JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Effect of Gluconic Acid Consumption during Simulation of Biological Aging of Sherry Wines by a Flor Yeast Strain on the Final Volatile Compounds

RAFAEL A. PEINADO,† JUAN J. MORENO,† JOSE M. ORTEGA,§ AND JUAN C. MAURICIO*,§

Departamento Química Agrícola y Edafología, Edificio C-3, and Departamento Microbiología, Edificio C-6, Campus Universitario Rabanales, Universidad de Córdoba, 14014 Córdoba, Spain

Flor yeast *Saccharomyces cerevisiae* (*capensis* G1) strain assimilates gluconic acid during the aerobic biological aging process of sherry wines and exerts significant changes on the final volatile compounds of wines, especially a decrease in volatile acidity and butanoic, isobutanoic, 2-methylbutanoic, and 3-methylbutanoic acids. This decrease may have a favorable effect on the quality of sherry wines.

KEYWORDS: Gluconic acid; flor yeast; wine; volatile compounds

INTRODUCTION

The presence of mycoses caused by *Botrytis cinerea* in the form of "noble rot" in grapes gives rise to special wines such as the French Sauternes or the Hungarian Tokay; very often, however, it is also the origin of severe problems in the wine-making process (*1*). Mycosis occurs during the grape ripening stage, and its development is influenced by meteorological factors such as moisture and rainfall and physiological factors such as vine variety and bunch shape (*2*, *3*).

Rot increases the activity of oxidase enzymes such as tyrosinase and laccase, which cause substantial color changes in wine; also, these enzymes increase the amount of dry extract (through the formation of glycerol and some polysaccharides), decrease titratable acidity, and increase volatile acidity as a result of *B. cinerea* infections being accompanied by the presence of acetic bacteria of the genera *Acetobacter* and *Gluconobacter* (*4*, *5*). The type of rot affecting grapes and its extent are usually determined from the gluconic acid content in the resulting must (*1*, *6*); thus, content above $\sim$1 g L^{-1} reflects a substantial proportion of rotten grapes. Recently, Couto et al. (*7*) have put in evidence that low levels of gluconic acid (up to 1$-$2 g L^{-1}) seem to indicate an initial stage of grape infection mainly by fungi (*B. cinerea*), whereas higher levels (up to 2$-$3 g L^{-1}) might be interpreted as the result of the activity of acetic acid bacteria.

Oxidase activity and bacterial contamination can be reduced by using appropriate amounts of sulfur dioxide; also, the filtering problems derived from the presence of polysaccharides can be lessened by using appropriate enzymes. However, the sensory properties of wine are altered by the presence of gluconic acid, which additionally renders it microbiologically unstable and results in long-term storage problems that can be solved only by reducing the concentration of the acid (*1*).

Sherry and Montilla-Moriles fino wines are biologically aged in American oak casks for several years in the presence of so-called "flor yeasts", which form a biofilm (flor velum) on the wine surface, where they grow on an aerobic metabolism (*8*). Such metabolism involves the consumption of ethanol, glycerol, acetic acid, and ethyl acetate and the production of acetaldehyde and some of its derivatives in addition to organic acids with four carbon atoms (*9*, *10*).

A decrease in the content of gluconic acid and an increase in volatile acidity during biological aging processes have been ascribed to the presence of lactic bacteria (*1*). These bacteria metabolize gluconic acid and produce lactic acid, glycerol, ethanol, acetic acid, and carbon dioxide (*11*). An increased volatile acidity is a recurrent result in wines from rotten grapes, which darken during the aging process, all of which has an adverse effect on the resulting wine.

Experiments with pure cultures of the flor yeast *Saccharomyces cerevisiae* (*capensis* G1) strain revealed consumption of gluconic acid when the yeasts were subjected to the aging conditions at their flor biofilm stage and hence adapted to glycerol consumption (*12*). In this respect, Peinado et al. (*13*) reported faster cell proliferation of *S. cerevisiae* in media containing glucono-δ-lactone as the sole carbon source when the yeasts were previously cultured in glycerol. Recently, a novel pathway for alcoholic fermentation of glucono-δ-lactone in the yeast *Saccharomyces bulderi* has been reported (*14*).

In this work, various tests were conducted on pure cultures of the flor yeast *S. cerevisiae* (*capensis* G1) strain with a view to identifying the conditions under which it consumes gluconic acid and determining the influence of such consumption on the contents in volatile compounds of fino wines obtained by biological aging.

* Corresponding author [e-mail mi1gamaj@uco.es; telephone (+34) 957218640; fax (+34) 957218650].
† Departamento Química Agrícola y Edafología.
§ Departamento Microbiología.

