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Improvement of volatile composition of wines by controlled addition of malolactic bacteria

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Abstract

The effect of malolactic fermentation (MLF) on the volatile composition of red wines was studied by inoculation with selected lactic acid bacteria. Four wines were inoculated with different *Oenococcus oeni* (syn. *Leuconostoc oenos*) strains, the major malolactic species found in wines, and one was inoculated with a *Lactobacillus* sp. strain. A non inoculated wine was also analyzed to act as a control. Malolactic fermentation and evolution of non volatile compounds were followed by HPLC and after the depletion of the malic acid present in wine the volatile compounds were extracted and analyzed by gas chromatography with flame ionization and mass spectrometry. Wines which had undergone the MLF showed a significant increment in total higher alcohols, esters and acids that are important in the sensory properties and quality of wine. © 2000 Canadian Institute of Food Science and Technology. Published by Elsevier Science Ltd. All rights reserved.

Keywords: *Leuconostoc oenos*; Malolactic fermentation; *Oenococcus oeni*; Volatile compounds

1. Introduction

Malolactic fermentation is the bioconversion of the malic acid in wine to lactic acid and carbon dioxide. It can be caused by various lactic acid bacteria although it is generally associated with three genera: *Oenococcus, Lactobacillus* and *Pediococcus*. Besides de-acidifying the wine, this fermentation improves the biological stability of wines by preventing the malic acid utilization by other non desirable species (Beelman, 1982; Davies, Wibowo, Eschenbruch, Lee & Fleet, 1985). Moreover, during MLF the bacteria also can affect the final aroma balance by modifying fruity aromas and maybe producing aroma active compounds by themselves (Davis et al., 1985; Henick-Kling, 1995).

Although there are contradictory results on the effect of MLF on wine flavour (Fleet, 1993) microbiological studies have established that MLF does noticeably change the wine aroma. Analysis of MLF and non-MLF wines by gas chromatography have found that wines fermented with selected strains of lactic acid bacteria had flavours better than those that had undergone spontaneous fermentation (Henick-Kling, 1995). Davies et al. (1985) reviewed studies which showed that different strains of malolactic bacteria could have different sensory effects on wines. While some strains produce beneficial volatile compounds, other strains did not contribute to the enhancement of wine flavours.

Nowadays, genetic engineering has given us new tools to accomplish malic acid consumption simultaneously with alcoholic fermentation, by introducing the malate transport and malolactic genes into *Saccharomyces cerevisiae*, avoiding the utilization of malolactic strains but not affecting the flavour composition (Denayrolles, Aigle & Lonvaud-Funel, 1995; Volschenk et al., 1997). The positive contribution of selected lactic acid bacteria strains to the final aroma in wines should encourage their utilization as starter cultures to inoculate red wines. The contribution of the aromas coming from the grape variety, yeasts fermentation and volatiles produced during the MLF should be evaluated as a whole towards a best final aromatic balance to obtain a quality wine.

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2. Materials and methods

2.1. Strains and culture conditions

Oenococcus oeni and *Lactobacillus* sp. strains were collected from Requena (Eastern Spain), isolated and identified as described by Pardo and Zúñiga (1992). Cells were routinely cultured in MP as previously described (Maicas, Pardo & Ferrer, in press).

2.2. Fermentations

Screw bottles (2000-ml) were sterilized (121°C 30 min), filled with 2000 ml of wine sterilized by filtration and inoculated with about 1×10^5 cfu ml^{-1} of each strain. Fermentations were carried out at 20°C and malic acid concentrations were monitored by HPLC till their concentrations dropped to 0.2 g l^{-1}.

2.3. Analysis of non volatile compounds

Sugars, organic acids and ethanol were quantified by HPLC (Merck-Hitachi). Samples were prepared and injected in two coupled HPX-87H Aminex columns (Bio-Rad Chemical Division, Richmond California) as previously described (Maicas, González-Cabo, Ferrer & Pardo, 1999). External standards were used to quantify the required compounds.

2.4. Isolation of volatile compounds

Extraction of volatile compounds from the wine was by continuous liquid–liquid extraction with 40:60 v/v dicloromethane:pentane mixture. For quantification, an internal standard (2-nonanol) was added to the wine (1.646 mg l^{-1}). Some anhydrous Na_2SO_4 was added after extraction to eliminate residual water. The solvent was then removed by distillation through a Vigreux column to a residual volume of 1 ml.

