

## Note: How do processing conditions affect the microflora of the sweet pomace of white grapes from Vinho Verde?

## Nota: Cómo afectan las condiciones de elaboración a la microflora del bagazo de uvas blancas del Vino Verde

M.L. Silva, F.X. Malcata\* and T.A. Hogg

*Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Porto, Portugal*

The independent and combined effects of the addition of tartaric acid, the addition of pectinases, and the time of fermentation (and the material of the container) on the specific rates of death of lactic acid bacteria and yeasts were experimentally evaluated through a full (or fractional) two-way factorial design for pomace fermentation from the Alvarinho (and the Loureiro) varieties. Microbes present in the pomace prior to fermentation were identified in a preliminary fashion. Knowledge of the behavior of the microflora in grape pomace as a function of each major processing parameter during the step of anaerobic fermentation is important in attempts to eventually standardize and optimize the manufacture of these distilled beverages.

**Keywords:** Sweet pomace, fermentation, yeasts, bacteria, vinho verde.

Se han evaluado los efectos combinados de la adición de ácido tartárico, de pectinasas, y del tiempo de fermentación (y el material del recipiente) sobre la velocidad específica de la muerte de las bacterias lácticas y de las levaduras en la fermentación del bagazo de uva procedente de las variedades Alvarinho y Loureiro. Antes de iniciarse la fermentación se identificaron los microorganismos presentes en el bagazo. El conocimiento del comportamiento de la microflora presente durante la fermentación anaerobia del bagazo de la uva en función de los parámetros de elaboración es importante para tratar de estandarizar y optimizar eventualmente la manufacturación de las bebidas destiladas.

**Palabras clave:** Bagazo de uva, fermentación, levaduras, bacterias, vino verde.

## INTRODUCTION

*Vinho Verde* (literally green wine) has been produced for at least five centuries in the northeast of Portugal, although the demarcated region was only officially recognized in 1929. This region, crossed by 5 of the 10

largest Portuguese rivers, has abundant water and extends over a surface of ~35 000 acres, and is worked by more than 160 000 farmers. The average year's production of the region is ~460 000 m<sup>3</sup>, which corresponds to approximately 15% of the overall wine production of Portugal (Gutiérrez, 1989). White *Vinho Verde* is characterized by high fixed acid content, particularly from malic and tartaric acids.

Instead of disposing of the pressed pomace (~15% w/w of total grape mass, constituted mainly by the skins and seeds and, to a varying extent, the stalks) as a low-value agricultural by-product, farmers often store

\* Address for correspondence: Escola Superior de Biotecnologia, Rua Dr. António Bernardino de Almeida, 4200 Porto, Portugal.

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it in carefully closed containers for several days without temperature control. During this period, spontaneous anaerobic fermentations of the sugars in the pomace effected by native microflora occur. The result is a complex mixture of volatile components, with ethanol as the predominant one. After fermentation, the pomace is manually removed into copper batch stills, and steam-distilled to remove the volatiles; the condensate obtained (called *bagaceira* in Portugal or *grappa* in Italy) has a unique flavor quite different from that of wine brandy.

The determination of the extent of fermentation is a completely empirical process based on the degree of maturity of the grapes upon harvest, the temperature and relative humidity of the environment, and the availability of container space. It is not feasible to sample the pomace along the fermentation to aid in monitoring the process, not only due to the heterogeneity of this feedstock but also because suitable sampling would disturb the anaerobic conditions. Furthermore, the information available on the microbial ecology of the pomace of white *Vinho Verde* upon storage is virtually nonexistent. For these reasons, an impetus exists for studies aimed at predicting (to some extent) the evolution of the microbial populations, and thus the microbial behavior throughout the storage period and the final flavor profile.

This note attempts to address this issue by, following an approximate identification of microbial strains present, postulating empirical models relating the numbers of the various microbial families with some of the more important (and more easily manipulated) processing parameters and by assessing the statistical validity of those models.

## MATERIAL AND METHODS

### Grape material and preparation

Alvarinho grapes were obtained from the Adega Cooperativa de Monção (Portugal), whereas Loureiro (dominant variety) grapes were obtained from the Estação Vitivinícola de Vairão (Portugal). Grapes were harvested at similar maturity stages and crushed and pressed in the usual fashion for the manufacture of white wine. For each variety, grape pomace from the same pressing batch was utilized for all experiments.