10.1021/jf034512j CCC: $25.00
Published on Web 09/03/2003

MATERIALS AND METHODS

Yeast Strain and Inocula. In this study a *S. cerevisiae* (*capensis* G1) strain (*15*) was used from the collection of the Microbiology Department of the University of Córdoba (Spain). This flor yeast prevails together with *Saccharomyces bayanus* in the biofilms formed on the surface of wines obtained by biological aging in the Montilla-Moriles region (Córdoba, southern Spain).

Two types of inocula were employed that were obtained from yeast cells growing on YM medium (0.3% yeast extract, 0.3% malt extract, and 0.5% peptone, pH 6.5) containing 50 g L^{-1} glucose (Merck) in one case and 30 g L^{-1} glycerol (Merck) in the other. Both were incubated at 27 ± 2 °C with shaking for 72 h. Yeast cells were collected by centrifugation at 5000 rpm and used to inoculate the different media with a population of 10^6 viable cells mL^{-1}.

The number of total and alive cells was determined by counting under a light microscope in a Thoma chamber following staining with Methylene Blue (*16*).

Culture Media. Synthetic culture media were used from the YNB without amino acids (Difco) containing 5 g L^{-1} glucose, 5 g L^{-1} gluconic acid (Sigma), and either 15% (v/v) ethanol (Merck) or 10 g L^{-1} glycerol, or both, as carbon source, and a control medium with 5 g L^{-1} gluconic acid as the sole carbon source. Wine from healthy Pedro Ximénez grapes was used for control experiments versus the same wine supplied with 5.1 g L^{-1} gluconic acid, because it is the highest quantity of acid reported in the study area. All of these media were sterilized by passage through Supra EK filters (Seitz, Germany). All tests were conducted in triplicate.

Experimental Conditions. All tests were carried out in 250 mL flasks, with or without shaking. Shaken samples were thermostated at 27 ± 2 °C and unshaken samples at 23 ± 2 °C. The volume of synthetic medium or wine used was that resulting in a surface/volume ratio of 17 cm^2 L^{-1}, which is the typical value for the traditional biological aging of sherry wines in oak casks.

Changes in the concentrations of gluconic acid and volatile compounds were studied in 15 flasks containing the same wine at 23 ± 2 °C without shaking. Nine flasks were supplied with the same amount of gluconic acid, and the other six, which were used as controls, had no gluconic acid. Control flasks were examined at 0 and 105 days (the end of the experiment), and the others at 40, 70, and 105 days. All wines were simultaneously inoculated with 10^6 viable cells mL^{-1} from a *S. cerevisiae* (*capensis* G1) culture grown in YM medium containing glycerol as the carbon source.

Exhaustive controls as visual in the culture medium flasks, microscopic examination, and samples pouring over the surface of a plate (Petri dish) were carried out during all of the experiences to detect possible contaminations of lactic bacteria that could consume gluconic acid.

Analyses. Ethanol was quantified by dichromate oxidation (*17*); titratable acidity, pH, residual sugars, and volatile acidity were determined with the official methods (*18*); gluconic acid was quantified using an enzymatic test from Boehringer-Mannheim (Germany) at pH 11; and the absorbances at 280, 420, and 520 nm were measured on a Beckman DU-640 UV spectrophotometer.

Major volatile compounds and polyols were determined by gas chromatography on an Agilent 6890 GC model (Palo Alto, CA), using the method of the OIV (*19*) as modified by Muñoz et al. (*20*). Capillary column CP-WAX 57 CB (60 m long; 0.25 mm i.d.; 0.4 μm film thickness) was used, and 0.5 μL aliquots of 10 mL wine samples previously supplied with 1 mL of 1 g L^{-1} 4-methyl-2-pentanol as internal standard were injected. Tartaric acid in the wine was removed by precipitation with 0.2 g of calcium carbonate, followed by centrifugation at 3000 rpm.

Quantification was based on the response factors obtained for standard solutions of each compound. A split ratio of 30:1, an FID, and a temperature program involving an initial temperature of 50 °C (15 min), a 4 °C min^{-1} ramp, and a final temperature of 190 °C (35 min) were used. The injector and detector temperatures were 270 and 300 °C, respectively. The flow rate of carrier gas (helium) was initially set at 0.7 mL min^{-1} (16 min) and followed by a 0.2 mL min^{-1} ramp to the final value (1.1 mL min^{-1}), which was held for 52 min.

