/mL of the strain in pure culture and 1×10^6 CFU/

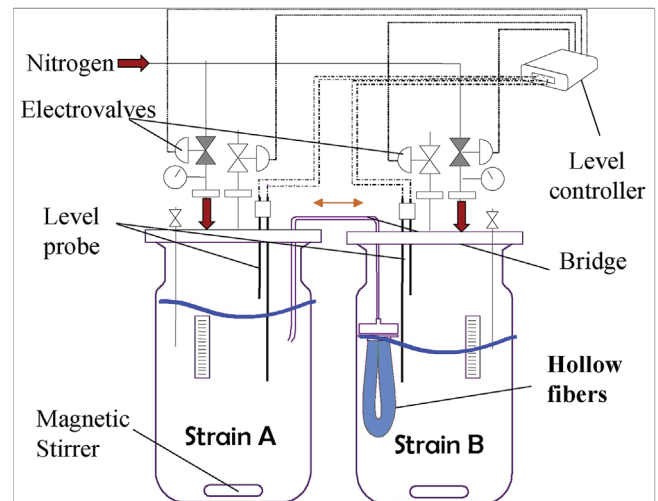


Fig. 1. Scheme of the membrane bioreactor used.

mL of each strain in mixed cultures. The experiments were stopped when L-malic acid was totally consumed in the medium culture.

2.3. Analytical methods

2.3.1. Growth

Bacterial growth was followed by measuring the optical density (OD) in a spectrophotometer (Hitachi U-2000) at 620 nm using a quartz cuvette with a 1 cm light path. Biomass was also determined by colony counts on MRS agar plates. The MRS agar was completed with 4 g/L L-malic acid and 5 g/L agar. Its pH was adjusted to 5.7 using a 10 M NaOH solution. A specific correlation between OD and number of colonies was determined for each bacterium and used to inoculate fermentations at 2×10^6 CFU/mL.

2.3.2. L-malic acid concentration

L-malic acid concentration was determined using an enzymatic assay (Roche Boehringer Mannheim/R-Biopharm, kit no. 10 139 068 035, Darmstadt, Germany) and the results were expressed in g/L.

3. Methodology for evaluation of interaction

Using the membrane bioreactor (MBR), it is possible to follow the development of each strain in mixed cultures with classical counting methods. Then, comparison of the growth of each strain in pure and mixed cultures is possible. Concerning L-malic acid consumption (MLF), experimentally it is not possible to measure the quantity of L-malic acid consumed by each one of the strains growing in the same medium. Only total malolactic activity of the two strains can be evaluated and compared with each strain activity in pure cultures.

However, it was demonstrated in previous study (Fahimi et al., 2014) the existence for one given strain of a link between bacteria growth and malic acid consumption. A mathematical model was proposed in pure cultures to represent the specific growth rate (μ) versus the specific L-malic acid consumption rate (ν). It allowed quantifying and comparing the link between these two activities for each of the strains:

$$\nu = ki \times \mu \times \frac{[mal]}{[kmal] + [mal]}$$

Table 1

Model parameter values determined in pure culture for the five strains.

Strain	A	B	C	D	E
k_i	35.8	54.5	45.7	70.8	62.2
k_{mal} (g.L ⁻¹)	1.1	1.23	1.14	0.47	0.8

v , specific ι -malic acid consumption rate:
 $\nu = \frac{1}{X} \times \frac{d[mal]}{dt}$ [(g/L/h)/(OD₆₂₀unit)]
 μ , specific growth rate: $\mu = \frac{1}{X} \times \frac{dX}{dt}$ (h⁻¹)

with [mal] the ι -malic acid concentration in g/L, X the biomass concentration in OD unit and t the time in hour.

For each pure strain, parameters k_i and k_{mal} was identified (Table 1). k_i was a parameter representing the proportional coefficient between v and μ , it informed about the intrinsic capacity of the cells of a strain to consume ι -malic acid, independently of its growth. k_{mal} was a substrate limitation parameter; a low value of k_{mal} signified that the bacteria is able to grow with a low concentration of ι -malic acid in the medium, in the opposite a high value of k_{mal} signified the bacteria require a high minimum threshold of ι -malic acid concentration to ensure its growth from the malate metabolism.