### Statistical design

The study encompassing the Alvarinho variety was arranged in a  $2^3$ -factorial design replicated twice with two levels of addition of tartaric acid, *T* (0 g and 80 g per 100 kg of grape pomace), two times of storage under anaerobic conditions, *t* (3 and 6 weeks), and two

levels of addition of pectinases, *E* (0 g and 2 g per 100 kg of grape pomace). The study encompassing the (dominant) Loureiro variety was arranged in a  $2^{4-1}$  factorial design replicated twice with two levels of addition of tartaric acid (0 g and 80 g per 100 kg of grape pomace), two times of storage under anaerobic conditions (3 and 5 weeks), two levels of addition of pectinases (0 g and 2 g per 100 kg of grape pomace), two types of container (plastic and wood), and an extra set of two experiments (replicated twice) run at the center's conditions (40 g of tartaric acid and 1 g of pectinases per 100 kg of grape pomace, and anaerobic maturation for 4 weeks), one using a plastic container and the other using a wood container. The experimental layout is depicted in Tables 1 and 2. The pectinases utilized (NOVO Nordisk, Bagsvaerd, Denmark) and the tartaric acid (commercial grade) were independently sprayed onto the pomace in the form of concentrated aqueous solutions so as to obtain the corresponding final ratios of additive per weight of pomace defined in the aforementioned experimental design.

### Sampling

Samples of 30 g of the grape pomace prior to anaerobic fermentation, and after 3 and 6 weeks (Alvarinho), or 3, 4, and 5 weeks (dominant Loureiro) as appropriate, were collected randomly from the pomace. The samples were homogenized for 10 min in a Stomacher with 270 ml of a 1/4-strength Ringer's solution (LAB M, Amer-sham). The homogenate was decimally diluted using the same diluent (serial dilutions ranging from  $10^{-1}$  to  $10^{-8}$ ).

### Enumeration and identification of yeasts

Diluted homogenates obtained at the various sampling times were inoculated on YM agar (Difco, Detroit, MI) previously acidified with commercial tartaric acid, and the plates were incubated at 25°C for 3 days. The various macroscopic colonies formed were counted. Representative colonies obtained from the initial sampling were isolated and subcultured on MEA (LAB M) for subsequent identification via conventional methods by Barnett et al. (1984). Assimilation tests were done via the API ID 32C kit method (BioMérieux), and the results were interpreted using the software by Barnett et al. (1990).

### Enumeration and identification of bacteria

Diluted homogenates were inoculated on WLN agar (LAB M) with 0.25 g L<sup>-1</sup> cycloheximide (Sigma, St. Louis, MO) added for the case of lactic acid bacteria, and 0.1 g L<sup>-1</sup> for the case of acetic acid bacteria. The plates were incubated at 30°C for 3 days under aerobic

(WLN<sup>+</sup>) or anaerobic (WLN<sup>-</sup>) conditions for acetic acid bacteria or lactic acid bacteria, respectively. The various macroscopic colonies formed were counted; the gram<sup>+</sup> positive catalase colonies were considered to be lactic acid bacteria, whereas the gram<sup>-</sup> catalase<sup>+</sup> colonies were (tentatively) assumed to be acetic acid bacteria. Acetic acid bacteria were classified based on the traditional procedures described by Buchanan and Gibbono (1974); tests performed at 30°C were growth at pH 4.5 and 30°C, oxidation of ethanol to carbon dioxide, and oxidation of lactate to carbon dioxide (Asai, 1971; Carr, 1968). Lactic acid bacteria were classified based on the traditional procedures described by Buchanan and Gibbono (1974); tests performed encompassed morphology of cells, homofermentative/heterofermentative character, and production of ammonia from arginine. The various strains of *Lactobacillus* were further identified using the API 50 CH kit (BioMérieux) method.

