Bacterial Inoculation Strategies for the Achievement of Malolactic Fermentation in High-alcohol Wines

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The purpose of this work was to study the induction of malolactic fermentation (MLF) in a wine that does not often support malic acid deacidification because of its high alcohol content. Amarone wine, known for its high alcohol content, served as a model. Simultaneous and sequential alcoholic and malolactic fermentation (AF/MLF) were conducted by direct inoculation of bacteria, which resulted in successful MLF in wines containing approximately 16% (v/v) alcohol. At higher ethanol contents, stuck MLF occurred because of growth inhibition. To overcome this technological problem, the performance of bacteria was tested in wine containing approximately 17% (v/v) ethanol using a starter preparation consisting of cells acclimatised in a wine-water solution (1:1) for 24 h and 48 h respectively. Total L-malic acid depletion was recorded when the bacterial cells that had been acclimatised for 48 h were inoculated simultaneously with yeast to conduct AF. The method by which the bacterial cultures are prepared and the time of inoculation affects the efficacy of MLF in high-alcohol wines. The inoculation of yeasts with acclimatised bacteria before AF seems to be a valid strategy to obtain complete MLF in high-alcohol wines.

Malolactic fermentation (MLF) in wine is characterised by the conversion of L-malic acid to L-lactic acid and carbon dioxide, and subsequent changes in aroma and taste profiles. This secondary fermentation is usually conducted by *Oenococcus oeni* (Lonvaud-Funel, 1999). MLF may occur spontaneously or may be induced by the inoculation of selected bacterial cultures that are commercially available as pure freeze-dried cultures. These strains tolerate high alcohol (> 13.0%, v/v) and low pH (< 3.20), traits which allow them to grow and survive in harsh environments such as wine. These two parameters, in combination with the presence of SO_2, low temperature and scarce nutrients, contribute to diminish the success of biological deacidification (Ribéreau-Gayon *et al*., 2006). The ethanol content in wine plays a critical role in MLF, as it disrupts membrane structures and affects many membrane-associated processes, including those involved in stress resistance and malolactic activity (Da Silveira *et al*., 2003, Chu-Ky *et al*., 2005).

The vinification of grape musts with a high sugar concentration (> 250 g/L) leads to potential high-alcohol wines, and this may cause problems with the induction of MLF. The inoculation of high-alcohol wines with starter cultures causes a substantial loss in viability prior to the adaptation of the cells to their "new" environment. This may cause sluggish or stuck MLF. Simultaneous AF/MLF offers the advantage of inducing MLF into such wines without having a negative impact on the quality of the wine and before the high alcohol content becomes inhibitory (Sieczkowski, 2004; Krieger, 2005; Murat *et al.,* 2007). Some authors are of the opinion that simultaneous inoculation interrupts AF (Alexandre *et al*., 2004). However, previous studies have shown that inoculation of grape juices with *O. oeni* together with yeast did not slow down or stop AF (Semon *et al*., 2001; Rosi *et al*., 2003). Jussier *et al.* (2006) described a method whereby they inoculated yeast and bacteria simultaneously in a low-pH white must and produced high levels of alcohol. Simultaneous inoculation led to a considerable reduction in the duration of MLF, because AF allowed sufficient time for the bacteria to become acclimatised to the increasing alcohol concentration before it reached toxic levels.

Due to the fact that spontaneous or induced MLF is often difficult to achieve in wine containing ethanol levels exceeding 15% (v/v), the development of strategies to favour the biological deacidification of high-alcohol wines is necessary to prevent sluggish or stuck MLF.

This study reports on the microvinification of simultaneous and sequential AF/MLF to evaluate MLF in high-alcohol wines such as Amarone. The performance of different bacterial starter preparations, bacteria for use in direct inoculation protocols and bacteria acclimated in wine before inoculation was evaluated. The viability of the cells and L-malic acid depletion were monitored during the fermentation of three different grape musts. The production of acetic and other organic acids was monitored.

MATERIALS AND METHODS

Strains

Oenococcus oeni strain VP41 (Lallemand Inc, Montréal, Canada) was used as the MLF starter culture because it possesses a high tolerance to ethanol. *Saccharomyces cerevisiae* strain VRB (Lallemand) was used to induce alcoholic fermentation. The compatibility of the two strains was verified in previous experiments (data not shown).