Thanks to the model, it is here possible to test the effect of the interaction on the link between growth and acid malic consumption established on pure cultures. In mixed cultures, the consumption of ι -malic acid can be calculated by using the experimental data of the biomass concentrations of each of the two strains according to the following equation:

$$\frac{d[mal]}{dt} = X_{s1,mixed} \times k_1 \times \mu_1 \times \frac{[mal]}{[mal] + k_{mal1}} + X_{s2,mixed} \times k_2 \times \mu_2 \times \frac{[mal]}{[mal] + k_{mal2}},$$

- s1: strain 1
- s2: strain 2

This predicted consumption in the mixed culture of a pair of bacteria was then compared to their experimental consumption and reveals if there is an effect of the presence of the other bacterium strain on the intrinsic capacity of the cells of a strain to consume ι -malic acid. If no interaction occurred on this link, modelled and experimental values would be identical.

4. Results

The methodology was applied on the ten pairs of bacteria studied in our work. Table 2 summarizes effects of interactions on

both growth and consumption of ι -malic acid of different pairs in the mixed cultures. The pairs could be classified into 3 kinds of interaction based on growth curves analysis.

4.1. Negative reciprocal interaction of both strain growth

This interaction concerned 6 pairs of strains (Table 2, lines 2 to 7). The example of the pair B/C was showed on Fig. 2. Fig. 2a shows the growth of B and C strains in pure and mixed cultures. Both strains were clearly affected negatively in the mixed culture. Growth began after 340 h approximately for the two strains in mixed cultures against 140 h in pure cultures. At the end of MLF, level of population was also 5 fold and 10 fold higher in pure cultures for strain B and for strain C.

The consumption duration of ι -malic acid in mixed culture was very long in comparison with the consumption of each strain in pure cultures (Fig. 2b); MLF of the mixed culture was affected negatively. However, Fig. 2b shows that the predicted and experimental ι -malic acid consumption are similar. These data indicate that if the growth or the acid malic consumption were affected negatively by the presence of the two strains, the intrinsic capacity of each cell to transformed malic acid into lactic acid was not affected compared to pure cultures.

In the same way, pairs A/B (Fig. 5 in Supplementary data), A/C, A/D, B/D (Fig. 6 in Supplementary data), and D/C showed interaction with negative effect on the growth of each strain in mixed cultures compared to their growth in pure cultures (Table 2, column 2). The level of growth inhibition was different from one pair to another and also from one strain to another but the tendency was the same.

MLF of these pairs was also slower in mixed culture than in each pure cultures, excepted for pairs A/D and B/D where MLF duration was identical to D pure culture (Table 2, column 3). As for the pair B/C, modelled values of ι -malic acid consumption were in accordance with experimental values for pair B/D. However, for pairs A/B, A/C, A/D, and D/C, experimental consumption of ι -malic acid was faster than the predicted one (Table 2, column 4). So, the intrinsic capacity of one (or two) strain(s) has been modified positively in mixed cultures compared to pure cultures.

4.2. Interaction that affect negatively the growth of one strain and positively the growth of the other

These interactions concerned three pairs where the E strain was always affected positively in the mixed culture (Table 2, lines 8 to 10).

Growth profile comparison for pair B/E are shown in Fig. 3a. In the mixed culture, the E strain was activated, with a lag phase shorter, while the B strain was strongly inhibited.

Fig. 3b presents results of ι -malic acid consumption of this pair B/E. The experimental consumption by the 2 strains in mixed culture was faster than the consumption by E strain cultivated alone

Table 2

Effects of interaction in mixed culture compared to pure culture for the ten pairs of bacteria A, B, C, D, and E. (+): positive effect, (=): neutral effect, (-): negative effect.

Mixed cultures	Effect on growth	Effect on MLF duration	Effect on the link between v and μ	Best developed strain
A/B	-A/-B	-A/-B	+	A
A/C	-A/-C	-A/-C	+	A
A/D	-A/-D	-A/=D	+	A
B/C	-B/-C	-B/-C	=	B
B/D	-B/-D	-B/=D	=	B
D/C	-D/-C	-D/-C	+	C
B/E	-B/+E	-B/+E	+	E
C/E	-C/+E	-C/+E	=	E
D/E	-D/+E	-D/+E	+	E
A/E	+A/=E	+A/=E	=	A