### Statistical analysis

The enumeration experimental data,  $y$ , were obtained as cfu g<sup>-1</sup> of original pomace after incubation on the appropriate agar or medium. It was assumed throughout the study that the viable microorganisms were dying according to a first-order law; namely,

$$-\frac{dy}{dt} = \mu y \quad (1)$$

$$t = 0, \quad y = y_0$$

where  $y$  is the concentration of viable microorganisms in the grape pomace,  $t$  the time elapsed since storing the grapes under anaerobiosis,  $\mu$  the specific rate of death, and  $y_0$  the initial concentration of viable organisms just prior to anaerobic storage. The foregoing assumption seems, at first sight, to be inconsistent with the well-established observation that *Saccharomyces cerevisiae* grows for at least 2 log cycles in most grape juice fermentations prior to undergoing any significant decrease in viability; however, it should be emphasized that  $y$  refers to the whole set of species grouped under a same family (e.g., yeasts), and so  $\mu$  denotes a lumped specific rate of decrease in viable microorganisms for the whole family rather than for a single species. The fact that a specific rate of death should be utilized instead of a specific rate of growth was confirmed by a posteriori analysis of the sign of the numerical estimates for such parameter obtained by statistical regression analysis. Equation (1) may be integrated to yield

$$\mu_{ap} = -\frac{\ln [y/y_0]}{t} \quad (2)$$

where  $\mu_{ap}$  is the average value of the specific death rate over time period  $t$ . All experimental data  $y$  were converted to the form  $\mu_{ap}$  prior to application of the statistical analysis. The normal distribution of the residuals of each set of replicates with respect to their average, and their independence from the average values themselves, for the data in the form  $\mu_{ap}$ , was checked as depicted in Fig. 1; because no apparent bias could be detected, the statistical regression analysis based on the classical contrast table method was followed safely (Box et al., 1978).

## RESULTS AND DISCUSSION

### Identification

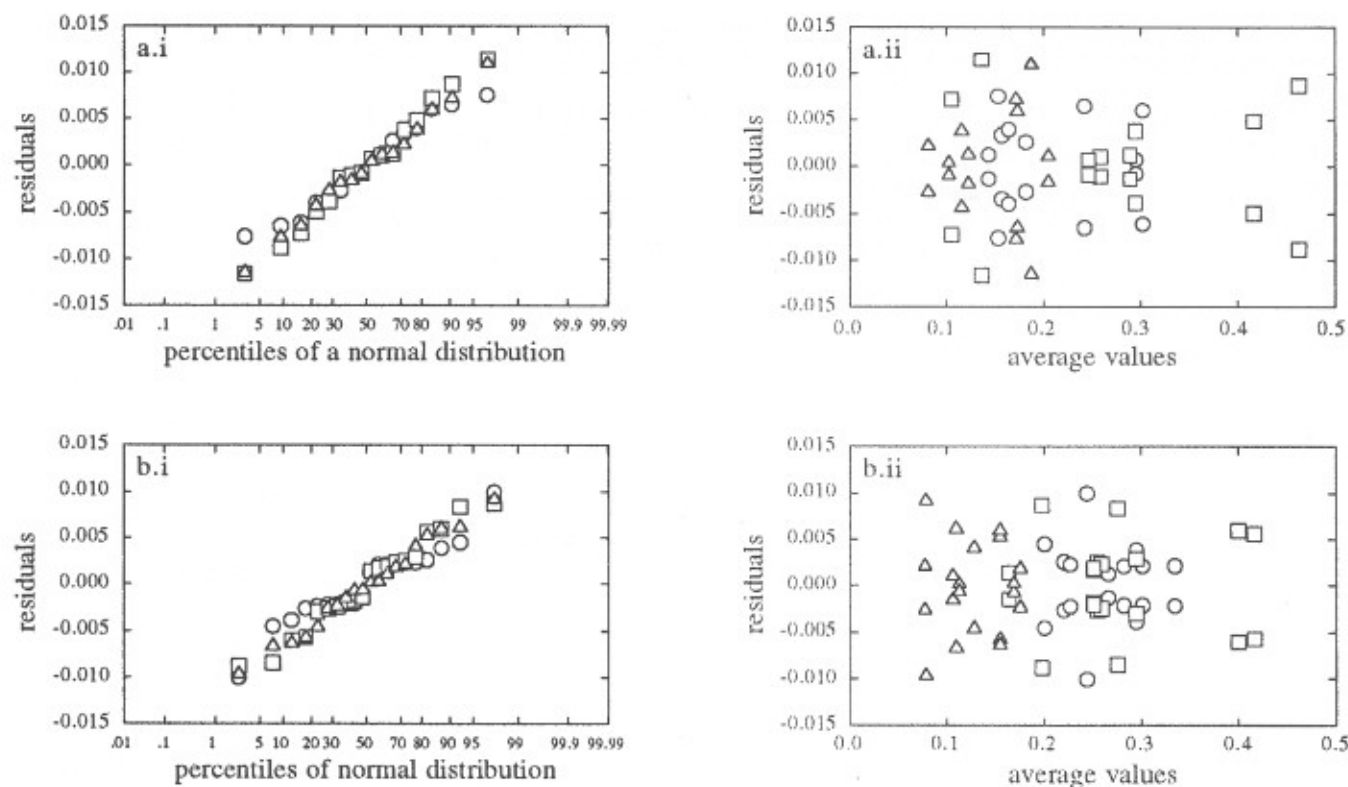
Of the species grown on YM, *Saccharomyces cerevisiae* (36% of all isolates), *Dekkera anomala* and *D. bruxellensis* (21%), *Rhodotorula auriantica* (14%), *R. mucilaginosa* (10%), *Pichia membranaefaciens* (10%), and *Candida versatilis* (9%) were identified. These results are somewhat expected in view of the dominant fermentative metabolism of these genera of yeasts (especially *S. cerevisiae*). These results are also consistent with several classical works pertaining to wine (rather than grape pomace) which indicate that there is a typical initial stage of macroscopic growth of apiculate yeasts, which is followed by a second stage where these genera clearly decline at the expense of the increase in numbers of *Saccharomyces cerevisiae* which will eventually dominate.