2.5. Analysis of volatile compounds

Gas chromatographic analysis was performed as described by Gil, Mateo, Jiménez, Pastor & Huerta, (1996). Identification of compounds was determined by comparing retention times with authentic compounds (Fluka) and using a Finnigan Mat 95 S mass spectrometric detector containing a Wiley library under the same chromatographic conditions.

3. Results and discussion

Table 1 shows the relative peak intensities of the volatile compounds found in the studied wines after the malolactic fermentation (MLF). Some of them were quantified when an appropriate standard compound was available (Table 2). All the *Oenococcus oeni* strains used in this work almost completely degraded the malic acid present in wine while *Lactobacillus* sp. CH4 left some quantities to be degraded (Table 3). Certain compounds detected in the wine before MLF remained in the same concentration in all the wines after the fermentation. These were 1-hexanol, 2-pentanol, isovaleric acid and hexanoic acid. They were considered varietal compounds, characteristic of the basal wine used (Table 1). Analysis of variance was performed to compare all wines obtained by inoculation with single cultures of malolactic bacteria with those that were not inoculated. The results obtained revealed differences in the rest of the volatile compounds (Table 1). In some cases these compounds were in higher concentrations in wines inoculated with any malolactic bacteria while some other compounds showed a variable result depending on the used strain; only the values obtained for one compound, ethyl 3-hydroxybutyrate, decreased significatively.

3.1. Higher alcohols

Higher alcohols are considered to be produced from aminoacids or from hexoses through pyruvate and appear to contribute to the flavor of wines. The total amounts of higher alcohols in table wines are about 0.14–0.42 g l^{-1} (Amerine, Berg, Kunkee, Ough, Singleton & Webb, 1982) which is in agreement with our results. Significant increments were recorded in all the wines which had undergone the MLF (except the one fermented with MA4) in comparison with controls (Table 2). Those increments were mainly due to benzyl alcohol, the production of which was detected in all the wines. Similar amounts (about 0.4 mg l^{-1}) were recorded in the four wines produced by *O. oeni* strains while a higher level (0.96 mg l^{-1}) was detected in the wine fermented by the homolactic species CH4. Isobutanol, 1-propanol, 1-butanol and isoamyl alcohol showed a characteristic result depending on the strain used to perform the MLF (Fig. 1). Data recorded for BM3 and MA4 strains were slightly lower than the basal wine while those values were higher for the rest of the strains. As expected, similar production patterns can be observed among these four alcohols because they have similar biosynthetic pathways. However, 2-phenylethanol results did not show significant diminution in all the final wines, which can be mainly due to physical adsorption to bacteria. As has been noticed before, 1-hexanol and 2-pentanol did not contribute to increase the total amounts of higher alcohols.

3.2. Esters

An important aroma compound produced during MLF is ethyl lactate. After MLF, we have found about

Table 1
Relative concentrations and coefficients of variation for the six extracts of each of analyzed wines