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Microvinifications

Three different microvinifications were conducted with partially dried grapes of the Corvina and Rondinella varieties, which are used in the production of Amarone wine. A different batch of grapes was used for each of the microvinifications. Each microvinification was prepared by crushing the grapes separately and, to obtain homogenous trials, without must clarification. The juice and solid fraction (grape pomace) were separated, mixed and subsequently divided into different fermentation volumes. The first microvinification (Must 1) was conducted with 40 L. Must 1 contained 254 g/L sugar. The second microvinification (Must 2) contained 267 g/L sugar and was divided into six volumes of 50 L each. The sugar concentration in two of these fermentation samples was corrected to 300 g/L, and in the other two samples to 320 g/L. This was done to increase the theoretical ethanol production to a minimum of 16 and 17% (v/v) respectively. These fermentations were designated low (L), medium (M) and high (H) in relation to their sugar content. The sugar additions mimicked the different contents achieved by grapes of different degree of drying normally used in Amarone winemaking. A control for spontaneous MLF was conducted separately by using must without sugar addition. The third vinification (Must 3) was conducted in 20 L volumes, with a sugar content to yield between 16.5 and 17.0% (v/v) alcohol after fermentation. The musts were sulphited by adding 50 mg/L SO_2 prior to yeast inoculation, and yeast additions were done according to the manufacturer's instructions.

Two different commercial freeze-dried preparations of the VP41 bacterial strain were used – the direct inoculation (MBR®, Lallemand) strain and the preparation (1-Step®, Lallemand) containing bacteria that require a short acclimatisation period. The bacteria for direct inoculation were used according to the manufacturer's instructions in the first two microvinifications. The third microvinification used bacteria which were acclimatised for 24 h (24-h cells) and 48 h (48-h cells). The lyophilised cells were resuspended in distilled water and incubated for 30 minutes at room temperature, and then an equal volume of wine (total acidity 6.00 g/L as tartaric acid, initial pH 3.38 adjusted to 3.50 with potassium bicarbonate, ethanol 14.5% (v/v), and residual sugar 11.25 g/L, total SO_2 of 45.0 mg/L and free SO_2 of 4.3 mg/L) was added. The wine-water solution with cell suspension was incubated at room temperature for 24 h and 48 h before the inoculation in must or wine.

To induce simultaneous AF/MLF, bacteria were inoculated 10 to 12 h after inoculation with yeast. To induce sequential AF/MLF, bacteria were inoculated at the end of alcoholic fermentation. Before every bacterial inoculation, the free SO_2 content was determined to be less than 10 mg/L. Commercial MLF nutrient (Optimaloplus, Lallemand) was added in each trial (simultaneous and sequential AF/MLF), according to the manufacturer's instructions.

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 3 g/L and L-malic acid was less than 0.1 g/L. The microvinifications were conducted without temperature control, and the temperature ranged between 15 and 20°C. Each microvinification was conducted at least in duplicate.

Microbiological analyses

Lactic acid bacteria (LAB) counts in the must and wine samples were enumerated on MRS media (Fluka, Seelze, Germany), to which was added 2% tomato juice broth (Difco, Detroit, MI) and 0.01% actidione (Fluka). The plates were incubated anaerobically at 28°C using an Anaerocult A kit (Merck, Darmstadt, Germany). After six days, colony counts were carried out and reported as colony-forming unit per mL (cfu/mL). LAB were monitored until the completion of AF and MLF. In the case of partial MLF, the determination of the LAB concentration ended when L-malic acid consumption ceased.

Analysis of must and wine

Musts were analysed at the time of crushing and wine samples were collected during and after AF and MLF. Ethanol was analysed by NIR spectroscopy using an Alcolyzer Wine apparatus (Anton Paar GmbH, Graz, Austria). Sugar content, total acidity and SO_2 were determined by titration according to standard analysis methods (Ough & Amerine, 1988). Yeast assimilable nitrogen (YAN) was determined by formol titration (Gump *et al*., 2000). Organic acids and acetaldehyde were quantified using enzyme kits (La Roche, Basel, Switzerland). The total soluble polyphenols in the wines were determined with Folin-Ciocalteu reagent by the Slinkard and Singleton methods (Slinkard & Singleton, 1977) and were expressed as mg of gallic acid per litre of wine through a calibration curve.

Must components are reported as a single value without standard deviation. Wine analyses are reported as the means of two determinations (one for each trial carried out at least in duplicate) ± standard deviation.

TABLE 1

Composition of the musts obtained from partially dried grapes utilised for the three microvinifications analysed before the inoculation of bacteria.