Of the species grown on WLN<sup>-</sup>, one was able to identify *Lactobacillus hilgardii* (58%), *L. brevis* (25%), and *Leuconostoc oenos* (17%); our results are in agreement with the conclusions by Vanderpoorten and Hogg (1994) using species clustering techniques.

The attempts to isolate acetic acid bacteria using the normally recommended selective media (i.e., WLN<sup>+</sup>) were seriously hampered by the ubiquitous presence of yeasts despite the inactivation of yeasts by cycloheximide. All colonies but one grown on the media in question were identified as yeasts; the exception proved to be *Acetobacter aceti*. For this reason, the enumeration data on WLN<sup>+</sup> refer almost exclusively to yeasts and will, as such, be considered hereafter.

### Enumeration

The initial counts for yeasts grown on WLN<sup>+</sup>, lactic acid bacteria grown on WLN<sup>-</sup>, and yeasts grown on YM for Alvarinho variety were  $1.9 \times 10^9$ ,  $1.8 \times 10^9$ , and



**Fig. 1.** Plot of the residuals of the replicates in the  $\mu_{ap}$  form pertaining to yeast grown on WLN<sup>+</sup> (○), lactic acid bacteria grown on WLN<sup>-</sup> (□), and yeasts grown on YM (△) versus the percentiles of a normal distribution (i) and versus their average (ii) for the Alvarinho variety (a) and for the dominant Loureiro variety (b).

**Fig. 1.** Representación de los valores residuales de las repeticiones de  $\mu_{ap}$  correspondiente a la levaduras crecidas en WLN<sup>+</sup> (○), bacterias lácticas crecidas en WLN<sup>-</sup> (□) y levaduras crecidas en YM (△) frente a los percentiles de una distribución normal (i) y frente a los valores medios (ii) para la variedad Alvarinho (a) y para la (dominante) variedad Loureiro (b)

$1.3 \times 10^9$ , respectively, whereas the corresponding counts for the (dominant) Loureiro variety were  $2.5 \times 10^8$ ,  $2.1 \times 10^8$ , and  $1.8 \times 10^8$ , respectively. Although it may be argued that the growth media utilized were not best suited for the enumerations in question because they were designed for the microflora of wine, it should be borne in mind that media specific for the microflora of grape pomace are not commercially available.