Table 1. Gluconic Acid Consumption by *S. cerevisiae* (Capensis G1) Flor Yeast Strain in Wines and YNB (without Amino Acids) Culture Media Containing Gluconic Acid, Different Sources of Carbon, and Conditions

medium[a]	glucose (g L^{-1})	glycerol (g L^{-1})	ethanol (% v/v)	pH	shaking	gluconic acid (g L^{-1})	consumption of gluconic acid (%)	days
1 (a)	5	0	0	5.5	no	5	0	90
2 (a)	5	10	0	5.5	no	5	0	90
3 (a)	5	0	15	5.5	no	5	0	90
4 (a)	5	10	15	5.5	no	5	0	90
5 (b)	0	0	0	3.0	yes	5	0	28
6 (b)	0	0	0	7.0	yes	5	0	28
7 (b)	0	10	0	4.0	yes	4.5	40	18
wine (a)	0	9.5	14.05	3.6	yes	4.5	43	100
wine (b)	0	9.5	14.05	3.6	yes	5	66	70

[a] Two inocula have been assayed with the yeast strain tested: (a) yeast grown in medium containing glucose and (b) yeast grown in medium containing glycerol.

Minor volatile compounds were determined following continuous extraction for 24 h of 100 mL of wine at pH 3.5, which was supplied with 5 mL of internal standard (30 mg L^{-1} 2-octanol) and 100 mL of Freon-11. The Freon extracts containing the volatile compounds were concentrated to 0.2 mL in a Kuderna−Danish microconcentrator, and 1.5 μL aliquots were injected into an HP-6890 chromatograph equipped with an HP MS 5972 mass detector from Agilent Technologies. An HP-Innowax 60 m long × 0.32 mm i.d. capillary column (0.25 μm film thickness) was used. The oven was held at 40 °C for 10 min, which was followed by a 1 °C min^{-1} ramp to 180 °C (held for 35 min). Helium at a constant flow rate of 0.9 mL min^{-1} was used as carrier gas. The split ratio was 30:1, and the MS detector was set at 1612 V in the scan mode in order to sweep the mass range from 39 to 300 amu.

Retention time, Wiley mass spectral library, and pure volatile compounds from Merck, Sigma-Aldrich, Riedel de Haën, and Fluka were used for identification, confirmation, and preparation of standard solutions of volatile compounds. Quantification was based on the response factors calculated from standard solutions that were subjected to the same extraction process as the samples, using the target and qualifier ions selected for each compound by the Hewlett-Packard Chemstation.

Statistical Treatment. The statistical software package Statgraphics Plus v. 2, from STSC, Inc. (Rockville, MD), was used to perform a single analysis of variance (ANOVA) to identify those compounds exhibiting significant differences with the controls at the end of the tests.

All results reported herein are the average of all three experiments.

RESULTS AND DISCUSSION

As can be seen from **Table 1**, no consumption of gluconic acid was observed in the tests with YNB without amino acid media to which 5 g L^{-1} glucose, 5 g L^{-1} gluconic acid, and either 15% (v/v) ethanol or 10 g L^{-1} glycerol or both were added, even though the yeast cells cultured previously in glucose or glycerol grew in all media, where they formed a thin surface film (media 1, 2, 3, and 4).

Tests with the same base media (media 5 and 6) but containing gluconic acid as sole carbon source, at pH 3.0 or 7.0, were also conducted with a view to determining the influence of the molecular form of the acid on its consumption by the yeast cells cultured previously in glycerol, because the acid occurs largely as glucono-δ-lactone (the cyclic form) at low pH values and as gluconate ion in neutral or alkaline media. Tests were finished after 28 days, with no apparent cellular yeast growth or gluconic acid consumption.

Only in the tests involving synthetic culture media with glycerol (medium 7) and wines enriched with 5 g L^{-1} gluconic

Table 2. Wine-Making Variables and Gluconic Acid Values in the Control Wines and Those Supplied with 5.1 g/L Gluconic Acid

compound	wine + gluconic acid, 0 days	control wine, 0 days	wine + gluconic acid, 105 days	control wine, 105 days	*p* value[a]
residual sugars (g L^{-1})	2.2 ± 0.2	2.2 ± 0.2	2.2 ± 0.3	2.2 ± 0.2	
ethanol (% v/v)	14.05 ± 0.03	14.05 ± 0.03	8.3 ± 0.1	8.25 ± 0.6	
viable yeast (10^6 cells mL^{-1})	1.0 ± 0.1	1.0 ± 0.1	8.8 ± 1.9	9.3 ± 2.2	
gluconic acid (g L^{-1})	5.1 ± 0.1	0.0 ± 0.0	3.73 ± 0.02	0.0 ± 0.0	
volatile acidity (mequiv L^{-1})	7.7 ± 0.3	4.3 ± 0.3	2.0 ± 0.2	2.8 ± 0.5	
pH	3.21 ± 0.02	3.34 ± 0.01	3.21 ± 0.05	3.51 ± 0.05	
titartable acidity (mequiv L^{-1})	56.5 ± 0.2	40.5 ± 0.2	47.2 ± 0.2	35.3 ± 0.1	
absorbance at 280 nm	8.91 ± 0.05	8.91 ± 0.05	8.27 ± 0.11	8.22 ± 0.05	
absorbance at 420 nm	0.161 ± 0.001	0.161 ± 0.001	0.106 ± 0.002	0.099 ± 0.003	*
absorbance at 520 nm	0.0374 ± 0.0004	0.0374 ± 0.0004	0.0108 ± 0.0005	0.013 ± 0.001	*