Compound	Ms	Before MLF		After MLF									
		Control		BM3[c]		MA4[c]		VV5[c]		TE3[c]		CH4[d]	
		Conc.	CV[a]	Conc.	CV	Conc.	CV	Conc.	CV	Conc.	CV	Conc.	CV
Alcohols													
1-Propanol	99.7	0.20	5.0	0.16	0.1	0.12	17.0	0.46	0.7	0.3	15.9	0.38	1.9
Isobutanol	96.2	1.59	2.3	1.25	0.0	0.98	3.3	2.65	0.6	2.11	2.6	2.24	2.4
2-Pentanol	90.8	0.16	1.6	0.15	1.0	0.13	1.6	0.18	0.3	0.17	8.3	0.16	1.3
1-Butanol	94.6	0.14	6.4	0.12	8.1	0.11	7.4	0.21	3.0	0.19	8.4	0.21	1.5
Isoamyl alcohol	95.9	43.49	0.8	39.13	0.3	37.15	0.8	50.47	0.3	46.21	1.6	48.28	1.0
1-Hexanol	92.3	0.40	2.3	0.40	0.8	0.39	2.4	0.39	3.8	0.39	0.1	0.40	1.0
Benzyl alcohol	[b]	0.14	0.7	0.23	0.5	0.22	2.7	0.21	7.1	0.21	0.1	0.54	2.8
2-Phenylethanol	86.1	16.95	4.2	17.45	0.3	15.83	2.0	15.84	2.7	15.66	4.9	16.74	3.0
Totals		63.07		58.89		54.92		70.41		65.24		68.95	
Esters													
Ethyl acetate	[b]	4.50	1.5	2.38	1.5	1.69	2.2	9.86	0.9	6.01	10.4	7.86	8.6
Ethyl caprilate	80.6	0.31	0.1	0.29	3.2	0.34	11.5	0.26	1.8	0.32	1.3	0.30	15.4
Isoamyl acetate	93.4	0.26	0.7	0.31	1.5	0.32	3.1	0.60	0.8	0.22	4.6	0.60	4.3
Ethyl caproate	90.9	0.16	1.6	0.15	0.3	0.14	2.3	0.19	1.7	0.17	1.5	0.18	1.2
Ethyl lactate	[b]	9.91	2.7	13.07	0.4	11.85	1.2	13.00	1.5	12.69	0.1	14.83	1.1
Ethyl 3-hydroxybutyrarate	92.9	0.04	5.8	0.04	0.7	0.04	3.0	0.04	1.0	0.03	3.1	0.04	1.7
Isoamyl caprilate	[b]	0.22	17.2	0.19	16.8	0.11	17.1	0.03	33.3	0.06	12.2	0.07	1.4
Diethyl succinate	93.6	0.82	3.9	0.98	6.8	0.82	6.9	0.80	0.3	0.69	2.7	0.88	0.3
Ethyl 4-hydroxybutyrarate	[b]	1.29	5.0	0.73	0.3	0.80	1.8	0.59	1.5	0.81	0.4	0.77	3.9
2-Phenethyl acetate	85.1	0.07	2.9	0.15	0.9	0.14	3.8	0.19	0.8	0.07	2.5	0.17	3.3
2-Phenethyl benzoate	[b]	0.46	1.6	1.04	2.5	0.74	8.9	0.64	6.3	1.04	7.5	0.63	13.9
Totals		18.07		19.34		16.99		26.21		22.11		26.32	
Acids													
Isovaleric acid	95.7	0.90	9.3	1.03	6.1	0.89	6.5	0.82	0.6	0.87	3.2	0.93	3.8
Isobutric acid	90.3	0.74	2.1	0.83	1.5	0.74	10.2	0.77	3.2	0.72	3.3	0.72	22.0
Hexanoicacid	95.3	1.32	5.8	1.32	1.5	1.19	0.7	1.18	1.2	1.19	0.6	1.25	2.1
10-Undecanoic acid	[b]	0.10	14.3	0.79	4.9	0.46	14.8	0.64	8.6	0.61	3.1	0.77	12.3
Capric acid	[b]	0.08	39.5	0.12	1.9	0.12	21.6	0.14	18.7	0.33	24.4	0.12	5.9
Caprilic acid	[b]	0.87	4.4	1.37	8.7	1.01	6.2	1.15	5.1	1.33	9.1	1.31	4.4
Totals		28.53		33.43		27.56		41.82		35.67		41.07	
Other compounds													
γ-Butirolactone	92.8	2.78	3.2	3.65	0.4	3.17	4.1	3.22	0.8	3.19	3.9	3.20	0.9
Threo-2,3-butanediol	93.1	11.86	4.9	22.82	0.0	14.33	2.1	13.81	0.4	15.64	4.4	13.53	2.1
Meso-2,3-butanediol	93.3	1.79	1.4	6.76	1.1	4.22	3.9	4.55	4.8	4.13	4.5	3.72	16.5
Erythro-2,3-butanediol	[b]	0.42	2.3	0.59	15.0	0.38	21.7	0.21	5.4	0.26	5.7	0.00	–
3-(Methylthio)-1-propanol	93.9	0.26	5.4	0.33	0.3	0.29	3.2	0.28	3.6	0.33	0.7	0.28	3.6

[a] CV = standard deviation/mean relative concentration (%).
[b] Compounds that were not identified by mass spectroscopy.
[c] *Oenococcus oeni*.
[d] *Lactobacillus* sp.

50 mg l^{-1} in wines fermented with *Oenococcus oeni* strains and 60 mg l^{-1} in wines where the homolactic CH4 strain was used. Valade and Laurent (1992) showed values significantly higher than ours (190 mg l^{-1}) but Fleet (1983) described standard concentrations for ethyl lactate in red wine up to 50 mg l^{-1}. Ethyl lactate production is coupled to lactic acid formation; so values in red wine depend on the MLF activity. The rise in ethyl acetate was also detected with some of the utilized strains but only strain VV5 showed remarkable levels at the end of the fermentation (Table 2). Ethyl acetate production during MLF has already been described and it can affect wine aroma when levels are over 200 mg l^{-1} (Amerine et al., 1982). This threshold for acetate was only exceeded by strain VV5.