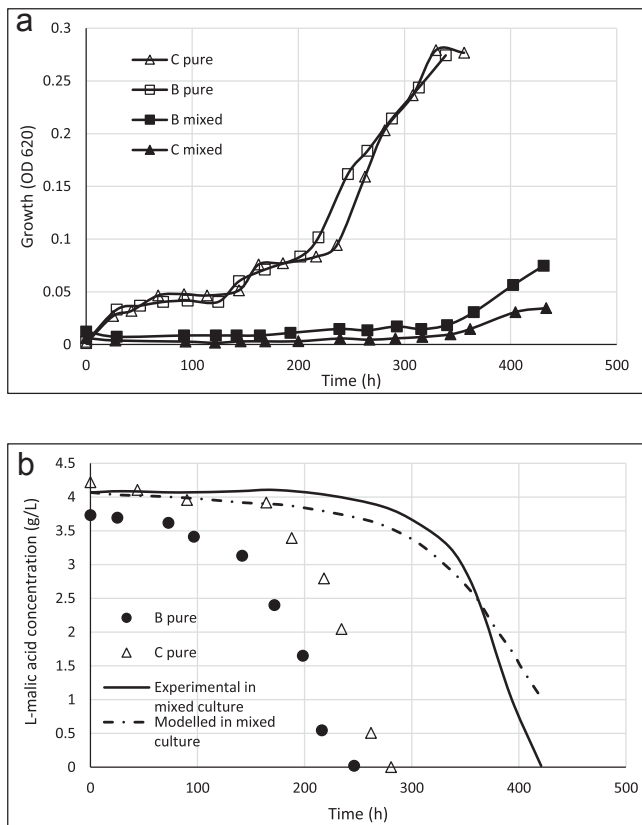


Fig. 2. Experimental values (dotted lines) for growth (2a) and consumption of L-malic acid (2b) by *O. oeni* strains B and C in pure and mixed cultures. Modelled values (dotted lines) for consumption of L-malic acid in mixed culture (2b).

while it was slower than consumption by B strain alone. The experimental consumption of L-malic acid for the mixed culture predicted was faster than the one predicted by the model. This could be interpreted as a different effect of interaction on growth and malolactic activity, and so a different link between these two activities for one strain.

This kind of interaction affecting negatively the faster strain in pure culture and positively the slowest one was also observed in the case of pairs C/E (Fig. 7 in Supplementary data) and D/E (Table 2, column 2). Strain E was always activated and reached quickly biomasses higher than those reached in pure culture. The stimulation was also remarkable on the duration of the latency phase that was shorter in mixed culture than in pure one. In fact, it seems that the presence of another strain (B, C, or D) activates the strain E. On the contrary, each one of strains B, C, and D was strongly inhibited in mixed culture in presence of strain E.

L-malic acid consumption in mixed culture for each of these 3 pairs was intermediate to the consumption of the two strains in pure cultures (Table 2, column 3). The experimental consumption of the 2 strains in mixed culture was faster than the predicted consumption in both cases of B/E and D/E pair. On the opposite for the C/E pair, they were similar indicating that in this case the link between growth and malolactic activity remained the same in pure and mixed cultures, not changing the intrinsic capacity of each cell of strain to do MLF (Table 2, column 4).

