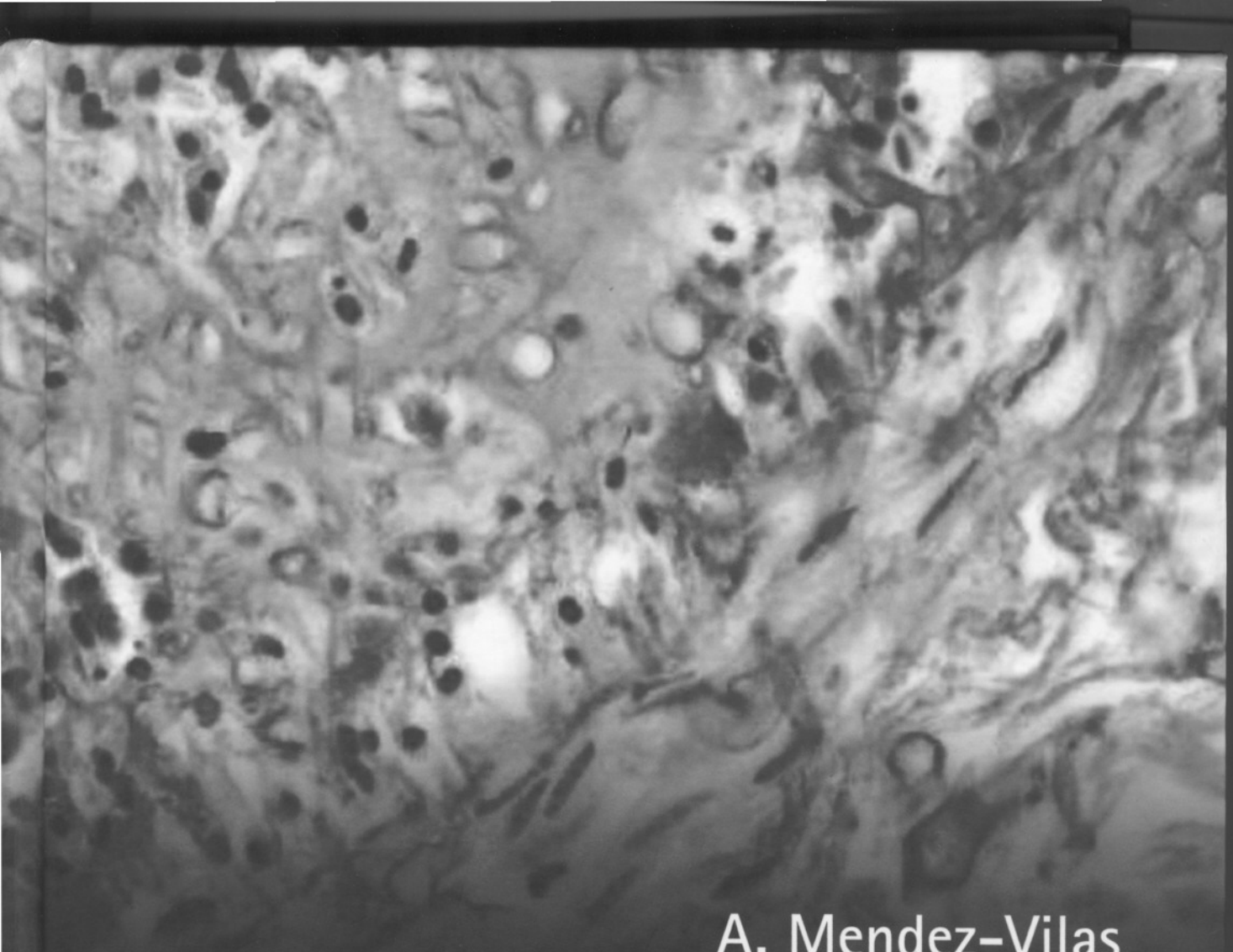

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Microorganisms in Industry and Environment

From Scientific and Industrial Research to Consumer Products

World Scientific

Selection of *Oenococcus oeni* as starter cultures to induce malolactic fermentation in Nebbiolo wine

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Malolactic fermentation (MLF) is the second fermentation of wine that causes a significant evolution of the product and significant changes in its sensory characteristics; this fermentation is principally carried out by *Oenococcus oeni*. Sometimes its performance has some problems and the use of commercial starter does not help to improve the management of the fermentation.

A selection of *O. oeni* strains, taken from the collection of the CRA- Centro di Ricerca per l'Enologia, was made to test their speed of adaptation in wine and the time for completing MLF.