The transformed experimental values are listed in Tables 1 and 2 for every set of experimental conditions. Based on the results depicted in Table 1, the empiric models to be entertained by the data generated with the Alvarinho variety on the 1% level of significance are as follows:

$$\hat{\mu}_{ap,A} = 0.2053 + 0.0503 \left( \frac{t - 31.5}{10.5} \right) - 0.0186 (E - 1) - 0.0247 \left( \frac{t - 31.5}{10.5} \right) (E - 1) \quad (3)$$

for yeasts grown on WLN<sup>+</sup>,

$$\hat{\mu}_{ap,L} = 0.2762 + 0.0805 \left( \frac{T - 40}{40} \right) - 0.0787 \left( \frac{T - 40}{40} \right) \left( \frac{t - 31.5}{10.5} \right) \quad (4)$$

for lactic acid bacteria grown on WLN<sup>-</sup>, and

$$\hat{\mu}_{ap,Y} = 0.1450 - 0.0395 \left( \frac{t - 31.5}{10.5} \right) \quad (5)$$

for yeasts grown on YM. Following a similar reasoning applied to the results depicted in Table 2, the empiric models to be entertained by the data generated with the (dominant) Loureiro variety on the 1% level of significance are as follows:

$$\hat{\mu}_{ap,A} = 0.2600 + 0.0289 \left( \frac{t - 28}{7} \right) - 0.0184 (E - 1) \quad (6)$$



**Table 1.** Experimental design and experimental data obtained for the apparent specific growth rate of yeasts on WLN<sup>+</sup> ( $\mu_{ap,A}$ ), lactic acid bacteria on WLN<sup>-</sup>, and yeasts on YM ( $\mu_{ap,Y}$ ) for pomace of the Alvarinho variety.

**Tabla 1.** Diseño experimental y datos botenidos del crecimiento específico aparente de levaduras crecidas en WLN<sup>+</sup> ( $\mu_{ap,A}$ ), de bacterias crecidas en WLN<sup>-</sup> ( $\mu_{ap,L}$ ), y de levaduras crecidas en YM ( $\mu_{ap,Y}$ ) del bagazo de uva variedad Alvarinho.

R	r	E&I								MV		
		I	1	2	3	12	13	23	123	$\mu_{ap,A}$	$\mu_{ap,L}$	$\mu_{ap,Y}$
1	1	+1	+1	+1	+1	+1	+1	+1	+1	0.2488	0.2576	0.1033
	2									0.2358	0.2597	0.1019
2	1	+1	+1	+1	-1	+1	-1	-1	-1	0.3084	0.2881	0.1244
	2									0.2962	0.2906	0.1212
3	1	+1	+1	-1	+1	-1	+1	-1	-1	0.1539	0.4211	0.1795
	2									0.1607	0.4112	0.1646
4	1	+1	+1	-1	-1	-1	-1	+1	+1	0.1427	0.4716	0.1764
	2									0.1452	0.4541	0.1988
5	1	+1	-1	+1	+1	-1	-1	+1	-1	0.1851	0.2455	0.0785
	2									0.1798	0.2471	0.0835
6	1	+1	-1	+1	-1	-1	+1	-1	+1	0.2947	0.2984	0.1116
	2									0.2962	0.2906	0.1198
7	1	+1	-1	-1	+1	+1	-1	-1	+1	0.1687	0.1483	0.1795
	2									0.1607	0.1251	0.1670
8	1	+1	-1	-1	-1	+1	+1	+1	-1	0.1615	0.0980	0.2065
	2									0.1463	0.1124	0.2038
$\mu_{ap,A}$		0.2053	0.0062	0.0503	-0.0186	0.0105	0.0070	-0.0247	0.0063			
$\pm t\sigma$		$\pm 0.0066$	$\pm 0.0166$	$\pm 0.0166$	$\pm 0.0166$	$\pm 0.0166$	$\pm 0.0166$	$\pm 0.0166$	$\pm 0.0166$			
$\mu_{ap,L}$		0.2762	0.0805	-0.0040	-0.0118	-0.0787	-0.0076	-0.0080	0.0119			
$\pm t\sigma$		$\pm 0.0088$	$\pm 0.0220$	$\pm 0.0220$	$\pm 0.0220$	$\pm 0.0220$	$\pm 0.0220$	$\pm 0.0220$	$\pm 0.0220$			
$\mu_{ap,Y}$		0.1450	0.0012	-0.0395	-0.0128	0.0059	0.0039	-0.0009	-0.0002			
$\pm t\sigma$		$\pm 0.0079$	$\pm 0.0198$	$\pm 0.0198$	$\pm 0.0198$	$\pm 0.0198$	$\pm 0.0198$	$\pm 0.0198$	$\pm 0.0198$			