4.3. Interaction with positive effect on the growth of one strain and no effect on the other

This kind of interaction concerned only the pair A/E. Comparing

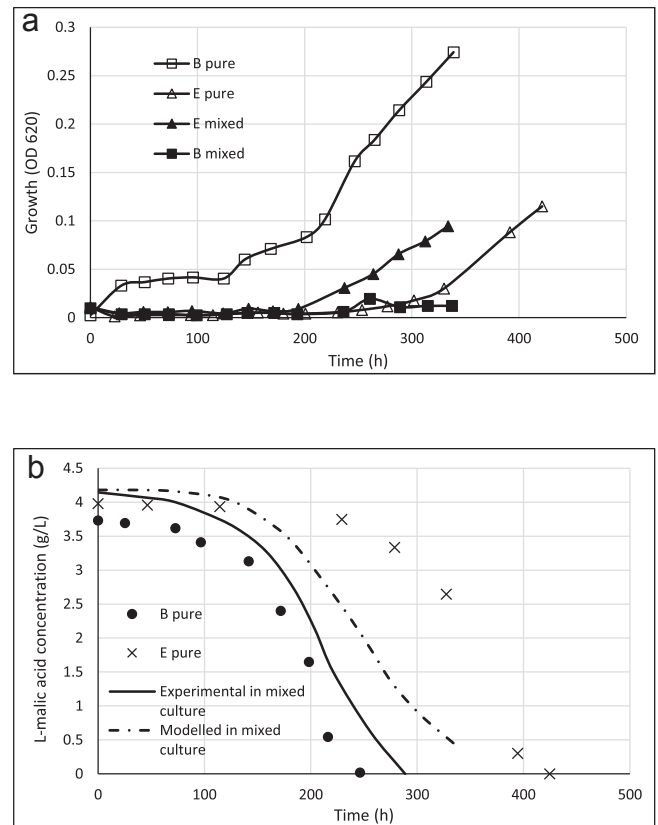


Fig. 3. Experimental values (dotted lines) for growth (3a) and consumption of L-malic acid (3b) by *O. oeni* strains B and E in pure and mixed cultures. Modelled values (dotted lines) for consumption of L-malic acid in mixed culture (3b).

growth profiles of the two strains in mixed culture (Fig. 4a), the development of A strain decreased during the first 120 h but after that the growth started, it reached rapidly higher biomasses than in pure culture. The strain E did not grow throughout the MLF in mixed culture. So growth of strain A was activated by the presence of strain E. There was no growth of strain E during 220 h of mixed culture. In fact consumption of L-malic acid was completed before the E strain started to grow.

Experimental consumption of L-malic acid for this pair was faster than the consumption of each strain in pure cultures (Fig. 4b). This can be justified by the activation of the growth of the strain A in mixed culture that we showed above. The mixing of these two strains in mixed culture seemed to lead to a gain on the duration of the MLF.

Modelled consumption of L-malic acid was almost similar to experimental consumption of the 2 strains A and E in mixed culture. This means that the global link between specific activities of growth and malolactic fermentation was the same in mixed culture as pure cultures.

5. Discussion

The study of the interactions between strains for the 10 pairs of *O. oeni* strains A, B, C, D, and E showed three different kinds of interactions on growth: negative reciprocal interactions of both strains in mixed culture (pairs A/B, A/C, A/D, B/C, B/D, and D/C), interactions that affect negatively the faster strain in pure culture and positively the slowest one (pairs B/E, C/E, and D/E), and interactions with positive effect on the fastest strain in pure culture

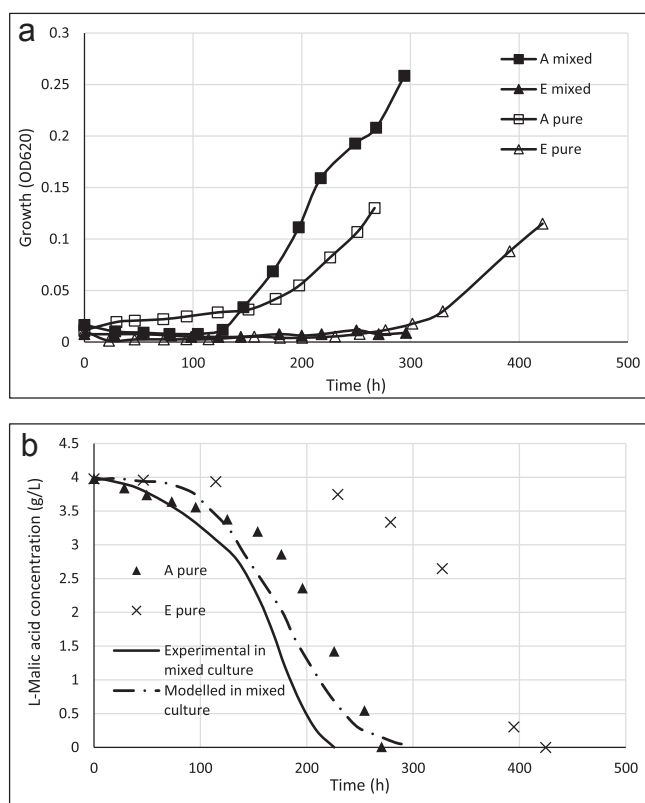


Fig. 4. Experimental values (dotted lines) for growth (4a) and consumption of L-malic acid (4b) by *O. oeni* strains A and E in pure and mixed cultures. Modelled values (dotted lines) for consumption of L-malic acid in mixed culture (4b).

(pair A/E).