Different assays were made to test the ability to grow in different media with increasing stressful conditions: low sugar content, malic acid (3g/L) and ethanol (10%); the aim of these steps was to adapt the bacteria to the wine environment. The last step was done in Nebbiolo wine. The two strains obtained at the end of the selection were characterized with molecular biology techniques and they were used as starters in Nebbiolo wine both individually and in pairs. The process of MLF was monitored and the sensory analysis of the obtained wines was made. The results showed that the starters used in the mixture allowed to obtain a wine with a quality comparable with the one obtained using a commercial strain.

Keywords: *Oenococcus oeni*, malolactic fermentation (MLF), starter selection, molecular characterization, sensory analysis.

1. Introduction

The conversion of L-malic into L-lactic acid and CO_2 is known as malolactic fermentation (MLF), and it is conducted in wine by some lactic acid bacteria, mainly *Oenococcus oeni* [1, 2]. This process is not a mere chemical conversion but it has a more complex meaning; there are three principal effects: reduction of the acidity, changes of the organoleptic properties and increased microbiological stability.

FML plays a decisive role in conferring to the wine a greater complexity of taste and aroma: the production of bacterial metabolites modifies wine flavour with attractive effects on sensory profile of the final product. These changes are appreciated by the market which tends to prefer wines with soft, intense and complex taste, with sensory notes possibly typical of the area of origin [3-7]. These aspects have made the FML particularly desired and increasingly sought after.

Induction of MLF offers microbiological stability by ensuring that the degradation of malic acid does not occur in the bottle, where the growth of LAB and the formation of CO_2 are undesirable. MLF occurs readily in high-pH wines if not actively discouraged by the use of inhibitory concentrations of SO_2 or sterilization by heat or filtration [2]. In low-pH wines, spontaneous MLF by indigenous LAB has been unreliable and induction of MLF by inoculation with commercially available strains of *O. oeni* has shown little success [2]. However, MLF is often difficult to conduct, even by inoculating with commercial strains of malolactic bacteria [8].

In this study, a selection procedure was applied to the *O. oeni* strains belonging to the collection of CRA-Centro di Ricerca per l'Enologia. Their capability to adapt in wine was tested and an evaluation of malic acid conversion both in synthetic medium and Nebbiolo wine was carried out. Moreover a sensory analysis of wine inoculated with the selected strains was performed to evaluate changes in aroma and organoleptic characteristics strain-dependent. These wines were also compared with the wine obtained with a widely used commercial starter.

2. Experimental

2.1 Bacterial strains

56 *O. oeni* belonging to the collection of CRA-Centro di Ricerca per l'Enologia in Asti were considered in this study. They are conserved in 50% glycerol at -80 °C.

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2.2 Adaptation and strain selection

The strain selection was performed with different adaptation steps using media with increasing stressful conditions.

In the first step each *O. oeni* was cultured in MRS broth (de Man Rogosa and Sharpe) pH 4.8 at 20 °C for 7 days; the strains which passed this step were transferred in a modified MRS (diluted 1:5 and added with 3g/L of malic acid, 10% ethanol, pH 3.5) and incubated at 20 °C for 7 days. In the third step, *O. oeni* strains were inoculated in diluted 1:10 MRS containing malic acid, ethanol and pH 3.5. In the forth step, 100 ml sterilized Nebbiolo wine were inoculated with the *O. oeni* which grew in the previous steps and incubated at 20 °C. This wine had 2.5 g/L malic acid and 13% ethanol.

During the experiments, L-malic acid was determined by HPLC according to Cane [9].

2.3 Microvinification trials

The selected strains were transferred in 5 L of Nebbiolo wine (vintage 2006). They were used individually and in mix, a control trial was made using the commercial starter Alpha (Lallemand).

The wine composition was the following: L-malic acid 2.5 g/L, ethanol 13% vol., total SO_2 35.2 mg/L, free SO_2 8.9 mg/L, pH 3.47. MLF was conducted at the temperature of 20 °C. MLF was followed by HPLC.

2.4 Sensory analysis

Sensory evaluation was conducted by a panel of 15 tasters on the four wines: the control inoculated with the commercial starter; the wine inoculated with the strain 5007; the wine inoculated with the strain 5060 and the wine inoculated with the mix 5007+5060.

The wines were presented in transparent coded glass (ISO 3591) [10] at room temperature and they were evaluated using ranking tests arranging wines according to the following parameters: colour acceptability, odour acceptability, taste acceptability, wine total acceptability. Data were analyzed using the Quade test and multiple comparisons (p=95%). [11]

Descriptive analysis was performed on the following descriptors: ruby red and violet reflexes for the colour; acidity, bitterness, softness, structure, taste-olfactory persistence, for the taste and the tactile sensations; the wine total acceptability was also measured. The judges scored each attribute on a relative intensity scale (0-100). These results were submitted to the analysis of variance (ANOVA), and Duncan's test, performed using XLSTAT 7.5.3 (p=95%).

