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Selection of lactic bacteria to induce malolactic fermentation in red wine of cv. Cencibel

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Summary

This paper describes the procedure for the selection of three lactic acid bacteria strains from 40 indigenous strains isolated in two cellars of the Spanish region of Castilla-La-Mancha.

The isolates were identified by classical microbiological and molecular techniques: Pulsed-Field Gel Electrophoresis (PFGE) of bacterial chromosome fragments obtained from restriction enzymes.

Selection was carried out considering the capacity to perform malolactic fermentation (MLF) in wine and the resistance to high alcoholic percentages and low pH levels. Three isolates (two *Lactobacillus plantarum* and one *Oenococcus oeni*) which were able to pass the above mentioned tests were selected. These isolates produced low volatile acidity, showed a moderate resistance to SO_2 and did not cause any degradation of residual sugars.

K e y w o r d s : Lactic bacteria, red wine, selection.

Introduction

In many wines, particularly red wines, after alcoholic fermentation, lactic acid bacteria perform malolactic fermentation (MLF). L-malic acid is converted into L-lactic acid and this leads to a decrease in wine acidity. The metabolism of lactic bacteria involves a significant number of compounds, many of them with relevant effects on taste and smell (De Revel *et al.* 1999). Moreover, this process contributes to wine stabilization (Davis *et al.* 1988). Wine lactic acid bacteria belong to three genera, *Lactobacillus*, *Pedicoccus* and *Oenococcus*.

Wine is a hostile medium for the growth of lactic bacteria; their developmental potential depends on the alcoholic percentage of pH, SO_2 and temperature as well as on other growth-related factors such as carbon and nitrogen sources and vitamins. Several research studies have shown that the resistance of lactic bacteria to wine constituents also depends on the bacterial strain involved (Britz and Tracey 1990, Izquierdo *et al.* 2003).

Molecular methods are used to classify strains. One of the most reproducible techniques, which also provides profiles quite easy to analyze, is total DNA restriction, as well as fragment separation by Pulsed-Field Gel Electrophoresis (PFGE) (Daniel *et al.* 1993).

From the first stages of winemaking to the final stage of the MLF process, several genera and species of lactic bacteria are developed (Izquierdo *et al.* 2000). However, not all of them are capable of degrading malic acid into lactic acid in an optimal way. Spontaneous MLF implies several risks, such as a considerable increase in volatile acidity, consumption of residual sugars and formation of undesirable metabolites leading to low quality wines, which are less standardized and of limited commercial value.

Commercial strains directly inoculated into wine improved significantly the process to control MLF (Nielsen *et al.* 1996). The use of lactic bacteria strains selected from the indigenous flora of each region as starters is an alternative to commercial strains (Masqué and Bordons 1996). This approach is based on the assumption that an improved adjustment of the strains to a given ecological environment might improve the quality of wine and maintain the typical regional peculiarities.

In this paper, we present results of a lactic acid bacteria selection procedure applied to 40 isolates obtained from two cellars of the Spanish winemaking region of Castilla-La-Mancha.

Material and Methods

I s o l a t i o n : In 2001, during harvest, a series of lactic bacteria isolates were obtained from red wine (cv. Cencibel) after MLF in two wine cellars located in the Designation of Origin (DO) La Mancha.

Decimal dilutions of the Ringer serum were inoculated into the surface of modified MRS Agar in Petri plates (4 $g \cdot l^{-1}$ malic acid, 10 $g \cdot l^{-1}$ fructose and 3 % of tomato juice). The plates were incubated at 30 °C for one week. After incubation colonies presenting different morphology on the plate were randomly selected and, in consecutive inoculations, pure cultures were obtained.

I d e n t i f i c a t i o n : After several identification tests such as microscopic observation of cell morphology, Gram tincture, catalase activity, and CO_2 production from glucose (heterofermentative), each isolate was proven to belong to the lactic bacteria group, the last test being the one that allowed us to link the isolates into their respective genus groups.

The technique used to link the isolates to their respective strain groups was Pulsed-Field Gel Electrophoresis (PFGE). The bacterial chromosomal fragments obtained af-

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ter digestion with the restriction enzyme are separated by PFGE (GINDREAU *et al.* 1997). For the isolates of the *Lactobacillus* and *Oenococcus* genus groups, the Sfi I y Apa I enzymes (Biolabs, England), respectively, were used.