[a] *p* values obtained by ANOVA for the wines at day 105: *, 0.05; **, 0.01; ***, 0.001.

acid and subjected to continuous agitation was the acid concentration found to decrease with time; the decrease amounted to 43% with the yeasts that were previously cultured in glucose and to 66% with those grown in glycerol. These results are consistent with those of Peinado et al. (*13*), who studied the adaptation of yeasts to the consumption of gluconic acid (added as glucono-*δ*-lactone), and those of Cortés et al. (*12*), who found the acid to be consumed in sherry type wines, by both pure and enriched cultures with the *S. cerevisiae* (*capensis* G1) strain.

As gluconic acid content may be decreased by lactic acid bacteria, the possible presence of these kinds of bacteria during all of the experiments have been strictly checked as described under Materials and Methods, so all of the studied experiments were lactic acid bacteria free.

In summary, the results obtained with regard to the consumption of gluconic acid can be ascribed to the following conditions: aerated media containing glycerol but no glucose or exhausted. The specific carrier for gluconic acid is not yet identified in *S. cerevisiae*. We suggest two possible hypotheses: first, the gluconic acid transporter of yeast could be repressed by glucose and derepressed on glycerol media; or, second, the gluconic acid lacks a specific carrier in *S. cerevisiae* and it can only enter in the yeast via some carrier. The physiological characterization of glycerol uptake in *S. cerevisiae* has revealed that glycerol can enter the cell in at least two different ways depending on the particular growth conditions. Thus, when glucose is present, glycerol can enter cells by facilitated transport, probably through the glycerol channel, encoded by the *FPS1* gene (*21*−*24*). By contrast, in cells that have been cultured in ethanol, glycerol, or acetate, a proton symport system ensures uptake of the glycerol (*23*, *25*, *26*). The last inducible active uptake may be the origin of gluconic acid entering *S. cerevisiae* cells in the absence of glucose.

To confirm whether gluconic acid consumption by *S. cerevisiae* cells growing as a biofilm (flor velum) on wine during its biological aging and to determine its effect on the volatile fraction of wine, a new experiment involving the addition of gluconic acid to healthy wines and their inoculation with pure cultures of yeast cells previously grown in glycerol was conducted over a period of 105 days with no shaking. In this way, the physicochemical and microbiological conditions under which wines with a high gluconic acid content are biologically aged on an industrial scale were reproduced in the laboratory.

Table 2 shows the wine-making variables and gluconic acid value of control wines and those supplemented with gluconic acid. Only pH and titratable and volatile acidity values were affected by the addition of gluconic acid. The effect on volatile acidity was a result of the commercially available gluconic acid used containing 1% acetic acid. All other compounds determined in the wines at the beginning of the tests exhibited no detectable changes by the effect of the addition of gluconic acid; their contents remained at the same levels as those in the control wine without gluconic acid addition (**Tables 3** and **4**).

Table 3. Final Contents of Major Volatile Compounds and Polyols in the Control Wines and Those Supplied with 5.1 g/L Gluconic Acid

compound	control wine, 0 days	wine + gluconic acid, 105 days	control wine, 105 days	*p* value[a]
acetaldehyde (mg L^{-1})	112 ± 4	222 ± 11	254 ± 22	
ethyl acetate (mg L^{-1})	34 ± 2	4 ± 1	4.3 ± 0.4	
1,1-diethoxyethane (mg L^{-1})	0 ± 0	1.0 ± 0.6	0.7 ± 0.5	
methanol (mg L^{-1})	49 ± 1	20 ± 1	20 ± 4	
1-propanol (mg L^{-1})	24.9 ± 0.7	24.0 ± 1.0	21.0 ± 1.0	*
isobutanol (mg L^{-1})	47 ± 1	31 ± 2	29.8 ± 0.6	
isoamyl alcohols (mg L^{-1})	316 ± 14	167 ± 7	154 ± 5	
acetoin (mg L^{-1})	21 ± 8	120 ± 8	129 ± 9	
ethyl lactate (mg L^{-1})	58 ± 4	35 ± 5	32 ± 1	
2,3-butanediol, levo (mg L^{-1})	615 ± 43	791 ± 110	668 ± 16	
2,3-butanediol, meso (mg L^{-1})	157 ± 14	296 ± 38	268 ± 3	
diethyl succinate (mg L^{-1})	10.4 ± 0.3	13.9 ± 2.0	14 ± 2	
2-phenylethanol (mg L^{-1})	74 ± 4	94 ± 9	96 ± 6	
glycerol (g L^{-1})	9.4 ± 1.4	0 ± 0	0 ± 0	