2.5 PFGE analysis

Intact genomic DNA was prepared following a modified version of the method described by Lucas et al. [12]. Plugs were washed once in 1X TE prior to incubation with 30 U of Apa I and Sfi I endonucleases (Sigma).

DNA fragments were resolved by pulsed-field electrophoresis in a CHEF DRIII apparatus (Bio-Rad) at 14 °C and 6 V/cm. Run protocols were described by Zapparoli et al. [13]. Gels were stained in ethidium bromide and visualized under UV lamp GelDoc 2000 (Bio-Rad). CHEF DNA marker Lambda Ladder (Bio-Rad) was used as molecular size standard.

3. Results and Discussion

3.1 Adaptation and strain selection

The work, presented schematically in figure 1, was conducted on 56 strains of *O. oeni* belonging to the collection of CRA-ENO, the first part of the work included four steps of adaptation of the bacteria in synthetic media with increasing stressful conditions.

20 strains were selected after the first step; after the second step were selected 8 strains which passed also the third step. At the end of fourth passage, the strains ISE 5007 and ISE 5060 were selected for their speed of growth and for malolactic fermentation capacity.

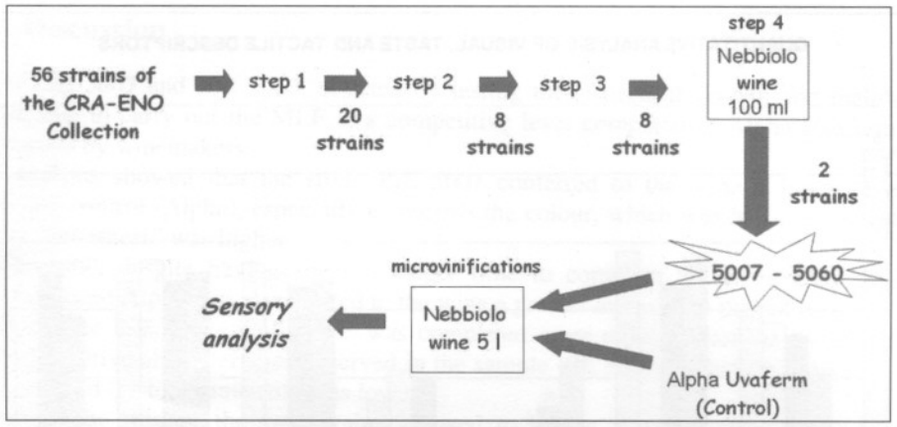

Fig. 1 Schematic representation of the results obtained during the adaptation.
In the step 1 the broth was MRS pH 4.8; in the step 2 MRS 1:5 with 10% ethanol, 3 g/L malic acid. pH 3.5; in the step 3 MRS 1:10 with 10% ethanol, 3 g/L malic acid, pH 3.5; in the step 4 Nebbiolo wine was used. Microvinifications trials were performed in 5L of Nebbiolo wine using the selected strains ISE 5007 and ISE 5060 individually and in mix; the widely used commercial starter Alpha Uvaferm was used as control. Sensory analysis was performed on the wines obtained at the end of MLF.

3.2 Microvinifications and sensory analysis

Malolactic fermentations in Nebbiolo wine lasted 3 months. The strain 5007 and the mix concluded the fermentation more quickly than Alpha, while the strain 5060 took a longer time.

Regarding the ranking test (figure 2), significant differences for the acceptability of the colour were found, in particular, the wine fermented with the strain 5007 appeared to be significantly less pleasant than the others. The sample 5060 resulted to have a more pleasant odour, but it was not statistically different from 5007+5060 and Alpha.