		Must 1	Must 2	Must 3
pH		3.24	3.31	3.29
Sugars	g/L	254	267	282
Total acidity †	g/L	7.75	7.70	7.83
L-malic acid	g/L	2.35	2.45	2.32
D-lactic acid	g/L	0.02	0.19	0.03
L-lactic acid	g/L	0.04	0.15	0.04
Acetic acid	g/L	0.05	0.14	0.05
Citric acid	g/L	0.29	0.41	0.39
Acetaldehyde	mg/L	4.3	2.5	1.6
Gluconic acid	g/L	0.35	0.57	0.57
Free SO_2	mg/L	<1.0	<1.0	<1.0
Total SO_2	mg/L	36	27	29
Total polyphenols§	mg/L	834	891	789
YAN	mg/L	258	261	330

†as tartaric acid
§as gallic acid

RESULTS

Must composition

The composition of the three musts used in this study is reported in Table 1. The gluconic acid content indicates that the musts were obtained from healthy grapes. The number of indigenous LAB in all musts, before the addition of SO_2, was found to be less than 3 000 cfu/mL.

Simultaneous and sequential AF/MLF

In Must 1, the kinetics of AF for all the trials were similar and the fermentation terminated after 16 days (data not shown). After AF, wines produced with simultaneous AF/MLF contained about 46% less L-malic acid than the other wines. As a consequence of L-malic acid depletion, pH, total acidity and L-lactic acid were affected (Table 2).

The density of bacteria in the wine produced by the simultaneous AF/MLF technique remained near 10^6 cfu/mL until the end of MLF. L-malic acid consumption began promptly a few days after the addition of bacteria and MLF terminated within 70 days (Figure 1).

In the wines inoculated after AF (sequential AF/MLF), the initial density of the bacteria decreased 10-fold, maintaining about 10^5 cfu/mL for several weeks, and then the cell population increased. In these wines, L-malic acid was completely converted to L-lactic acid within 112 days.

Spontaneous MLF did not occur in the non-inoculated wine (data not shown). Table 2 shows the composition of the wines after MLF.

TABLE 2

Composition of the wines after alcoholic fermentation (AF) and malolactic fermentation (MLF), produced with Must 1 by the induction of simultaneous or sequential AF/MLF.

		After AF		After MLF	
		Simultaneous	**Sequential**	**Simultaneous**	**Sequential**
Ethanol	% (v/v)	14.86 ± 0.02	14.81 ± 0.06	14.80 ± 0.05	14.78 ± 0.04
Residual sugars	g/L	2.30 ± 0.14	2.65 ± 0.35	2.28 ± 0.09	2.63 ± 0.17
pH		3.24 ± 0.00	3.19 ± 0.01	3.33 ± 0.01	3.34 ± 0.01
Total acidity†	g/L	7.30 ± 0.11	7.72 ± 0.03	6.48 ± 0.01	6.31 ± 0.02
L-malic acid	g/L	1.46 ± 0.02	2.29 ± 0.02	0.05 ± 0.03	0.10 ± 0.03
D-lactic acid	g/L	0.21 ± 0.00	0.19 ± 0.00	0.29 ± 0.01	0.27 ± 0.01
L-lactic acid	g/L	0.73 ± 0.01	0.02 ± 0.00	1.36 ± 0.02	1.45 ± 0.02
Acetic acid	g/L	0.14 ± 0.01	0.13 ± 0.00	0.19 ± 0.03	0.20 ± 0.01
Citric acid	g/L	0.28 ± 0.02	0.29 ± 0.02	0.27 ± 0.00	0.25 ± 0.01