[a] *p* values obtained by ANOVA for the wines at day 105: *, 0.05; **, 0.01; ***, 0.001. No detectable change by gluconic acid addition was observed in the studied compounds at 0 days.

Fifteen days after inoculation with 10^6 viable cells mL^{-1}, the control wines and those containing gluconic acid exhibited a rough, ivory biofilm ∼3 mm thick virtually throughout their surface. This velum is typically formed by *S. cerevisiae* (*capensis* G1) strain growing on an aerobic metabolism and contributes to the special features of biologically aged sherry wines (*9*).

At the end of the tests (day 105), an overall 1.38 g L^{-1} gluconic acid (27% of the amount added to the wines) was found to have been consumed. The wines supplied with gluconic acid also exhibited significant differences at the 95% confidence level in titratable acidity, pH, A_{420}, and A_{520} that can be ascribed to the effect of residual gluconic acid. In addition, the wines supplied with gluconic acid exhibited a more marked reduction in volatile acidity (from 7.7 to 2.0 mequiv L^{-1}) than the control wines (from 4.2 to 2.8 mequiv L^{-1}).

The contents in methanol, major higher alcohols (viz., isobutanol, isoamyl alcohol, and 2-phenylethanol), ethyl esters of acetic, lactic, and succinic acids, and carbonyl compounds (acetaldehyde, 1,1-diethoxyethane, and acetoin) were not influenced by the presence of gluconic acid in the wines. Nor were the contents in polyols (viz., levo- and meso-2,3-butanodiol

Table 4. Initial and Final Contents in Minor Volatile Compounds in the Control Wines and in Those Supplied with 5.1 g/L Gluconic Acid

compound	control wine, 0 days	wine + gluconic acid, 105 days	control wine, 105 days	*p* value[a]
ethyl propanoate (μg L^{-1})	163 ± 16	956 ± 93	1303 ± 95	*
propyl acetate + 2,3-butanedione (μg L^{-1})	318 ± 31	183 ± 22	106 ± 15	**
isobutyl acetate (μg L^{-1})	48 ± 3	15 ± 3	19 ± 2	
2-butanol (μg L^{-1})	1964 ± 200	1069 ± 113	1140 ± 276	
ethyl butanoate (μg L^{-1})	150 ± 6	53 ± 5	89 ± 19	*
2,3-pentanedione (μg L^{-1})	0 ± 0	2003 ± 185	2829 ± 601	
isoamyl acetate (μg L^{-1})	592 ± 50	26 ± 3	47 ± 12	*
1-butanol (μg L^{-1})	2683 ± 202	1948 ± 124	2283 ± 326	
limonene (μg L^{-1})	128 ± 17	147 ± 24	120 ± 15	
ethyl hexanoate (μg L^{-1})	116 ± 3	0 ± 0	0 ± 0	
3-methyl-1-pentanol (μg L^{-1})	178 ± 7	98 ± 4	158 ± 5	***
hexanol-1 (μg L^{-1})	764 ± 15	264 ± 26	214 ± 27	
3-ethoxy-1-propanol (μg L^{-1})	0 ± 0	566 ± 50	507 ± 50	
furfural (μg L^{-1})	83 ± 3	0 ± 0	0 ± 0	
ethyl octanoate (μg L^{-1})	11 ± 2	120 ± 11	93 ± 6	**
ethyl 3-hydroxybutyrate (μg L^{-1})	687 ± 43	1215 ± 130	1255 ± 68	
linalool (μg L^{-1})	0 ± 0	28 ± 3	26 ± 5	
1-octanol (μg L^{-1})	531 ± 10	223 ± 27	221 ± 3	
isobutanoic acid (μg L^{-1})	1604 ± 72	6906 ± 449	10303 ± 548	**
γ-butyrolactone (μg L^{-1})	19380 ± 1327	32653 ± 3894	27393 ± 3562	
butanoic acid (μg L^{-1})	1741 ± 97	3779 ± 83	4416 ± 146	**
2-methylbutanoic acid + 3-methylbutanoic acid (μg L^{-1})	1175 ± 42	3618 ± 505	6608 ± 292	***
furfuryl alcohol (μg L^{-1})	239 ± 14	498 ± 39	498 ± 188	
neral (μg L^{-1})	56 ± 9	93 ± 12	146 ± 11	**
methionol (μg L^{-1})	4783 ± 432	3905 ± 330	8473 ± 1121	**
β-citronellol (μg L^{-1})	37 ± 3	0 ± 0	0 ± 0	
2-phenylethyl acetate (μg L^{-1})	347 ± 11	56 ± 10	212 ± 21	***
hexanoic acid (μg L^{-1})	1400 ± 45	0 ± 0	0 ± 0	
benzyl alcohol (μg L^{-1})	383 ± 49	452 ± 30	474 ± 51	
4-ethylguaiacol (μg L^{-1})	90 ± 3	89 ± 11	88 ± 11	
pantolactone (μg L^{-1})	392 ± 8	1671 ± 63	2058 ± 293	
Z-nerolidol (μg L^{-1})	27.05 ± 0.05	76 ± 12	64 ± 2	
diethyl malate (μg L^{-1})	525 ± 23	406 ± 45	487 ± 54	
octanoic acid (μg L^{-1})	2582 ± 68	0 ± 0	0 ± 0	
4-ethylphenol (μg L^{-1})	12.9 ± 0.4	15.7 ± 0.6	14 ± 2	
decanoic acid (μg L^{-1})	537 ± 57	0 ± 0	0 ± 0	