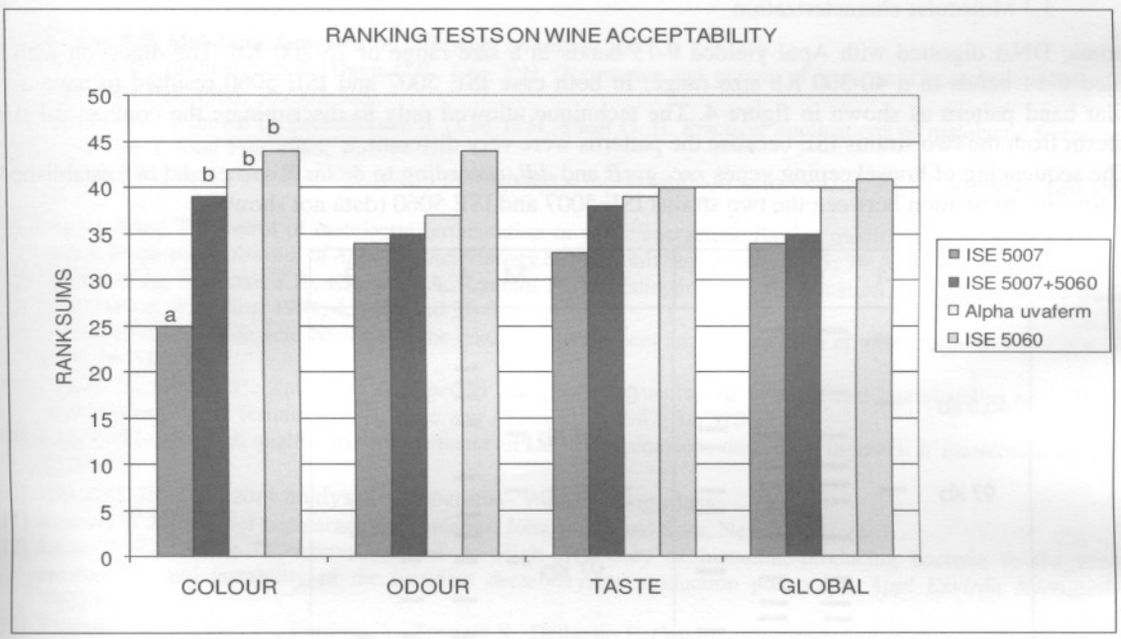

Fig. 2 Ranking test for the evaluation of the colour, odour, taste and global acceptability.
The higher is the rank sum and the higher is the acceptability of considered parameter.
Different letters indicate significant differences with Quade test ($p < 0,05$) and multiple comparison.

The quantitative analysis of the visual, gustative and tactile descriptors showed significant differences ($p < 0.05$) for the intensity of the ruby red colour and of the bitter taste (figure 3). In particular, the descriptor "ruby red" was significantly less intense in the wine with ISE 5007 than in the samples ISE 5007+5060 and ISE 5060. This could explain the lower pleasantness of the colour obtained with the ranking test. The same wine ISE 5007 was significantly more bitter than Alpha Uvaferm and ISE 5060. For no other measured descriptors significant differences were found among the wines.