Other esters contribute to wine odor; e.g. isoamyl acetate and ethyl caproate and are especially important for a pleasant fruity note (Gil et al., 1996). The production of these two compounds depended on the assayed

Table 2
Total concentrations (mg l^{-1}) and coefficients of variation for the six extracts of each of analyzed wines

Compound	MS[b]	Before MLF		After MLF									
		Control		BM3[d]		MA4[d]		VV5[d]		TE3[d]		CH4[e]	
		Conc.	CV[a]	Conc.	CV	Conc.	CV	Conc.	CV	Conc.	CV	Conc.	CV
Alcohols													
1-Propanol	99.7	3.35	5.0	2.80	0.1	1.99	17.0	7.79	0.7	5.23	5.9	6.38	1.9
Isobutanol	96.2	9.68	2.3	7.58	0.0	5.93	3.3	16.12	0.6	12.84	2.6	13.64	2.4
1-Butanol	94.6	0.13	6.4	0.12	8.1	0.11	7.4	0.20	3.0	0.19	8.4	0.20	1.5
Isoamyl alcohol	95.9	96.46	0.8	86.79	0.3	82.39	0.8	111.94	0.3	102.48	1.6	107.07	1.0
1-Hexanol	92.3	0.68	2.3	0.68	0.8	0.66	2.4	0.67	3.8	0.66	0.1	0.69	1.0
Benzyl alcohol	[c]	0.25	0.7	0.41	0.5	0.39	2.7	0.37	7.1	0.38	0.1	0.96	2.8
2-Phenylethanol	86.1	22.75	4.2	23.43	0.3	21.25	2.0	21.27	2.7	21.02	4.9	22.48	3.0
Esters													
Isoamyl acetate	93.4	0.30	0.7	0.35	1.5	0.37	3.1	0.68	0.8	0.25	4.6	0.67	4.3
Ethyl acetate	[c]	98.69	1.5	52.23	1.5	36.94	2.2	216.04	0.9	131.69	10.4	172.11	8.6
Ethyl caproate	90.9	0.24	1.6	0.23	0.3	0.21	2.3	0.28	1.7	0.25	1.5	0.26	1.2
Ethyl lactate	[c]	39.90	2.7	52.62	0.4	47.71	1.2	52.33	1.5	51.10	0.1	59.69	1.1
Ethyl caprilate	80.6	1.16	0.1	1.05	3.2	1.27	11.5	0.97	1.8	1.17	1.3	1.11	15.4
Ethyl 3-hydroxybutyrarate	92.9	0.11	5.8	0.10	0.7	0.09	3.0	0.09	1.0	0.08	3.1	0.09	1.7
Diethyl succinate	93.6	1.05	3.9	1.25	6.8	1.05	6.9	1.02	0.3	0.88	2.7	1.12	0.3
Acids													
Isobutric acid	90.3	1.70	2.1	1.90	1.5	1.69	10.2	1.77	3.2	1.65	3.3	1.66	22.0
Isovaleric acid	95.7	1.24	9.3	1.42	6.1	1.23	6.5	1.13	0.6	1.20	3.2	1.29	3.8
Caprilic acid	[c]	1.71	4.4	2.70	8.7	1.98	6.2	2.27	5.1	2.62	9.1	2.58	4.4
Capric acid	[c]	0.14	39.5	0.22	1.9	0.22	21.6	0.26	18.7	0.61	24.4	0.22	5.9
Other compounds													
γ-butirolactone	92.8	2.82	3.2	3.71	0.4	3.22	4.1	3.27	0.8	3.23	3.9	3.25	0.9
Theo-2,3-butanediol	93.1	431.29	4.9	829.91	0.0	521.22	2.1	502.31	0.4	569.02	4.4	492.30	2.1
Meso-2,3-butanediol	93.3	123.34	1.4	465.02	1.1	290.25	3.9	312.62	4.8	283.97	4.5	255.92	16.5
Erythro-2,3-butanediol	90.5	33.85	2.3	48.15	15.0	30.63	21.7	16.78	5.4	21.50	5.7	0.0	[c]
3-(Methylthio)-1-propanol	93.9	0.90	5.4	1.14	0.3	0.98	3.2	0.98	3.6	1.14	0.7	0.97	3.6

[a] CV = standard deviation/mean concentration (%).
[b] MS = mass spectrometry similarity (%).
[c] = Compounds that were not identified by MS.
[d] = *Oenococcus oeni*.
[e] = *Lactobacillus* sp.