Determination of the strain species based on the criteria established by the BERGEY'S MANUAL OF SYSTEMATIC BACTERIOLOGY (1986), using the API 50 CH gallery as a simplified technique to identify species of lactic bacteria strains (HOFER 1976). The gallery consists of 50 micro-tubes with an anaerobiotic area (the tube portion) in order to perform fermentation studies, and an aerobiotic area (cupola portion) for oxidation and assimilation studies. Each micro-tube contained a dehydrated substrate of the carbohydrate family and its derivatives (heterosides, polyalcohols, and uronic acids) as indicated by the manufacturer (Biomerieux).

Selection: The tests were carried out using 100 ml of wine. The strain inoculation process was performed using a young modified MRS agar culture, which was subsequently sown in a modified MRS culture medium, which was then incubated at 28 °C for 3 d. After incubation, 3 ml of the culture were centrifuged at 3,500 rpm for 20 min. Then, the pellet was resuspended in a 10 ml Ringer solution and the centrifugation process was repeated at 3,500 rpm for another 10 min. The initial population colony sown for each strain was determined by counting all the viable cells on Petri plates; values ranging from 1.00×10^7 to 1.00×10^8 cfu·ml^{-1} (colony forming units per ml) were obtained. The pellet obtained in this process was added to 100 ml of wine and incubated at 22 °C for 10 d.

The evolution of L-malic and L-lactic acid was enzymatically determined after 3, 7, and 10 d. Also, on day 10 the formed lactic acid was analysed, as well as volatile acidity, pH, and total acidity. The isolate selection process based on the following criteria:

Ability to perform the MLF in wine: Wine of cv. Cencibel was used in the experimental cellar of the IVICAM. The physico-chemical parameters of this wine were: 12.3 % v/v alcohol; 4.30 g·l^{-1} total acidity in tartaric acid; pH 3.70; 0.30 g·l^{-1} volatile acidity in acetic acid; 60 mg·l^{-1} of total sulfurous dioxide and 2.038 and 0.650 g·l^{-1} malic acid and lactic acid, respectively.

Resistance to alcohol: The amount of alcohol in the wine used as a basis in the last stage was raised to 13.5 % v/v, which is a high value for most lactic bacteria. The resistance to alcohol was determined, and values of 12.3 and 13.5 % v/v were obtained.

Resistance to low pH level: By addition of phosphoric acid the pH level of the wine was modified. Then, the lactic bacteria resistance was determined at pH levels of: 3.7, 3.5, and 3.3.

Results and Discussion

Isolation and identification: During the first stage of isolation, 40 lactic bacterial isolates were obtained, 15 (8 *Lactobacillus* and 7 *Oenococcus*) from the so-called cellar A and 25 (19 *Lactobacillus* and 6 *Oenococcus*) from cellar B. The Table shows the results obtained after grouping the isolates according to their respective genera.

T a b l e

Identification of the selected strains species

Strain	4	5	35
Glycerol	-	-	-
Erytritol	-	-	-
D-Arabinose	-	-	-
L-Arabinose	+	+	+/-
Ribose	+	+	+
D-Xylose	-	+	-
L-Xylose	-	-	-
Adonytol	-	-	-
β-metil D-Xyloside	-	-	-
Galactose	+	+	+
D-Glucose	+	+	+
D-Fructose	+	+	+
D-Mannose	+	+	+
L-Sorbose	-	-	-
Rhamnose	-	-	-
Dulcytol	-	-	-
Inosytol	-	-	-
Manytol	+	-	+
Sorbytol	-	-	-
α-metil-D-Mannoside	-	-	-
α-metil-D-Glucoside	-	-	-
N-Acetil Glucosamine	+	+	+
Amigdaline	+	+	+
Arbutine	+	+	+
Esculine	+/-	+	-
Salicine	+	+	+
Celobiose	+	+	+
Maltose	+	+	+
Lactose	-	-	-
Melibiose	+	-	+
Sacarose	+	-	+
Trehalose	+	+	+
Inuline	-	-	-
Melyzitose	-	-	-
D-Rafinose	-	-	-
Starch	-	-	-
Glycogen	-	-	-
Xylitol	-	-	-
Gentiobiose	+	-	+
D-Turanose	-	-	-
D-Lixose	-	+	-
D-Tagatose	-	-	-
D-Fucose	-	-	-
L-Fucose	-	-	-
D-Arabytol	+/-	-	+/-
L-Arabytol	-	-	-
Gluconate	+/-	-	+/-
2-ceto gluconate	-	-	-
5-ceto gluconate	-	-	-
Identification	*Lactobacillus plantarum*	*Oenococcus oeni*	*Lactobacillus plantarum*