† as tartaric acid

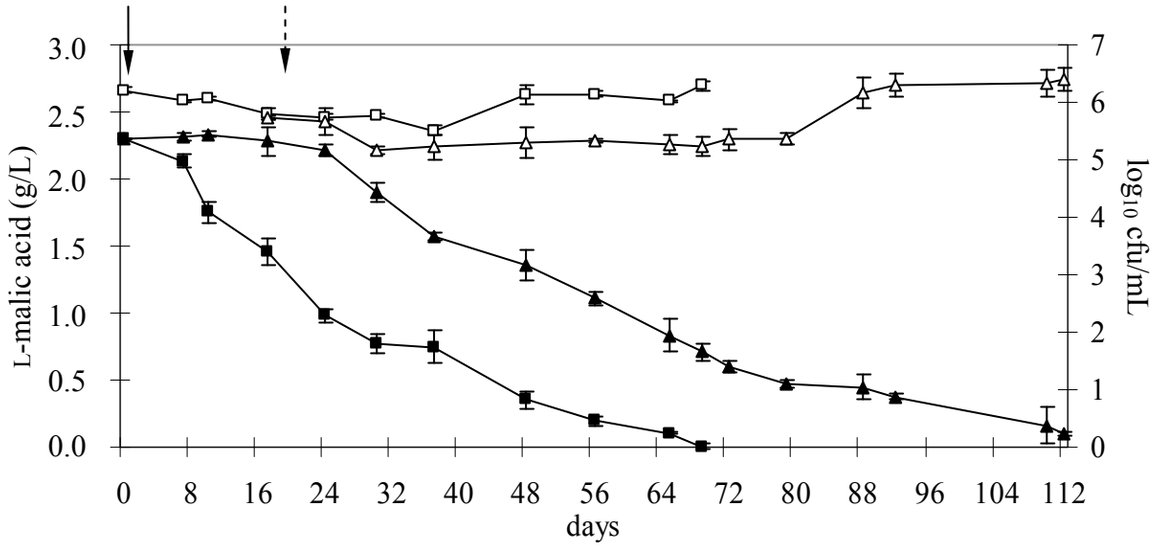

FIGURE 1

Lactic acid bacteria concentration (log_{10} cfu/mL, white symbols) and L-malic acid depletion (g/L, black symbols) determined in trials with simultaneous AF/MLF (squares) and with sequential AF/MLF (triangles). Arrows indicate the time of bacterial inoculation (fill, simultaneous AF/MLF; dotted, sequential AF/MLF).

Simultaneous AF/MLF in musts with high sugar content

In Must 2, the rate of sugar consumption and ethanol production was similar in all the trials and AF completed within three weeks, reaching the maximal expected ethanol content after 15, 17 and 21 days in L (low sugar), M (medium sugar) and H (high sugar) wines (Table 3). Acetic acid production increased in relation to ethanol content. In all the wines, the L-malic acid content was approximately 37% less than what was present in the initial grape Must 2 (Table 3).

At the end of AF, the bacterial cell viability decreased differently in all the trials, as shown in Fig. 2. Indigenous bacteria did not grow in the control wine (data not shown). The viable cell differences between wines of increasing alcohol levels could be due to the toxic effect of ethanol in spite of favourable temperatures (18 to 20°C) and the absence of free SO_2 (< 1 mg/L). After fermentation, the M and H wines experienced a slow and constant decline in the bacteria population, while cell viability was maintained in the L wine, and even increased by about 1 $\log_{10}$ cfu/mL after a transitory decline.

L-malic acid was completely depleted in the L wine, while some remained in the other wines. In the L wine, the fastest L-malic acid depletion was observed during AF. Table 3 shows the composition of the wines after MLF.

TABLE 3

Composition of wines after the alcoholic (AF) and malolactic fermentation (MLF) produced with grape Must 2, containing low (L), medium (M) and high (H) sugar content, by the induction of simultaneously AF/MLF.

		After AF			After MLF		
		L	**M**	**H**	**L§**	**M‡**	**H‡**
Ethanol	% (v/v)	15.72 ± 0.16	16.92 ± 0.10	17.84 ± 0.98	15.68 ± 0.10	16.89 ± 0.14	17.80 ± 0.63
Residual sugars	g/L	2.71 ± 0.25	2.97 ± 0.11	2.75 ± 0.32	2.65 ± 0.17	2.94 ± 0.13	2.70 ± 0.12
pH		3.23 ± 0.02	3.27 ± 0.01	3.27 ± 0.01	3.40 ± 0.02	3.31 ± 0.01	3.31 ± 0.02
Total acidity†	g/L	7.42 ± 0.25	7.30 ± 0.12	7.44 ± 0.08	6.38 ± 0.02	6.44 ± 0.08	6.46 ± 0.03
L-malic acid	g/L	1.51 ± 0.16	1.55 ± 0.04	1.56 ± 0.15	0.09 ± 0.03	1.29 ± 0.03	1.37 ± 0.02
D-lactic acid	g/L	0.24 ± 0.01	0.22 ± 0.01	0.23 ± 0.02	0.21 ± 0.01	0.22 ± 0.00	0.21 ± 0.02
L-lactic acid	g/L	0.36 ± 0.02	0.34 ± 0.05	0.32 ± 0.01	1.23 ± 0.02	0.42 ± 0.00	0.33 ± 0.01
Acetic acid	g/L	0.26 ± 0.02	0.41 ± 0.03	0.51 ± 0.04	0.31 ± 0.01	0.40 ± 0.02	0.52 ± 0.03
Citric acid	g/L	0.41 ± 0.01	0.40 ± 0.01	0.39 ± 0.01	0.32 ± 0.01	0.39 ± 0.01	0.38 ± 0.01
Acetaldehyde	mg/L	6.0 ± 0.3	6.0 ± 0.2	5.9 ± 0.1	5.7 ± 0.4	6.0 ± 0.3	6.0 ± 0.1
Gluconic acid	g/L	0.56 ± 0.00	0.57 ± 0.02	0.55 ± 0.03	0.51 ± 0.02	0.53 ± 0.04	0.52 ± 0.02