NOTE: EE—estimated effects; E&I—effects and interactions; I—grand average; MV—measured values; r—replicate; R—run; t—probability point of Student's *t*-distribution on the 1% level of significance with 16 degrees of freedom (mean) or 8 degrees of freedom (effects); 1—normalized level of addition of tartaric acid, defined as  $(T-40)/40$ ; 2—normalized time of fermentation, defined as  $(t-31.5)/10.5$ ; 3—normalized level of pectinase addition, defined as  $(E-1)$ ; and  $\sigma$ —standard deviation evaluated with 16 degrees of freedom (mean) or 8 degrees of freedom (effects).

for yeasts grown on WLN<sup>+</sup>,

$$\hat{\mu}_{ap,L} = 0.2767 + 0.0530 \left( \frac{T-40}{40} \right) - 0.0602 \left( \frac{T-40}{40} \right) \left( \frac{t-28}{7} \right) \quad (7)$$

for lactic acid bacteria grown on WLN<sup>-</sup>, and

$$\hat{\mu}_{ap,Y} = 0.1268 - 0.0350 \left( \frac{t-28}{7} \right) \quad (8)$$

for yeasts grown on YM. In the above statistical correlations,  $\mu_{ap,A}$  (in d<sup>-1</sup>) denotes the estimated average specific death rate of yeasts grown on WLN<sup>+</sup>,  $\mu_{ap,L}$  (in d<sup>-1</sup>) the estimated average specific death rate of lactic acid bacteria grown on WLN<sup>-</sup>,  $\mu_{ap,Y}$  (in d<sup>-1</sup>) the estimated average specific death rate of yeasts grown

on YM, *T* the amount of tartaric acid added (in g per 100 kg of grapes), *t* the fermentation time (in days), and *E* the amount of enzyme added (in g per 100 kg of grapes). The statistical adequacy of using least squares to estimate the various adjustable parameters in the aforementioned empiric models can be assessed through diagnostics of residuals (Fig. 1). Because no major deviations from linearity are observed in the normal plots, nor funnel-shaped tendencies in the residual versus average plots, it can be concluded that the errors are normally distributed and the variance is constant, which are two of the major assumptions underlying the validity of the reasoning utilized.

It is interesting to note that in the case of yeasts, both models [Eqs. (3) and (6)] yield similar results to a considerable extent. For intermediate conditions, it can

**Table 2.** Experimental design and experimental data obtained for the apparent specific growth rate of yeasts on WLN<sup>+</sup> ( $\mu_{ap,A}$ ), lactic acid bacteria on WLN<sup>-</sup>, and yeasts on YM ( $\mu_{ap,Y}$ ) for pomace of the (dominant) Loureiro variety.**Tabla 2.** Diseño experimental y datos botenidos del crecimiento específico aparente de levaduras crecidas en WLN<sup>+</sup> ( $\mu_{ap,A}$ ), de bacterias crecidas en WLN<sup>-</sup> ( $\mu_{ap,L}$ ), y de levaduras crecidas en YM ( $\mu_{ap,Y}$ ) del bagazo de uva variedad dominante Loureiro.

