A pre-spoilage marker for bacterial activity in fortified wine, conversion of L-malic acid to L-lactic acid

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G. DE REVEL, A.B. CAPELA AND T. HOGG. 1994. Sixty-one different fortified wines, from the Douro valley, inoculated with *Lactobacillus hilgardii* previously isolated from a spoiled fortified wine, were sampled progressively for 280 d. Samples were analysed for volatile acidity (acetic acid) and frozen for subsequent analysis where appropriate. After 280 d, 7/61 wines showed increases in volatile acidity of $>0.5 \text{ g l}^{-1}$ and were considered spoiled. Retrospective analysis of frozen samples of spoilage-positive wines showed that all had apparently undergone a malolactic conversion before the onset of heterolactic activity (acetate production). It is suggested that the monitoring of L-lactic acid might be a method of detecting activity of spoilage lactobacilli before the onset of spoilage.

INTRODUCTION

The bacterial deterioration of sweet fortified wine, predominantly by heterofermentative lactobacilli, is a recognized phenomenon within the wine industry (Goswell 1986). In the case of wines from the Douro demarcated region in the north of Portugal (the source of Port) the scale of this problem is generally perceived to be small, but occasionally (and unpredictably) much larger. Spoilage of Douro fortified wine is normally detected as an increase in the oenological parameter of volatile acidity (basically equivalent to acetic acid concentration), and occurs most often, but not exclusively, during the first and second years of maturation in wooden barrels. Indeed volatile acidity has classically been used as the parameter of choice for the monitoring of spoilage, this normally being measured by standard methods in batch, although semi-continuous, automated methods are becoming more common. The disadvantage of using volatile acidity in the control of spoilage is that any increase in concentration implies that spoilage has already started. The work presented here suggests that the conversion of L-malic acid to L-lactic acid might be a pre-spoilage marker for bacterial activity in fortified wine.

The results presented here were obtained from an experiment in which fortified wines were inoculated with a known spoilage strain of *Lactobacillus hilgardii* (which was previously shown to be the most prevalent ethanol-tolerant organism isolated from Douro fortified wine (Couto and Hogg 1994)). Wines were sampled at regular intervals, part of each sample being analysed for volatile acidity and the

remainder frozen for retrospective analysis of spoiling wines.

MATERIALS AND METHODS

Wines and bacteria

Sixty-one different red wines, from a range of viticultural sub-regions of the Douro demarcated region and all from the 1991 vintage, were obtained from two companies. All wines were screened for the presence of spoilage bacteria by inoculating the centrifuged pellet (10 000 g, 10 min) from 30 ml of wine into 10 ml of modified MRS broth (MRS + 10 g l^{-1} fructose, 15% v/v ethanol, pH 4.5). After covering with 1 cm depth of vaspar, tubes were incubated at 25°C. Following screening, wines were adjusted to pH 3.8 (with 1 mol l^{-1} NaOH) and 18% v/v ethanol (with H_2O).

The wines were then inoculated with a strain of *Lact. hilgardii*, previously grown in modified MRS medium at 25°C for 3 d, washed in 18% v/v ethanol solution and inoculated at 10 ml of $A = 0.7$ (640 nm) per 1.5 l of wine. Inoculated wines were stored at 25°C under a cover of sterile liquid paraffin and samples (20 ml) taken at monthly intervals (2 weekly for the first 3 months); all samples were analysed for volatile acidity and stored at -25°C for retrospective analysis of 'positives'. Ethanol-tolerant organisms were also isolated from all wines which showed signs of spoilage and identified using SDS-PAGE (Couto and Hogg 1994).