§ completed MLF
‡ partial MLF
† as tartaric acid

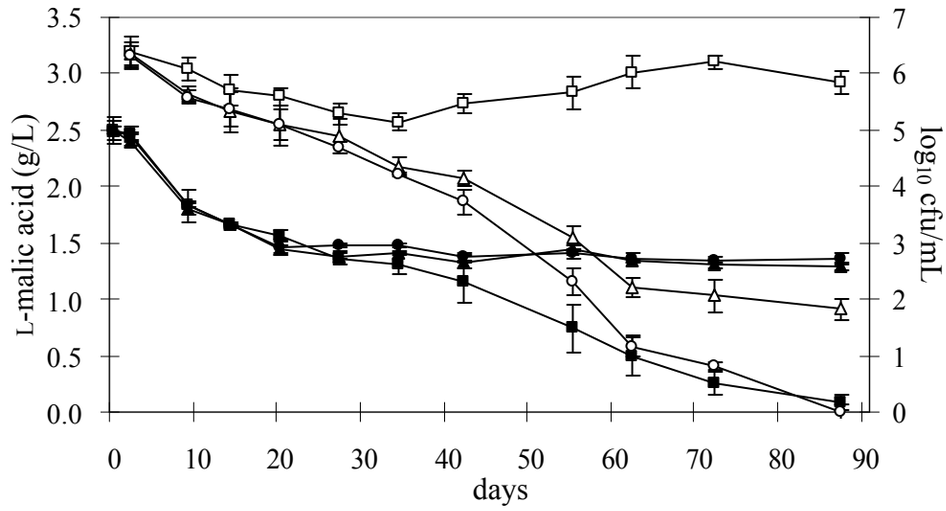

FIGURE 2

Lactic acid bacteria concentration ($\log_{10}$ cfu/mL, white symbols) and L-malic acid depletion (g/L, black symbols) determined in trials prepared with must containing low (square), medium (triangle) and high (circle) sugar content.

Simultaneous and sequential AF/MLF using acclimatised bacteria

Since the results from the second microvinification indicated that the inoculation of bacteria in must with a theoretical ethanol content of over 16% (v/v) failed to complete L-malic acid depletion, a different strategy to successfully induce MLF under high-alcohol conditions was investigated.

In this third microvinification, with Must 3, the VP41 strain was utilised in a different manner from its use in the two previous microvinifications. Before inoculation of *O. oeni* in the must or wine, the cells were acclimatised following a specific protocol (see Materials and Methods). After the addition of approximately the same volume of wine to the rehydration solution, the cell concentration diminished by less than half (4.8 x 10^8 cfu/mL). This level remained unchanged after 4 h of incubation in the acclimation wine, while it increased to 7.7 x 10^8 cfu/mL after 24 h and maintained a similar concentration after 48 h.

Simultaneous AF/MLF was induced in the must containing sugar for the production of wine with ethanol above 16% (v/v), using bacteria acclimated for 24 h (24-h cells) and 48 h (48-h cells). These two preparations yielded different results. As shown in Figure 3, the 48-h cells were able to complete MLF. More than 80% of the L-malic acid present in the must was consumed during

TABLE 4

Composition of wines after alcoholic fermentation (AF) and malolactic fermentation (MLF), produced with grape Must 3 with simultaneous AF/MLF by inoculation of acclimatised bacteria for 24 h (24-h cells) or 48 h (48-h cells).

		After AF		After MLF	
		24-h cells	**48-h cells**	**24-h cells‡**	**48-h cells§**
Ethanol	% (v/v)	16.74 ± 0.12	16.71 ± 0.09	16.72 ± 0.16	16.68 ± 0.17
Residual sugars	g/L	2.56 ± 0.43	2.60 ± 0.27	2.53 ± 0.21	2.57 ± 0.32
pH		3.20 ± 0.00	3.26 ± 0.01	3.23 ± 0.00	3.28 ± 0.00
Total acidity†	g/L	7.49 ± 0.04	6.73 ± 0.03	7.42 ± 0.04	6.64 ± 0.01
L-malic acid	g/L	1.76 ± 0.05	0.33 ± 0.02	1.69 ± 0.06	0.15 ± 0.01
D-lactic acid	g/L	0.18 ± 0.00	0.18 ± 0.00	0.19 ± 0.01	0.22 ± 0.00
L-lactic acid	g/L	0.17 ± 0.05	0.92 ± 0.30	0.26 ± 0.05	1.23 ± 0.03
Acetic acid	g/L	0.29 ± 0.00	0.31 ± 0.04	0.32 ± 0.00	0.37 ± 0.00
Citric acid	g/L	0.40 ± 0.02	0.38 ± 0.02	0.41 ± 0.02	0.33 ± 0.01
Acetaldehyde	mg/L	16.2 ± 0.3	16.3 ± 1.4	15.4 ± 0.8	14.4 ± 1.3
Gluconic acid	g/L	0.52 ± 0.03	0.53 ± 0.01	0.54 ± 0.01	0.53 ± 0.01