+ positive result; - negative result; +/- undecided result

Under microscope, *Lactobacillus* isolates had a bacterial shape, while *Oenococcus* isolates had a coconut shape. Moreover, *Oenococcus* isolates produced CO_2 from glucose (heterofermentative), while *Lactobacillus* isolates did not (homofermentative).

After applying the PFGE technique to each isolates, isolates from cellar A were divided into 6 different profile groups, and those from cellar B were divided in 11 different profile groups. Fig. 1 shows the percentage of each strain, as well as their genera. Note that strains such as the 4th and 5th strain from cellar A and the 18th and 35th strain from cellar B, are more isolated than the others. This suggests a higher level of implantation of these strains in the two cellars. Furthermore, it can be obeserved that no strain seems to be simultaneously present in both cellars.

Ability to perform the MLF in wine: Fig. 2 shows the percentage of L-malic acid for each strain after 7 d of degradation. Out of the 17 strains studied, only 12 strains were selected; those that had degraded less than 50 % of malic acid after 7 d were rejected. Eight strains completed MLF after 7 d with a degradation of malic acid >80 %; in some cases, even an almost complete degradation of malic acid was observed. When chemical analyses were carried out after 10 d no excessive increase in volatile acidity was observed for any strain (data not shown).

Resistance to alcohol: Alcohol can be a limiting factor for lactic bacteria growth, as well as for the onset of MLF (Carbó *et al*. 1998 a). The higher amount of alcohol, the slower MLF in all the studied strains. Fig. 3 shows the percentage of malic acid degraded after 7 d in wines with 12.3 and 13.5 % alcohol (v/v), respectively. For all the strains, a lower content of degraded malic acid was observed after 7 d if the amount of alcohol increased. However, this process was much slower in the 10th, 14th, 18th, 26th and 27th strain. In the case of the 26th and 27th strain, the percentage of degraded malic acid was very low: approximately 10 %. Therefore, only 7 out of the 12 initial strains were selected.

Resistance to low pH levels: Although a similar tendency was observed for all strains with decreasing pH, the behaviour of lactic bacteria isolates varied. In most of the strains degraded malic acid decreased significantly when the pH level of wine decreased from 3.7 to 3.5, and in some cases, even prevented MLF at these pH levels. Fig. 4 shows the percentage of degraded malic acid after 7 d

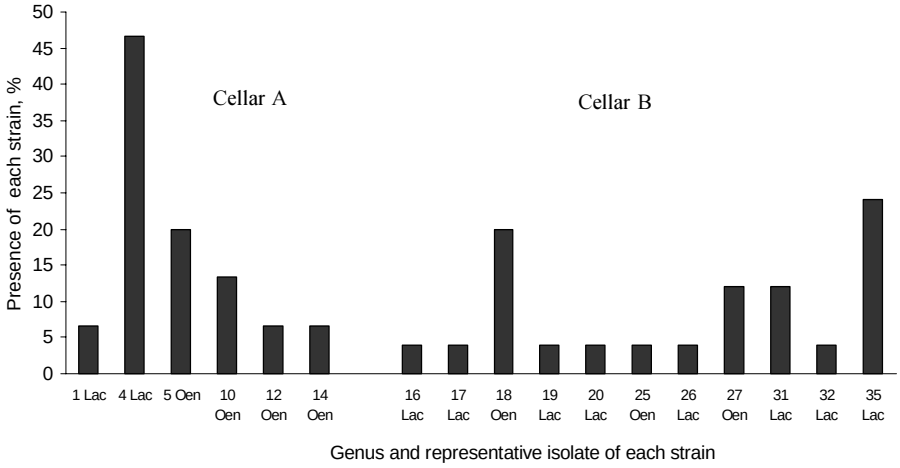

Fig. 1: Genera and percentage of appearance of each strain in the two studied cellars.