Table 3
Malolactic fermentation data: viable bacteria and evolution of organic acids

Wine	Viable bacteria in wine after inoculation (cfu ml^{-1})	Malolactic fermentation complete (days)	Remaining malic acid (g l^{-1})	Lactic acid produced (g l^{-1})	Acetic acid produced (g l^{-1})
Sterile (non inoculated)	–	–	3.87	–	–
Oenococcus oeni MA4	1.9×10^5	30	0.09	2.29	0.21
Oenococcus oeni VV5	1.8×10^5	23	0.13	2.22	0.20
Oenococcus oeni TE3	4.6×10^5	35	0.09	2.20	0.13
Oenococcus oeni BM3	1.4×10^5	29	0.10	2.16	0.16
Lactobacillus sp. CH4	3.0×10^5	[a]	0.81	1.62	0.23

[a] Fermentation was stopped after 60 days.

strain (Table 1). The most significant increments were detected with CH4 and VV5, while the other strains did not produced remarkable levels of those esters. Among the other identified esters, 2-phenethyl acetate and 2-phenethyl benzoate were those that showed more significant increments. The total amounts of esters found after the MLF in these red wines suggest their beneficial contribution to the wine's final aroma (Table 1).

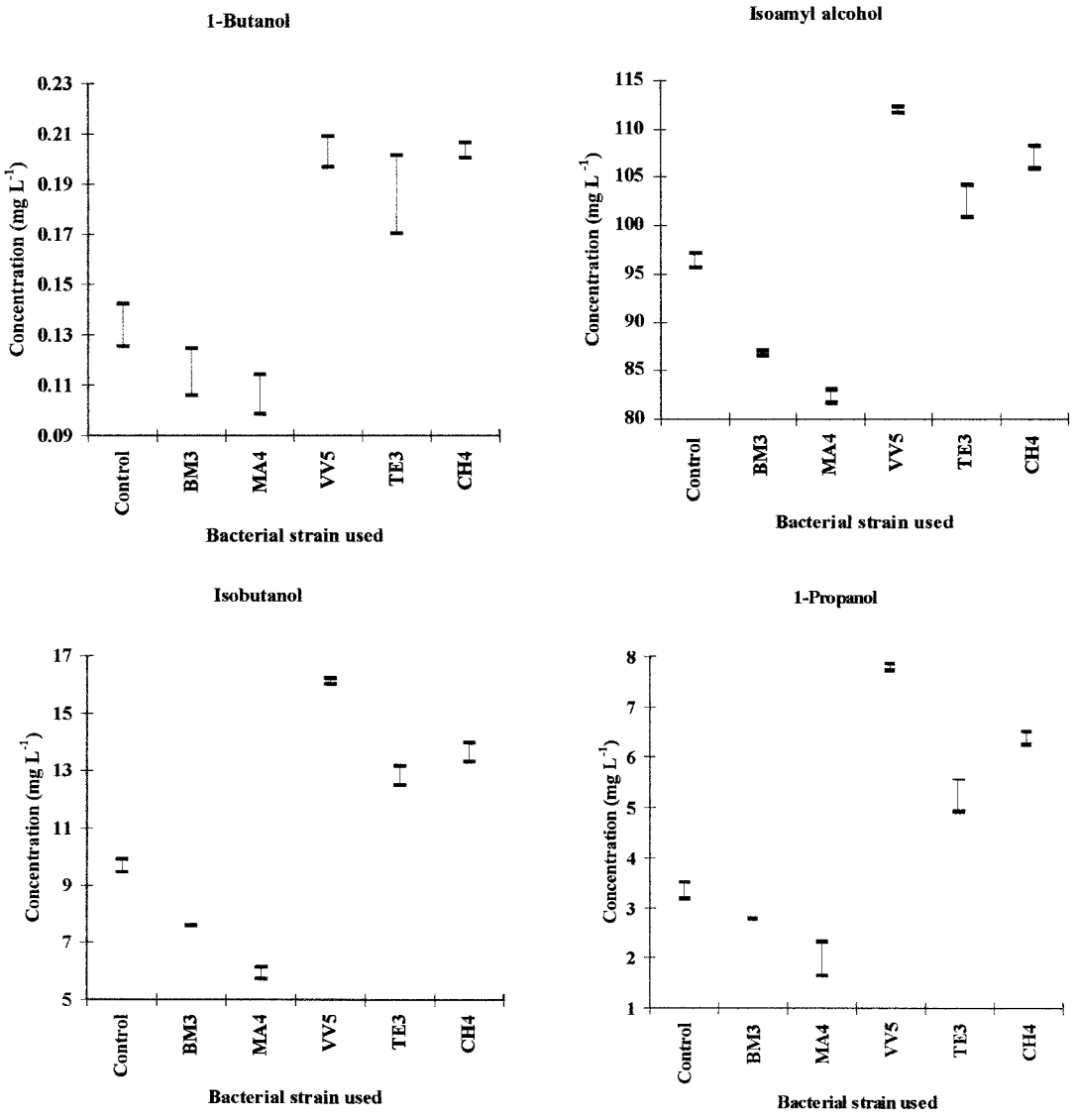

Fig. 1. Concentration of some alcohols in different wines. Values are means of three determinations.

3.3. Acids

The acetic acid concentration in wines increased about 0.2 g l^{-1} during MLF (Table 3), bringing the final concentration in wine to 0.60–0.65 g l^{-1}. *Oenococcus oeni* is an heterofermentative lactic bacteria and under favourable conditions acetic acid is not surprisingly produced from acetyl-CoA. The values we have recorded are according to those previously found by some authors. It has been suggested that wines should be rejected as faulty when they contain more than 0.8 g l^{-1} of acetic acid (Davies et al., 1985; Henick-Kling, 1995).

Although the volatile fatty acids are present in wines in only trace quantities, their low odor thresholds and their pungent odors may make them important odor contributors. Isovaleric, isobutiric and hexanoic acids did not show high values in any of the studied wines after the MLF. Although capric and caprilic acids were higher in all the wines once the MLF had finished, the sum of their concentrations was never over 2 mg l^{-1}. Higher concentrations of these acid are reported to be negative for final aroma (Miranda-López, Libbey, Watson & McDaniel, 1992). Very important increments were detected in peak at RT = 66.16. Mass spectrometry analysis indicated it is 10-undecanoic acid but no previous reports in wine fermentations have been found.

3.4. Another compounds