Analyses

Volatile acidity (VA as g l^{-1} , acetic acid equivalent) was determined by distillation/titration using segmented flow analysis (SFA) in a Technicon Autoanalyser 2 SL Colorimeter (Technicon Instruments Corp, USA). Hexose sugars and mannitol were determined by HPLC (Spectra Physics 8800) with an NH_2 -Spherisorb[®] column, mobile phase: acetonitrile/water 90:10; RI detection (Hewlett Packard 1047A) (Tusseau and Bourniol 1984). Malic and lactic acids by HPLC (Beckman Gold 168, 2 columns C18-Merck[®] 12.5 and 25 cm; mobile phase KH_2PO_4 0.2 mol l^{-1} , pH 2.4, flow 0.6 ml min^{-1} ; injection 20 μl ; detection u.v. 210 nm (Beckman 126)) (Tusseau and Benoit 1986). D- and L-lactic acids were determined using enzymatic test kits for food analysis (Boehringer Mannheim).

RESULTS

During 1 year of incubation at 25°C, 7/61 wines showed obvious increases ($>0.5 \text{ g l}^{-1}$) in volatile acidity (Table 1), the remaining wines showing no or very slight increases in this parameter ($<0.07 \text{ g l}^{-1}$) over this period. Wines showing obvious volatile acidity increases were considered as positive for spoilage. The presence of the inoculated strain of *Lact. hilgardii* as the unique ethanol-tolerant (15% v/v) organism was confirmed in all positives by analysis of the SDS-PAGE profiles of total cell proteins.

Analysis of frozen samples of all positive wines showed that malic acid was consumed and L-lactic acid produced prior to an increase in volatile acidity; Table 2 shows this for three positive wines. The typical pattern of consump-

tion of malic and production of L-lactic acids is shown in Fig. 1; this graph represents the changes in one wine (6b). The sequence of acid conversions was found to be identical for all wines, the only differences being in the time of onset of malic acid degradation (between 0 and 300 d, data not shown), and the rate of production and final values (Table 1) of volatile acidity. In all positive wines, pyruvic and citric acids were also consumed during the apparent malolactic phase (data not shown). In the case of citric acid, consumption began when more than half of the malic present had been converted.

Fig. 1 Relationship between malolactic activity and evolution of volatile acidity in a spoiling fortified wine. ■, L-Malic acid; □, L-lactic acid; ●, volatile acidity

Table 1 Comparison of volatile acidity at $t = 0$, $t = 280$ d for seven wines showing spoilage to that of 54 unspoiled wines

Sampled	Time (d)	54 non-spoiled wines		Spoiled wines						
		Mean	S.D.	6b	15c	4a	6a	15b	13e	186
April 1992	0	0.153	0.050	0.16	0.10	0.11	0.14	0.06	0.45	0.16
March 1993	280	0.221	0.056	1.6	0.88	0.66	1.76	2.2	2.4	2.1

Volatile acidity as g l^{-1} acetic acid.

Time (d)	Wine 186		Wine 6b		Wine 15c	
	Malic*	VA†	Malic	VA	Malic	VA
75	1.14	0.17	1.05	0.2	1.42	0.2
137	1.02	0.24	0.90	0.2	1.36	0.24
181	0.32	0.24	0.04	0.18	1.34	0.18
216	0.16	0.46	0	1.0	0	0.34
280	0.05	2.1	0	1.6	0	0.88

* Malic acid expressed as g l^{-1} .

† Volatile acidity expressed as g l^{-1} acetic acid equivalent.

Table 2 Change in concentration of L-malic acid and volatile acidity (VA) in three wines showing spoilage

Fig. 2 Fructose and glucose utilization and production of mannitol, D-lactic acid and volatile acidity (g l^{-1} acetic acid equivalent) by *Lactobacillus hilgardii* during spoilage of a fortified wine. ■, Fructose; ◆, glucose; ●, mannitol; ○, volatile acidity; □, D-lactic acid

Figure 2 represents the alterations occurring in the phase following the apparent malolactic conversion in another of the spoiling wines (6a). The decrease in fructose and glucose concentrations and the evolution of mannitol are both consistent with heterolactic degradation of glucose and fructose under anaerobic conditions. The same graph (but with different y-axes) shows the increases in volatile acidity and D-lactic acid for the same wine. Mannitol and volatile acidity increases (up to 8 g l^{-1} for mannitol) were noted in all positive wines (data not shown); these products constitute a recognized spoilage condition known as 'mannite' (Fornachon 1949; Dittrich 1977). Glucose and fructose were apparently co-utilized, there being no measurable differences between the percentage utilization of these sugars. These results also show the evolution D-lactic acid only during this mannite spoilage stage. A good correlation between D-lactic acid concentration and volatile acidity was noted in all positive wines throughout spoilage.