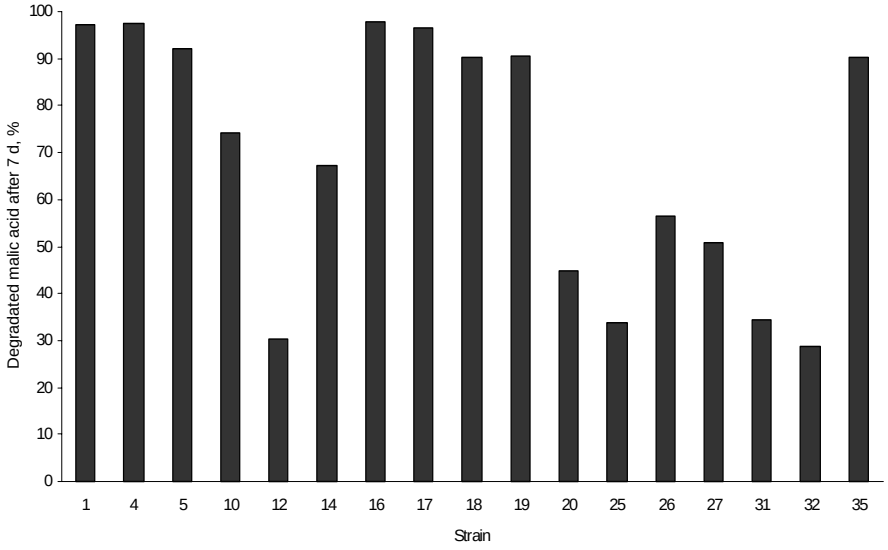

Fig. 2: Ability to perform MLF in wine (pH = 3.7; alcohol = 12.3 % v/v; total SO_2 = 50 mg·l^{-1}).

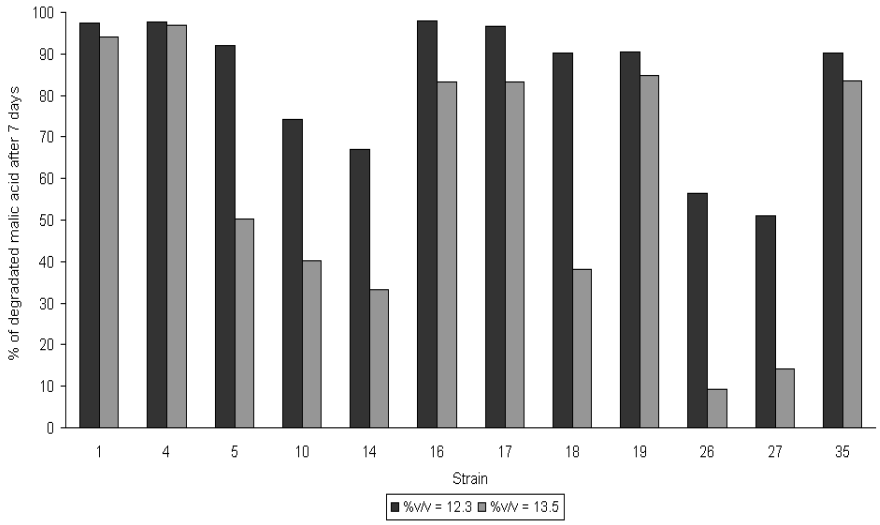

Fig. 3: Ability to perform MLF in wine with different amounts of alcohol.

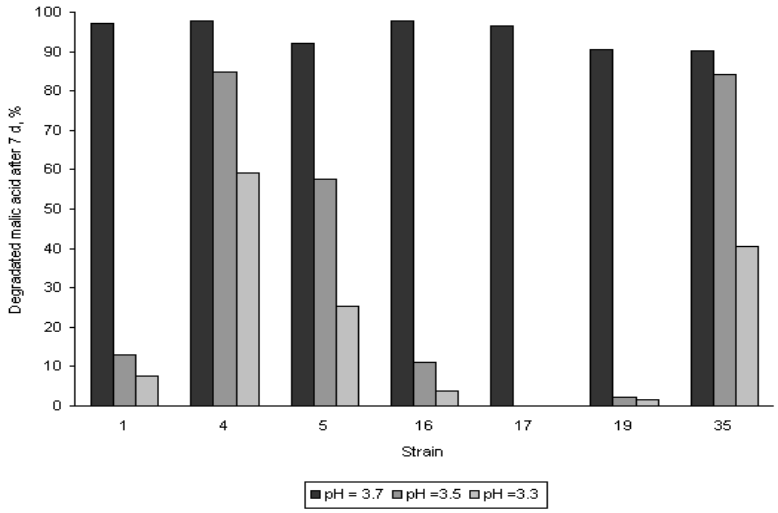

Fig. 4: Ability to perform the MLF in wine at different pH levels.

for each strain in wines with a pH level of 3.7, 3.5 and 3.3, respectively. At pH 3.3, only a few isolates gave satisfactory values in terms of malic acid degradation. Therefore, only three of the 7 initial strains were selected.