R	r	I	E & I							MV		
			1 (+234)	2 (+134)	3 (+124)	4 (+123)	12 (+34)	13 (+24)	23 (+14)	$\mu_{ap,A}$	$\mu_{ap,L}$	$\mu_{ap,Y}$
1	1	+1	+1	+1	+1	+1	+1	+1	+1	0.2655	0.2530	0.0751
	2									0.2681	0.2494	0.0799
2	1	+1	+1	+1	-1	-1	+1	-1	-1	0.2238	0.2530	0.1074
	2									0.2285	0.2582	0.1048
3	1	+1	+1	-1	+1	-1	-1	+1	-1	0.2989	0.3936	0.1604
	2									0.3031	0.4056	0.1495
4	1	+1	+1	-1	-1	+1	-1	-1	+1	0.2842	0.4216	0.1688
	2									0.2799	0.4103	0.1697
5	1	+1	-1	+1	+1	-1	-1	-1	+1	0.2052	0.2844	0.1134
	2									0.1962	0.2676	0.1126
6	1	+1	-1	+1	-1	+1	-1	+1	-1	0.2183	0.2618	0.0879
	2									0.2235	0.2571	0.0692
7	1	+1	-1	-1	+1	+1	+1	-1	-1	0.3329	0.1898	0.1489
	2									0.3372	0.2073	0.1611
8	1	+1	-1	-1	-1	-1	+1	+1	+1	0.2299	0.1626	0.1778
	2									0.2254	0.1655	0.1736
9	1	+1	0	0	0	-1	0	0	0	0.2912	0.2980	0.1325
	2									0.2988	0.2920	0.1239
10	1	+1	0	0	0	+1	0	0	0	0.2347	0.2494	0.1032
	2									0.2547	0.2532	0.1160
$\mu_{ap,A}$			0.2600	0.0115	0.0289	-0.0184	0.0099	0.0064	-0.0035	-0.0132		
$\pm t_{\sigma}$			$\pm 0.0050$	$\pm 0.0146$	$\pm 0.0146$	$\pm 0.0146$	$\pm 0.0119$	$\pm 0.0146$	$\pm 0.0146$	$\pm 0.0146$		
$\mu_{ap,L}$			0.2767	0.0530	-0.0170	0.0038	-0.0014	-0.0602	-0.0090	-0.0008		
$\pm t_{\sigma}$			$\pm 0.0060$	$\pm 0.0176$	$\pm 0.0176$	$\pm 0.0176$	$\pm 0.0144$	$\pm 0.0176$	$\pm 0.0176$	$\pm 0.0176$		
$\mu_{ap,Y}$			0.1268	-0.0018	-0.0350	-0.0036	-0.0088	-0.0002	-0.0071	0.0051		
$\pm t_{\sigma}$			$\pm 0.0058$	$\pm 0.0170$	$\pm 0.0170$	$\pm 0.0170$	$\pm 0.0138$	$\pm 0.0170$	$\pm 0.0170$	$\pm 0.0170$		

NOTE: EE—estimated effects; E&I—effects and interactions (including additive aliases); I—grand average; MV—measured values; r—replicate; R—run; t—probability point of Student's t-distribution on the 1% level of significance with 20 degrees of freedom (mean) or 8 degrees of freedom (all effects except 4), and 10 degrees of freedom (effect 4); 1—normalized level of addition of tartaric acid, defined as (T-40)/40; 2—normalized time of fermentation, defined as (t-28)/7; 3—normalized level of pectinase addition, defined as (E-1); 4—type of container (-1: plastic; +1: wood);  $\sigma$ —standard deviation evaluated with 20 degrees of freedom (mean), 8 degrees of freedom (all effects except 4), or 10 degrees of freedom (effect 4).

be said that the specific death rates range from  $\sim 0.21$  to  $0.26 \text{ d}^{-1}$  for yeasts grown on WLN<sup>+</sup>, from  $\sim 0.13$  to  $0.15 \text{ d}^{-1}$  for yeasts grown on YM, and  $\sim 0.28 \text{ d}^{-1}$  for lactic acid bacteria grown on WLN<sup>-</sup>. These nominal values yield approximate half-lives of 2.7–3.3 days for yeasts (WLN<sup>+</sup>), 2.5 days for lactic acid bacteria (WLN<sup>-</sup>), and 4.6–5.3 days for yeasts (YM).

The addition of tartaric acid plays a statistically significant role only for lactic acid bacteria grown on WLN<sup>-</sup>. The effect of pectinase action appears to be relevant only for yeasts (WLN<sup>+</sup>), in which case it is negative; one possible explanation would be that the

increasing availability of fermentable monosaccharides brought about by the hydrolytic action of the aforementioned enzyme upon pomace pectins enhances the metabolism of part of the strains present and hence balances, to a considerable extent, their death rate. The effect of fermentation time is ubiquitous for both grape varieties and all processing conditions. The container material does not affect to any considerable extent the microbial ecology prevailing in the fermenting grape pomace. Therefore, the interpretation of the experimental data generated for the (dominant) Loureiro variety may be redone based on a

posteriori assumption that the experiments were laid out as a full  $2^3$  factorial design (i.e., variable 4 in Table 2 would take the value 0 for both runs in experiment 10). Such analyses yield the same estimates for all parameters except for interaction 123 (which changes to 0.0187, 0.0037, and  $-0.0087$  for  $\mu_{ap,A}$ ,  $\mu_{ap,L}$ , and  $\mu_{ap,Y}$ , respectively) and yield narrower 99% confidence intervals for all effects and interactions (i.e.,  $\pm 0.0133$ ,  $\pm 0.0161$ , and  $\pm 0.0154$  for  $\mu_{ap,A}$ ,  $\mu_{ap,L}$ , and  $\mu_{ap,Y}$ , respectively) but not for the averages; in any case, the conclusions on the truly significant effects remain essentially the same.