[a] *p* values obtained by ANOVA for the wines at day 105: *, 0.05; **, 0.01; ***, 0.001. No detectable change by gluconic acid addition was observed in the studied compounds at 0 days.

and glycerol) affected by the metabolization of the acid by yeast cells (**Table 3**). In fact, only the 1-propanol contents exhibited significant differences at the confidence level of $p \leq 0.05$.

The content changes shown in **Table 3** are typical of a biological aging process and fall within reported ranges (*9*, *27*); however, acetaldehyde was produced in lesser amounts on average, and the opposite was true of the isomers of 2,3-butanediol obtained in the wines containing gluconic acid. These results suggest that gluconic acid may somehow influence the equilibrium involving acetaldehyde and 2,3-butanediol, both of which play prominent roles in biological aging processes (*28*).

It should also be noted that the populations of living yeast cells at the end of the tests were similar in the control wine and in the wine supplied with gluconic acid (**Table 2**).

Table 4 shows the contents in minor volatile compounds in the wines. The 38 minor compounds determined at day 105 exhibited the same variations with respect to the initial contents, both in the control wines and in those supplied with gluconic acid. Only methionol (3-methylthio-1-propanol) exhibited a decrease in its content during the aging process in the wines containing gluconic acid and an increase in control wines.

Only 14 minor volatile compounds exhibited significant differences between the two types of wine at the end of the study. Thus, ethyl propanoate, ethyl butanoate, isoamyl acetate, 3-methyl-1-pentanol, isobutanoic acid, butanoic acid, 2-methylbutanoic acid + 3-methylbutanoic acid, methionol, neral, and 2-phenylethyl acetate were found in higher contents and propyl acetate + 2,3-butanedione and ethyl octanoate in lower concentrations in the control wines.

Butanoic acid and isobutanoic acid, and the combination of 2-methylbutanoic and 3-methylbutanoic acids, are typical products of the metabolism of flor yeasts in their aerobic development stage (*9*). The fact that these acids were obtained in lesser amounts in the wines that were supplied with gluconic acid must be related to the presence of the acid and its metabolism.

The significant differences observed in the above-mentioned volatile compounds may be ascribed to the involvement of gluconic acid in the metabolic pathways of *S. cerevisiae* flor yeast. Thus, yeast cells previously cultured in glycerol-containing media probably metabolize some of the gluconic acid via the pentose phosphate pathway (*29*), similarly to other wine microorganisms such as heterofermentative lactic bacteria (*30*) or some yeast species such as *Schizosaccharomyces pombe* (*31*, *32*); in this way, they can inhibit the formation of the above-described byproducts. In fact, glucono-δ-lactone is known to induce enzymes of the oxidative part of the pentose phosphate pathway in *S. cerevisiae* (*33*).

Table 5 shows the variation of the contents in those compounds that exhibited significant differences at the end of the study between the control wines and those supplied with gluconic acid.