§ completed MLF
‡ partial MLF
† as tartaric acid

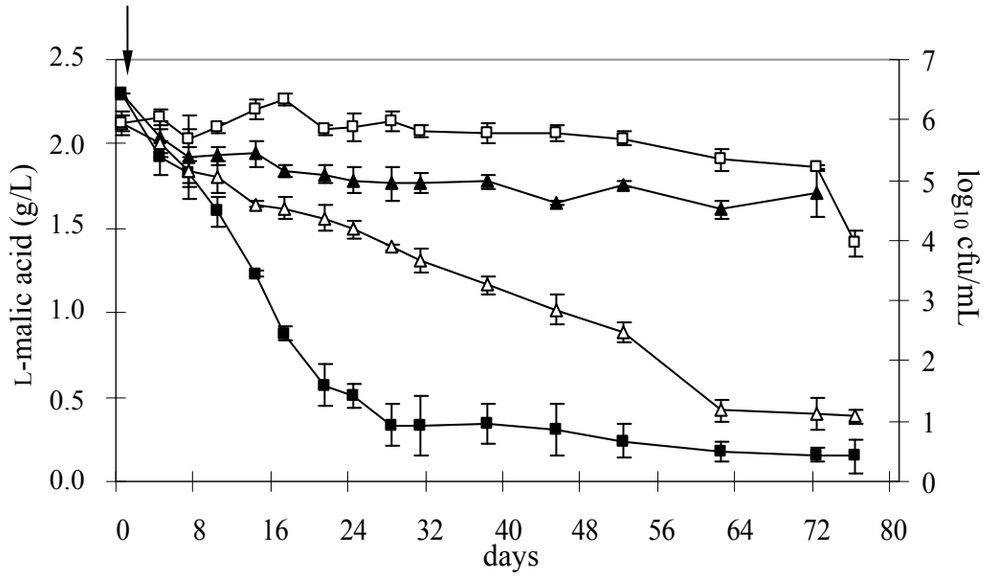

FIGURE 3

Lactic acid bacteria concentration (log_{10} cfu/mL, white symbols) and L-malic acid depletion (g/L, black symbols) determined in trials inoculated with 24-h cells (triangle) and 48-h cells (square) before AF (simultaneous AF/MLF). Arrow indicates the inoculation with bacteria.

AF by 48-h cells. In the 24-h wine, only 24% of L-malic acid of the must had disappeared at the end of AF (Table 4). Cells acclimated for 24 h underwent a rapid mortality that caused the cessation of MLF (Fig. 3).

Sequential AF/MLF was induced in wine containing 16.7% (v/v) ethanol, obtained by the fermentation of the same must utilised for simultaneous AF/MLF. The inoculation of 24 h- and 48 h-acclimatised bacteria was carried out after drawing the wine off. In both cases, MLF failed because of cell mortality that reduced the populations by 2 to 3 $\log_{10}$ cfu/mL within two weeks (data not shown).

DISCUSSION

The practice of yeast-bacteria co-inoculation is often criticised 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 acid production, causing a reduction in wine quality (Alexandre *et al.,* 2004). The rapid proliferation of yeast cells in the must inoculated with bacteria indicated the absence of an inhibitory effect on yeasts by bacteria. These results confirm previous observations concerning interference in the fermentative activity of yeasts by bacteria when inoculated together in grape must (Semon *et al*., 2001; Rosi *et al*., 2003; Sieczkowski, 2004; Jussier *et al*., 2006). Since D-lactic acid is a final product of sugar metabolism by heterolactic bacteria, the determination of this acid in wines produced by simultaneous AF/MLF proves that sugar catabolism by the inoculated bacteria was inhibited. Previously, it was shown by comparison between simultaneous and sequential AF/MLF that acetic acid did not increase or did not increase significantly in wines inoculated with bacteria before AF (Semon *et al*., 2001; Rosi *et al*., 2003; Jussier *et al*., 2006). In the second microvinification experiment with Must 2, the acetic acid increase was probably caused mainly by yeast metabolism, as acetic the acid concentration was proportional to the ethanol content. It is well known that yeast under stress conditions produces acetic acid in quantities higher than under standard growth conditions (Erasmus *et al.,* 2004).