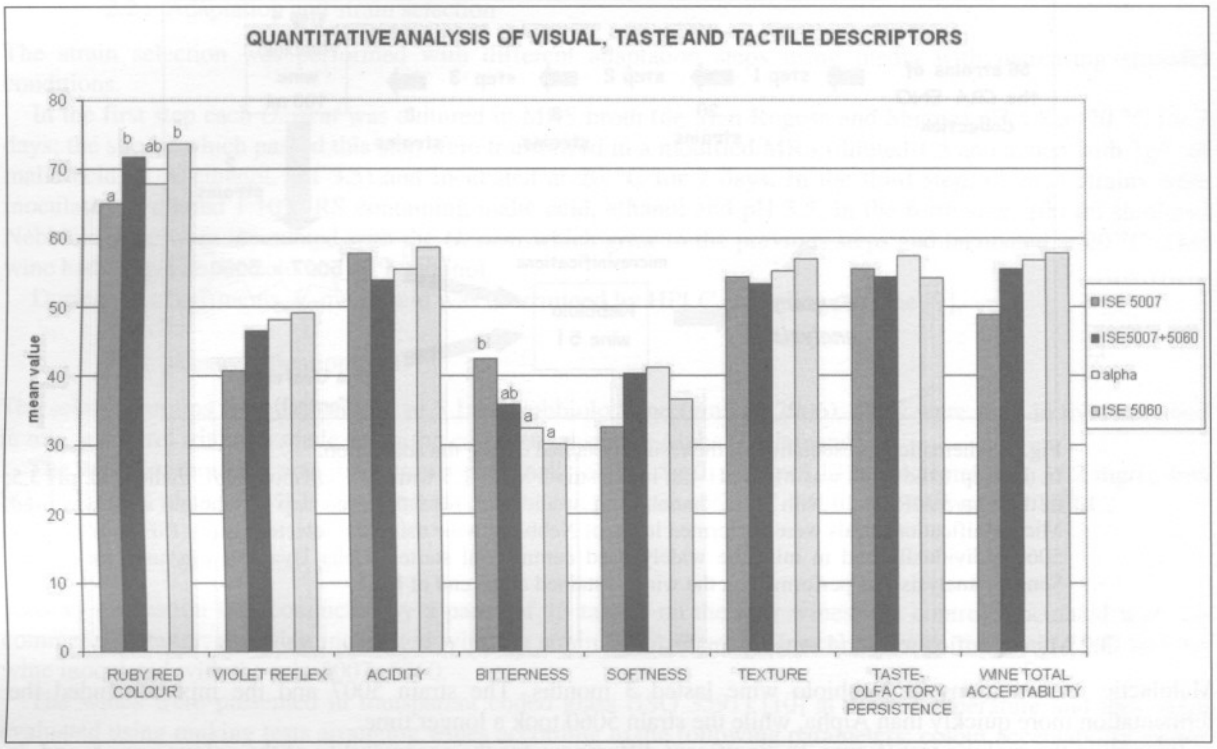

Fig. 3 Average values of the descriptors of colour, taste and global assessment.
Different letters indicate significant differences with ANOVA and Duncan test ($p < 0.05$).

3.3 Molecular characterization

Genomic DNA digested with ApaI yielded 9-15 bands in a size range of 25-200 Kb. The digestion with SfiI yielded 6-14 bands in a 40-300 Kb size range. In both case ISE 5007 and ISE 5060 resulted to have a very similar band pattern as shown in figure 4. The technique allowed only to discriminate the commercial Alpha Uvaferm from the two strains ISE because the patterns were very different.

The sequencing of housekeeping genes *rec, gyrB* and *ddl*, according to de las Rivas et al. [14], established an effective discrimination between the two strains ISE 5007 and ISE 5060 (data not shown).

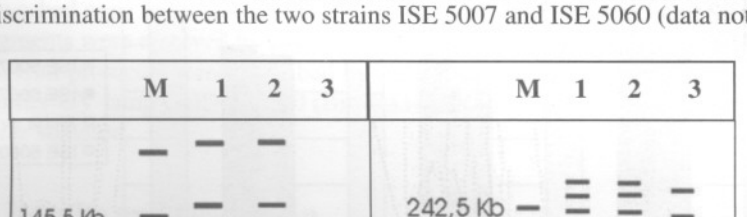

Fig. 4 PFGE patterns obtained after *Oenococcus oeni* DNA digestion with the enzymes ApaI and SfiI.
M: molecular maker λ ladder (Bio-Rad), lane 1: ISE 5007, lane 2: ISE 5060, lane 3: Apha Uvaferm.

4. Discussion

The *O. oeni* ISE 5007 and ISE 5060, selected by testing their speed of growth and malolactic fermentation capacity, are able to carry out the MLF at a competitive level compared to Alpha Uvaferm, one of the most employed starters by winemakers.

Sensory analysis showed that the strain ISE 5007 conferred to the wine a lower level of pleasantness compared to the control (Alpha), especially as regards the colour, which was less intense, and the taste, where the descriptor "bitterness" was higher.

Strain ISE 5060, despite having spent a longer time to complete the fermentation, gave no particular differences compared Alpha, but it conferred to the wine a greater intensity to the red ruby colour.

With the mixture ISE 5007+5060, MLF was completed more quickly than the commercial strain, without presenting the negative characteristics observed in the sample ISE 5007: in fact the colour acceptance was very similar to Alpha and the bitter intensity was lower.

In conclusion the mix of the two strains allowed to obtain a wine with a quality comparable to the commercial strain.

Moreover the study showed that the adaptation of bacterial strains using media gradually poorer in nutrients, avoid the typical problem of industrial strains, which presented problem of growth in a difficult environment such as wine.

This research allowed us to acquire knowledge aimed at achieving a technological innovation and improvements in wine making, to develop and extend a new process which can be realized in a service for businesses. In fact the ability to select indigenous bacterial strains for MLF can be a useful tools to increase the value of the link between wine and its territory, while protecting the typicality of DOC and DOCG.

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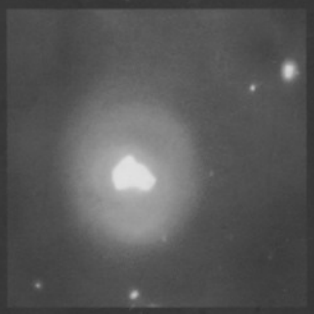

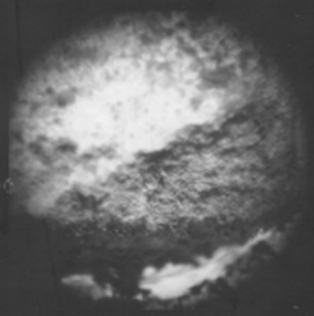

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