In the experimental ranges studied, the specific rate of death of yeasts ( $WLN^+$ ) increases with time (probably due to the increasing toxicity of the grape pomace with time arising from the rising presence of alcohols). For the same microbial family, the second-order interaction between time and pectinase addition is statistically significant for the Alvarinho variety but not for the Loureiro (dominant) variety (although this fact may be the result of the combination of interaction 23 with the alias 14, which could be of opposite sign and hence lead to a result slightly below the threshold of statistical significance). The specific death rates of lactic acid bacteria increase with the tartaric acid content (probably due to the toxicity of this compound, especially in the undissociated form which prevails at the usual pH of grape pomace), but the interaction of the presence of tartaric acid with the fermentation time has an opposite effect (i.e., the effect of the presence of tartaric acid becomes attenuated as time elapses). The specific death rates of yeasts decrease significantly with storage time; it might be that the alcohol tolerance of some of the wild strains becomes apparent as the concentration of the alcohols increase with time. For similar times, the specific rates of death for both grape varieties remain virtually the same, irrespective of the amounts of tartaric acid or pectinases added.

Finally, it should be noted that two alternative methodologies may be followed in attempting to establish cause-effect relationships in natural (and thus unique) wine products: either prepare a synthetic medium which resembles the grape pomace matrix, or use the grape pomace from a given harvest, and in both cases, test the effect of one (or more) processing factor(s) using a convenient experimental design. Although the former may allow more fundamental conclusions to be obtained, extrapolation to reality is often poor because there is always a multitude of factors one is unaware of and which cannot be exactly paralleled in the artificial matrix. The latter does not suffer from this shortcoming, provided that experiments are duly replicated, because the effects of any factors which are not

considered per se will show up in lumped form in the estimates of variability. Although it may be argued that different conclusions may be drawn for grape pomace obtained from different harvests or handled in different ways, it should be noted that our models (and hence the conclusions obtained therefrom) have always associated confidence intervals; furthermore, the general conclusions encompassing two grape varieties obtained from two completely different locations are not too different from one another.

## FINAL REMARKS

It should be emphasized that the microbiological results available to date in the literature pertain only to wine of fresh grapes, and so complete critical assessment of the validity of our quantitative results cannot be made at this point. This communication attempts to shed some light into the characterization of the dynamic ecology prevailing in grape pomace in terms of lumped microbial genera; however, further in-depth work is required in order to fully understand the growth/death behavior of every microbial genus and correlate them with the depletion of sugars and the concomitant production of flavor compounds. Full success of this work is not anticipated unless specific media paralleling the intrinsic factors of grape pomace are duly developed.

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## REFERENCES

1. Asai, T (1971) The classification and biochemistry of acetic acid bacteria. In *Biochemical and Industrial Aspects of Fermentation* (K. Sakakuchi, T. Kemura, and S. Kuoshito, Eds.). Tokyo, Japan: Kodansha.
2. Barnett JA, Payne RW, Yarrow D (1984) *Yeasts—Characterization and Identification*. Cambridge: Cambridge University Press.

3. Barnett JA, Payne RW, Yarrow D (1990) *Yeasts Identification Program—Version 2*. Cambridge: Cambridge University Press.
4. Buchanan RE, Gibbono NE (1974) *Bergey's Manual of Determinative Bacteriology*. Baltimore, MD: Williams & Wilkins.
5. Box GEP, Hunter WG, Hunter JS (1978) *Statistics for Experimenters*. New York: Wiley.
6. Carr JG (1968) Identification of acetic acid bacteria. In: *Identification Methods for Microbiologists, Part B* (Gibbs BM and Shapton DA, eds.). London: Academic Press.
7. Gutiérrez J (1989) O primeiro vinho a ser exportado. *Alimentar* 4(19/20): 33–34.
8. Vanderpoorten W, Hogg TA (1994) Diversity of lactic acid bacterial strains in grape pomace. *IUMS Meeting (Applied Bacteriology and Microbiology Section)*.