It is important to emphasise that the success of MLF depends to the occurrence of oenological parameters (i.e. pH, temperature, alcohol content and SO_2 concentration) close to the optimal values for malolactic activity (Valliant *et al.,* 1995; Ribéreau-Gayon *et al*., 2006). Jussier *et al*. (2006) observed a significant reduction in time to end L-malic acid depletion from Chardonnay at a pH of 3.53 and ethanol over 13% (v/v) when simultaneous AF/MLF was induced with respect to sequential AF/MLF. Three different experimental conditions were tested, in which pH and ethanol represented the limiting factors for MLF. Simultaneous inoculation resulted in a valid strategy to overcome sluggish and stuck MLF. This method of inoculation is a simplification of the procedure known as "*pied de cuve malo*", where cells progressively adapting to wine acquire resistance to stresses, thus improving their performance (Laurent & Valade, 1993). In the second microvinification, with the combination of pH 3.23 and 15.72% (v/v) ethanol, the *O. oeni* VP41 strain completed the conversion of L-malic acid to L-lactic acid. Using the same strain, Loubser (2004) reported the completion of MLF in a wine of similar ethanol content (15.62%, v/v), but higher pH (3.7). While testing 11 *O. oeni* strains in wine at pH 3.5, Nannelli *et al.* (2004) observed that only five completed MLF in the presence of 14.0% (v/v) ethanol, while only one completed MLF at 15.4% (v/v) alcohol.

The success of MLF by non-acclimatised cells in wines with ethanol levels in excess of 16.0% (v/v) seems to be improbable despite concurrent AF/MLF induction. The simultaneous yeast-bacteria inoculation failed in wines containing 16.9 and 17.8% (v/v) ethanol. These results show that high levels of ethanol in combination with low pH exert negative effects upon the survival of *O. oeni* in wine. It is possible that these factors damage cytoplasmic membrane function, which has a deleterious effect upon malolactic activity (Da Silveira *et al.,* 2002; Chu-Ky *et al.,* 2005). Under such difficult conditions, the acclimation of the bacteria assumes a fundamental role in the management of MLF. The rehydration of lyophilised cells in wine-water solution constitutes a favourable condition of adaptation before inoculation in must or wine. The importance of acid adaptation to optimise the survival and growth of *O. oeni* in wine was highlighted previously (Drici-Cachon *et al*., 1996). Beltramo *et al*. (2006) confirmed the value of stress pre-adaptation for malolactic starter cells, particularly at low pH, and the better survival of acid-adapted cells than of non-adapted cells was verified on wine-like medium at pH 3.5 and 10% (v/v) ethanol. The incubation of cells in wine-water solution (which constitutes an acidified medium) before the inoculation in wine induces a phenomenon of cross-protection against various stresses (Beltramo *et al*., 2006). Nevertheless, the failure of acclimatised bacteria in sequential AF/MLF with wine at an ethanol level of over 16% (v/v) (the case of the third microvinification) demonstrates that this procedure does not guarantee an adequate cell survival rate for successful MLF under such winemaking conditions.

The performance of the bacteria when they are inoculated into grape must along with the yeast (simultaneous AF/MLF) is enhanced by allowing for a period of bacterial adaptation to the gradual ethanol concentration produced during AF. The combination of an acclimatisation by rehydration step and adaptation to ethanol by co-inoculation increases the chances for successful MLF in high-alcohol wine. The acclimatisation period drastically affects bacterial malic acid consumption before alcohol toxicity becomes problematic for the bacteria. Nevertheless, the incubation time of cells in the wine-water solution seems to be crucial for the outcome of MLF. Zapparoli (2004) reported that *O. oeni* acquires stress resistance during the stationary growth phase and that 10-day-old cells were more resistant to ethanol and pH than three-day-old cells. The different behaviour observed in the 24-h and 48-h cells is explained by the effects of starvation on the selection of stress-resistant cells.

CONCLUSIONS

We analysed the results of MLF management during the production of high-alcohol wines. High ethanol and low pH are two stress factors that, when combined with other oenological factors, influence the survival of LAB, and hence the MLF. The strategy of inoculation, as well as the preparation of the culture starter, determined the ease of MLF. The acclimatisation of bacteria is a crucial step. Further investigations would be necessary to provide better information on the molecular and biochemical mechanisms responsible of the acquisition of stress resistance by the cells during this step.

LITERATURE CITED

Alexandre, H., Costello, P.J., Remize, F., Guzzo, J. & Guilloux-Benatier, M. 2004. *Saccharomyces cerevisiae-Oenococcus oeni* interaction in wine: current knowledge and perspectives. Int. J. Food Microbiol. 93, 141-154.

Beltramo, C., Desroche, N., Tourdot-Maréchal, R., Grandvalet, C. & Guzzo, J. 2006. Real-time PCR for characterizing the stress response of *Oenococcus oeni* in a wine-like medium. Res. Microbiol. 157, 267-274.

Chu-Ky, S., Tourdot-Marechal R., Marechal, P-A. & Guzzo J. 2005. Combined cold, acid, ethanol shocks in *Oenococcus oeni*: effects on membrane fluidity and cell viability. Biochim. Biophys. Acta 1717, 118-124.