The greatest variations in the contents of volatile compounds were normally observed during the first 40 days after inoculation with *S. cerevisiae* (*capensis* G1) strain, while the yeast cells

Table 5. Variation of the Contents in Gluconic Acid, Glycerol, and the Minor Volatile Compounds That Exhibited Significant Differences at the End of the Biological Aging Experience between Control Wine and Those Supplemented with Gluconic Acid

compound	0 days	40 days	70 days	105 days
gluconic acid (g L^{-1})	5.1 ± 0.1	4.83 ± 0.06	4.40 ± 0.05	3.73 ± 0.02
glycerol (g L^{-1})	9 ± 1	1.5 ± 0.4	0 ± 0	0 ± 0
propyl acetate + 2,3-butanedione (μg L^{-1})	318 ± 31	149 ± 12	121 ± 9	183 ± 22
isoamyl acetate (μg L^{-1})	592 ± 50	101 ± 10	62 ± 5	26 ± 3
2-phenethyl acetate (μg L^{-1})	347 ± 11	227 ± 17	116 ± 10	56 ± 10
ethyl butanoate (μg L^{-1})	150 ± 6	122 ± 12	82 ± 7	53 ± 5
ethyl octanoate (μg L^{-1})	11 ± 2	104 ± 12	121 ± 10	120 ± 11
ethyl propanoate (μg L^{-1})	163 ± 16	2353 ± 231	1517 ± 132	956 ± 93
neral (μg L^{-1})	56 ± 9	272 ± 33	87 ± 8	93 ± 12
3-methyl-1-pentanol (μg L^{-1})	178 ± 7	213 ± 2	152 ± 13	98 ± 4
methionol (μg L^{-1})	4783 ± 432	3104 ± 118	3926 ± 341	3905 ± 330
butanoic acid (μg L^{-1})	1741 ± 97	3954 ± 223	3232 ± 281	3779 ± 83
isobutanoic acid (μg L^{-1})	1604 ± 72	8762 ± 625	6914 ± 601	6906 ± 449
2-methylbutanoic acid + 3-methylbutanoic acid (μg L^{-1})	1175 ± 42	3086 ± 190	3371 ± 293	3618 ± 505

formed the flor velum on the wine surface. During that period, the contents in propyl and isoamyl acetates, and that of 2-phenylethanol, decreased markedly, and so did that of ethyl butanoate, albeit to a lesser extent. By contrast, the contents in other esters such as ethyl octanoate and (especially) ethyl propanoate increased during this period.

The above-mentioned esters exhibited an identical behavior in all wines subjected to the same aging conditions with the same yeast; this is consistent with previous results (*9*, *10*) and can be ascribed to the activities of esterases (hydrolysis and synthesis), which are especially active at the early flor velum formation stages (*34*).

The content in neral, which is a monoterpene involved in the early stages of sterol formation by the yeasts, also increased over the first 40 days and then decreased to levels twice as high as the initial ones. Flor yeasts synthesize monoterpenes in wines from healthy grapes (*35*). On the other hand, the content in the sulfur-containing alcohol methionol decreased by 35% during the first few days and then leveled off at concentrations similar to the starting values. One other alcohol (3-methyl-1-pentanol) exhibited a slight tendency to decrease in content during the tests.

The compounds exhibiting the greatest changes under the effect of flor yeasts were butanoic and isobutanoic acids, and the combination of 2-methylbutanoic and 3-methylbutanoic acids, the contents of which grew by a factor of 2 or 4 within the first 40 days and then remained virtually unchanged throughout the rest of the study. Gluconic acid was consumed at a rate of 7 mg L^{-1} day^{-1} during the first 40 days and at a rate of 14 mg L^{-1} day^{-1} afterward, coinciding with the leveling off in the contents of the previous acids and the disappearance of glycerol from the medium (**Table 5**).

The contents in acids with four and five carbon atoms increase during the aging of wine containing no gluconic acid in the presence of the same yeast strain [*S. cerevisiae* (*capensis* G1)], particularly between days 30 and 120 after formation of the flor velum (*10*); their contents were higher than those obtained in this work, and this decrease can be considered a favorable effect on wine quality as these compounds are associated with cheese, butter, and sweat odors, which are detrimental to the aroma of sherry-type fino wines.

This paper investigates a new area of sherry wine elaboration by flor yeasts. In fact, assimilation of gluconic acid during such aerobic process seems, from the obtained results, to exert a significant impact on the final volatile compounds of fino wines. From this point of view, future investigations on the studied subject for a possible application of the results to the wine industry will be carried out with higher volumes in a designed and patented device by our research group (Patent P9702139), which produces an acceleration of the process of biological aging by aeration without destroying the flor velum (*10*).