In Table 2, we can observe that the interactions existing in the six pairs A/B, A/C, A/D, B/E, D/C, and D/E had a positive effect on the global link between the specific consumption L-malic acid activity v and the specific growth activity μ . Hypothesis are an activation of the consumption of L-malic acid and/or an inhibition of the growth of the strains. The interaction effect also resulted in a loss of initial biomass that was observed experimentally in the first part of the cultures. Presumably to adapt to the conditions of mixed culture, the cells ability to consume L-malic acid increased but does not necessarily led to an increase in growth. Therefore, the overall correlation between v and μ rates was affected in the mixed culture. For the four other pairs (B/C, B/D, C/E, A/E), the link between specific consumption and specific growth was the same in mixed cultures as in pure cultures.

In wine medium many factors can affect the onset and/or the progress of MLF; physicochemical conditions such as a high concentration of ethanol (Ingram & Buttke, 1984; King & Beelman, 1986; Rosa & Sa-Correia, 1992), low pH (Henick-Kling, 1990), low temperature (Asmundson & Kelly, 1990; Maicas, Pardo, & Ferrer, 2000), nutrient depletion (Remize et al., 2006), fatty acids presence (Guerrini, Bastianini, Granchi, & Vincenzini, 2002; Guilloux-Benatier, Le Fur, & Feuillat, 1998) and sulfur dioxide addition (Romano & Suzzi, 1993). In addition to those factors, this work shows clearly the importance of interactions factors in their effect on the physiology of strains, being in the same stressed conditions (20 °C, pH 3.5, and 10% of ethanol), bacteria behave differently in mixed culture in comparison to the pure culture. Indeed it have been demonstrated that inadequate biological conditions may cause the failure of MLF by release of some common inhibitory metabolites from yeasts such as SO₂ (Carreté,

Vidal, Bordons, & Constanti, 2002; Henick-Kling & Park, 1994; Osborne, Dube Morneau, & Mira de Orduna, 2006), specific inhibitory metabolites produced by some strains of *Saccharomyces cerevisiae* (Nehme et al., 2010) and probably inhibitory metabolites produced by indigenous strains of LAB (Knoll, Divol, & Du Toit, 2008).

Comparing the five studied strains, strain A was always the best developed one in the presence of one of the other strains B, C, D, or E; followed by strain E that was the best developed one in mixed culture with B, C, or D, while it doesn't grow in the presence of the strain A. In this last case the interaction could be a competition phenomenon since the difference of growth rate is very high. Then the B strain is the best developed one in presence of strains C or E. Finally, strain C is the best one in mixed culture of the pair C/D. In terms of best growth, the following order was found: A, E, B, and C. Growth of strain D is always disadvantaged in mixed culture. Nevertheless it is a strain that present a very low growth in pure culture but has the highest specific consumption activity of L-malic acid (Fahimi et al., 2014). This means that although a strain growth is affected in mixed culture its malolactic activity may be higher than the other strain.

It also was observed that for the majority of cases, the presence of 2 strains in the same culture medium led to an extension of the duration of the lag phase. Let to suppose that in mixed culture, after a duration corresponding to the duration of the latency phase in pure culture, strains activate their defense system and produce extra cellular metabolites (indirect interaction) that inhibit reciprocally their development. For most of the mixed cultures, after this long latency phase we observed that their maximal specific growth rates become higher than those reached in pure cultures. On one hand, this result can be explained by the fact that there is a loss of cells during the lag phase and culture medium only cells that acquire resistance and become able to withstand environmental conditions (mixed culture) survive. In the other hand, activation of growth can also be explained by reaching a certain concentration of extra cellular molecule(s) produced in conditions of stress and promote(s) growth activity.

In this work we evaluated the globally consumption of L-malic acid in mixed culture. Regarding the effects of interactions on the growth and on the consumption of L-malic acid, a large variability between pairs was showed. In our study we focused on indirect interactions between microorganisms (use of Membrane Bioreactor). Effects we have seen, both positive and negative, are not due to direct contact between strains, so they are only due to the excretion of extra cellular metabolite(s) or due to a potential competition phenomena in some cases. Then it still mandatory to continue research in this way to identify the nature of the agents responsible of the identified interactions.

From a practical point of view, this study brings to the fore difficulties for winemakers to manage MLF. The diversity of behaviours prevents predicting MLF with good certainty. Even the used of bacteria selected for their MLF efficiency, the success is not assured in case of natural presence of other bacteria strains that could interact negatively.

Acknowledgements

The study is conducted within the framework of the ANR program DIVOENI biodiversity n°ANR-07 BDIV 011-01. We thank all partners for the provision of strains of *Enococcus* Collection.

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