Da Silveira, M.G., San Romão, M.V. Louriero-Dias, M.C., Rombouts, F.M. & Abee, T. 2002. Flow cytometric assessment of membrane integrity of ethanol-stressed *Oenococcus oeni* cells. Appl. Environ. Microbiol. 68, 6087-6093.

Da Silveira, M.G., Golovina, E.A., Hoekstra, F.A., Rombouts, F.M. & Abee. T. 2003. Membrane fluidity adjustments in ethanol-stressed *Oenococcus oeni* cells. Appl. Environ. Microbiol. 69, 5826-5832.

Drici-Cachon, Z., Guzzo, J., Cavin, J.-F. & Diviès, C. 1996. Acid tolerance in *Leuconostoc oenos*. Isolation and characterization of an acid-resistant mutant. Appl. Microbiol. Biotech. 44, 785-789.

Erasmus, D.J., Cliff, M. & Van Vuuren, H.J.J. 2004. Impact of yeast strain on the production of acetic acid, glycerol, and the sensory attributes of icewine. Am. J. Enol. Vitic. 55, 371-378.

Gump, B.H., Zoecklein, B.W. & Fugelsang, K.C. 2000. Prediction of prefermentation nutritional status of grape juice. *In:* Spencer, J.F.T. & Ragout de Spencer, A.L. (eds.). Methods in biotechnology, vol 14. Humana Press, Totowa, New York. pp. 283-296.

Jussier, D. Morneau, A.D. & Mira de Orduña, R. 2006. Effect of simultaneous inoculation with yeast and bacteria on fermentation kinetics and key wine parameters of cool-climate Chardonnay. Appl. Environ. Microbiol. 72, 221-227.

Krieger S. 2005. Determining when to add malolactic bacteria. In: Morenzoni, R. & Specht, K.S. (eds). Malolactic fermentation. Understanding the science and practice. Lallemand Inc Montréal, Canada. pp. 12.1-12.9.

Laurent, M. & Valade, M. 1993. La réactivation des bactéries lyophilisées sur mout pour líensemencement de la fermentation malo-lactique en Champagne. In Proc. Les aspects microbiologiques de la fermentation malo-lactique, April 1993, Montpellier, France, pp. 49-55.

Lonvaud-Funel, A. 1999. Lactic acid bacteria in the quality improvement and depreciation of wine. Antonie van Leeuwenhoek 76, 317-331.

Loubser P.A. 2004. Familiarize yourself with malolactic fermentation. Wynboer Technical Yearbook 5, 32-33.

Murat M.-L., Gindreau, E. & Augustin, C. 2007. MLF management part II: yeast-bacteria interaction, the application of sequential and co-inoculations. Australian and New Zealand Grapegrower Winemaker 522, 45-51.

Nannelli, F., Creatini, F. & Rosi, I. 2004. Aptitude of selected and wild strains of *Oenococcus oeni* to induce malolactic fermentation in harsh wines. *In* Proc. Wine quality and malolactic fermentation conf., May 4-5 2004, Porto, Portugal. pp 19-24.

Ough, C.S. & Amerine, M.A. 1988 (2nd ed). Methods Analysis of Musts and Wines. Wiley and Sons, Chichester, UK.

Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B. & Lonvaud-Funel, A. 2006 (2nd ed). Handbook of enology: the microbiology of wine and vinifications. Wiley and Sons, Chichester, UK.

Rosi, I., Fia, G. & Canuti V. 2003. Influence of different pH values and inoculation time on the growth and malolactic activity of a strain of Oenococcus oeni. Australian J. Grape Wine Research 9, 194-199.

Semon, M.J., Edwards, C.G., Forsyth, D. & Dinn C. 2001. Inducing malolactic fermentation in Chardonnay musts and wines using different strains of Oenococcus oeni. Australian J. Grape Wine Research 7, 52-59.

Sieczkowski, N. 2004. Maîtrise et intérêts de la co-inoculation «levures-bactéries». Revue Française d'OEnologie 207, 24-28.

Slinkard, A.K. & Singleton, V.L. 1977. Total phenol analysis: automation and comparison with manual methods. Am. J. Enol. Vitic. 28, 49-55.

Valliant, H., Formisyn, P. & Gerbaux, V. 1995. Malolactic fermentation of wine: study of the influence of some physico-chemical factors by experimentation design assay. J. Appl. Bacteriol. 79, 640-650.

Zapparoli, G. 2004. Colony dimorphism associated with stress resistance in *Oenococcus oeni* VP01 cells during stationary growth phase. FEMS Microbiol. Lett. 239, 261-265.