The wines which had undergone the MLF showed significant increments in the levels of γ-butirolactone; about 0.4 mg l^{-1} were produced in the assayed strains. Those increases were similar to those found by Valade and Laurent (1992) who also detected increments in wines after the MLF. The different quantities recorded can be explained on the basis of individual strain characteristics.

3-Methionol or 3-(methylthio)-1-propanol was identified as the principal sulfur compound in wine. Only wines fermented with TE3 and BM3 strains showed high concentrations but never over 1.14 mg ml^{-1}. Although the contribution of 3-(methylthio)-1-propanol to the overall flavour of wine should be take into account the levels we have detected are not too high in comparison with the range of values established as acceptable for this compound in red wines, 0.5–2 mg l^{-1} (Miranda-López et al., 1992).

But perhaps, one of the most interesting compounds detected in wine is 2,3-butanediol. Three racemic species have been found in these wines (*erythro*, *threo*, and *meso*) (Table 1). Lactic acid bacteria metabolize pyruvate anaerobically yielding varying amounts of carbon dioxide, acetoin, diacetyl, 2,3-butanediol, acetic acid, ethanol and lactic acid (Davis et al., 1985; Wibowo, Eschenbruch, Davis & Lee, 1985). However, no detectable levels of diacetyl were detected in these wines, probably because it has been enzymatically reduced to 2,3-butanediol. This spontaneous reduction was described by Martineau and Henick-Kling (1995) who also found lower levels of diacetyl (< 0.005 mg l^{-1}) in wines after the MLF. Acetoine, the other compound involved in the same production pathway, can also be reduced to 2,3-butanediol by the enzyme acetoin reductase. These two reductions can explain the higher levels of 2,3-butanediol found in these wines (Table 1).

As can be ascertained, lactic acid bacteria implications in wine-making are not only due to malolactic fermentation itself. In addition to malic acid degradation, which was accomplished by all the assayed *Oenococcus oeni* strains and only partially by *Lactobacillus* sp. CH4, these microorganisms were able to noticeably modify the final wine volatile composition. Increments in total higher alcohols, ethyl esters and acids contribute to enhance the sensory properties and quality of wines that have undergone MLF (Tables 1, 2). Moreover, it should be noticed that only some of the inoculated strains can contribute to the production of beneficial volatile compounds. This is the reason to suggest the induction of MLF in red wines with selected lactic acid bacteria strains that can offer a positive contribution to the final aroma in wines.

Acknowledgements

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