DISCUSSION

The data presented above suggests that, in the case of red fortified wines of the Port style inoculated with the most represented ethanol-tolerant organisms isolated from wines of the Douro demarcated region, a malolactic conversion precedes the development of spoilage characteristics. For dry table wines, it is generally accepted that the organisms responsible for the desired malolactic conversion will perform this concurrently with the degradation of hexose

sugars (sometimes preceding), the sugar metabolism often continuing after malolactic has finished (Pilone and Kunkee 1972; Radler 1975; Tracey and Britz 1989). This has not been reported in the case of spoilage of fortified wine of the Port type.

Spoilage of Port-type fortified wines is normally monitored, by consensus rather than rule, by increase in volatile acidity. If the phenomenon noted here proves to be general for this type of wine, as is suggested by an initial screen of spoiled wines in our laboratory, then a reduction in the concentration of L-malic acid, or perhaps more feasibly the monitoring of L-lactic acid, might offer significant advantages over current practices. Certainly the results presented here suggest that a systematic study of this phenomenon would be most appropriate.

The levels of L-malic acid in musts and wines from the Douro region are normally relatively low ($\text{ca } 2 \text{ g l}^{-1}$). The increase in concentration of L-lactic acid is relatively easy to determine against background levels of an average of 0.1 g l^{-1} in non-spoiled wines in this study. In our laboratory the use of enzymic detection for this parameter both in batch and in Flow Injection Analysis (FIA) systems (Lima and Rangel 1992) has proved most satisfactory.

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REFERENCES

- Couto, J.A. and Hogg, T.A. (1994) Diversity of ethanol-tolerant lactobacilli isolated from Douro fortified wine: clustering and identification by numerical analysis of electrophoretic protein profiles. *Journal of Applied Bacteriology* 76, 487-491.
- Dittrich, H.H. (1977) *Mikrobiologie des Weines* Stuttgart: Verlag Eugen Ulmer.
- Fornachon, J. (1949) *Bacterial Spoilage of Fortified Wines*. Adelaide: The Griffing Press.
- Goswell, R.W. (1986) Microbiology of fortified wines. In *Development in Food Microbiology* ed. Robinson, R.K. Ch. 1. London: Elsevier Applied Science Publishers.
- Lima, J.L.F.C. and Rangel, A.O.S.S. (1992) Enzymatic determination of L(-) malic and L(+) lactic acids in wine by flow injection analysis. *American Journal of Enology and Viticulture* 43, 58-62.

- Pilone, G.J. and Kunkee, R.F. (1972) Characterization and energetics of *Leuconostoc oenos* ML34. *American Journal of Enology and Viticulture* 23, 61-70.
- Radler, F. (1975) The metabolism of organic acids by lactic acid bacteria. In *Lactic Acid Bacteria in Beverages and Food* ed. Carr, J.G. pp. 17-27. London: Academic Press.
- Tracey, R.P. and Britz, T.J. (1989) The effect of amino acids on malolactic fermentation by *Leuconostoc oenos*. *Journal of Applied Bacteriology* 67, 589-595.
- Tusseau, D. and Benoit, C. (1986) Analyse collaborative des acides organiques des moûts et des vins par HPLC. *Feuille Vert OIV* 813, 1-15.
- Tusseau, D. and Bourniol, D. (1984) Détermination des glucides dans les moûts, vins et champagnes par HPLC. *Feuille Vert OIV* 776, 1-10.