S e l e c t e d s t r a i n s : Only three strains, the so-called 4th, 5th, and 35th strains,were selected. The first two had been isolated in cellar A, and the third one in cellar B.

The Table shows the results obtained after determining the genus and species of each strain using API 50 CH test galleries. Fig. 5 shows the PFGE profile of the three selected strains.

Bacterial viability was significantly affected by the presence of the total sulfurous dioxide in wine, higher concentrations of sulfur, leading to higher rates of dead bacteria. While the highest viability occurred at 70 $mg \cdot l^{-1}$ SO_2 the lowest was found at 100 and 120 $mg \cdot l^{-1}$ (CARBÓ *et al*. 1998 b).

The three isolates showed a similar tendency, increasing sulfur content in wine always leading to a decrease in the quantity of degraded malic acid after 7 d (Fig. 6).

Usually, MLF is related to an increase in volatile acidity due to a slight degradation of residual sugars and of citric acid present in wine. No excessive increase in volatile acid-

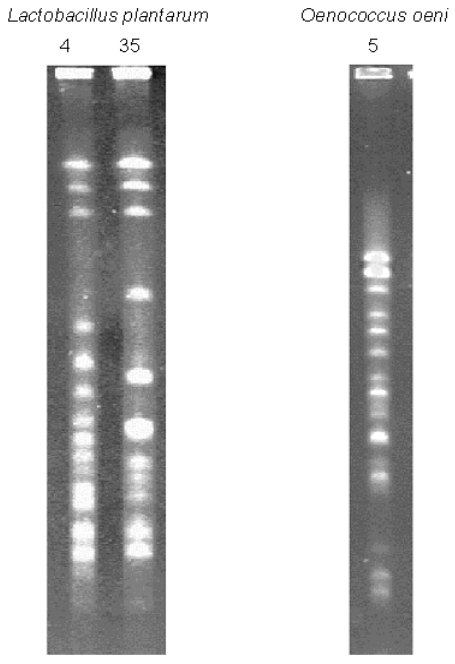

Fig. 5: PFGE profiles of the three selected strains.

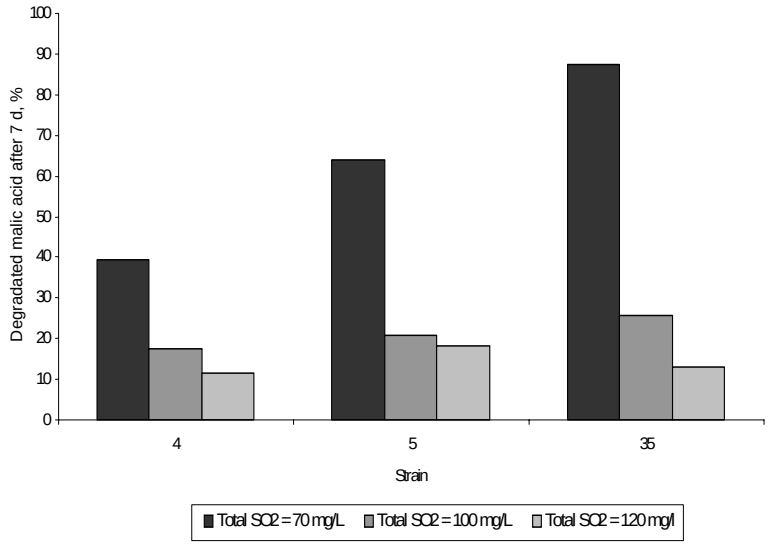

Fig. 6: Ability to perform the MLF in wine with different content in total SO_2.

ity was observed for any of the selected strains in wines after MLF.

As far as sugar degradation is concerned, no significant decrease in the level of residual sugars were observed when comparing the initial values with those obtained in the final stage of MLF.

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