LITERATURE CITED

(1) Pérez, L.; Valcarcel, M. J.; González, P.; Domecq, B. Influence of *Botrytis* infection of the grapes on the biological aging process of Fino sherry. *Am. J. Enol. Vitic.* **1991**, *42*, 58−62.

(2) Suárez, J.; Iñigo, B. Microbial alterations in wines. Yeasts and molds. In *Enological Microbiology*; Mundi-Prensa: Madrid, Spain, 1990; pp 331−347.

(3) Reynier, A. Leaf and clusters diseases. In *Viticulture Manual*; Mundi-Prensa: Madrid, Spain, 2002; pp 405−464.

(4) Joyeux, A.; Lafon-Lafourcade, S.; Ribereau-Gayon, P. Evolution of acetic acid bacteria during fermentation and storage of wine. *Appl. Environ. Microbiol.* **1984**, *48*, 153−156.

(5) Joyeux, A.; Lafon-Lafourcade, S.; Ribéreau-Gayon, P. Metabolism of acetic acid bacteria in grape must: consequences on alcoholic and malolactic fermentation. *Sci. Aliment.* **1984**, *4*, 247−255.

(6) Chauvet, S. Application of isotachophoresis to analyses of the major acids in wine. Contribution to the study of the relationship between the intensity of the development of the noble rot and the chemical composition of the sweet white wines. Ph.D. Thesis, University of Bordeaux II, 1981.

(7) Couto, J. A.; Graça, A. R.; Soares-Franco, J. M.; Hogg, T. The use of gluconic acid level as an indicator of the activity of acetic acid bacteria in grapes. Presented at the VII Symposium International d'Oenologie, Bordeaux, France, 2003.

(8) Martínez, P.; Valcárcel, M. J.; Pérez, L.; Benítez, T. Metabolism of *Saccharomyces cerevisiae* flor yeasts during fermentation and biological aging of fine sherry: by-products and aroma compounds. *Am. J. Enol. Vitic.* **1988**, *49*, 240−250.

(9) Cortés, M. B.; Moreno, J.; Zea, L.; Moyano, L.; Medina, M. Changes in aroma composition of sherry wines during their biological aging carried out by *Saccharomyces cerevisiae* races *bayanus* and *capensis*. *J. Agric. Food Chem.* **1988**, *46*, 2394−2398.

(10) Cortés, M. B.; Moreno, J.; Zea, L.; Moyano, L.; Medina, M. Response of the aroma fraction in sherry wines subjected to accelerated biological aging. *J. Agric. Food Chem.* **1999**, *47*, 3297−3302.

(11) Ribéreau-Gayon, J.; Peynaud, E.; Ribéreau-Gayon, P.; Sudraud, P. *Treatise of Oenology, Sciences and Techniques of Wine*; Dunod: Paris, France, 1976.

(12) Cortés, M. B.; Moreno, J.; Zea, L.; Moyano, L.; Medina, M. Biological aging of sherry wines with high content in gluconic acid. *Proceedings, V National Congress of Enologists*, Mérida, Spain; Extremadura Enologists Association: Almendralejo, Merida, Spain, 1999; pp 141−144.

(13) Peinado, J.; Florindo, J.; López-Barea, J. Glutathione reductase from *Saccharomyces cerevisiae* undergoes redox interconversion *in situ* and *in vivo*. *Mol. Cell. Biochem.* **1992**, *110*, 135−143.
(14) Van Dijken, J. P.; van Tuijl, A.; Luttik, M. A. H.; Middelhoven, W. J.; Pronk, J. T. Novel pathway for alcoholic fermentation of δ-gluconolactone in the yeast *Saccharomyces bulderi*. *J. Bacteriol.* **2002**, *184*, 672−678.
(15) Kurtzman, P.; Fell, J. W. *The Yeast: a Taxonomic Study*; Elsevier Science Publishers: Amsterdam, The Netherlands, 1998.
(16) European Brewery Convention. Analytical microbiology. *J. Inst. Brew.* **1977**, *83*, 115−117.
(17) Crowell, E.; Ough, C. A modified procedure for alcohol determination by dichromate oxidation. *Am J. Enol. Vitic*. **1979**, *30*, 61−63.
(18) EEC. *Official Report of the European Community, L-272*; Mundi-Prensa: Madrid, Spain, 1990.
(19) OIV. Compendium of international methods of analysis of spirituos drinks, alcohols and aroma compounds of drink. *Off. Int. Vigne Vin. Paris* **1994**, 123−124.
(20) Muñoz, D.; Peinado, R. A.; Moreno, J.; Medina, M. Determination of major volatile compounds and polyols in wine by GC in capillary column and direct injection. Presented at the XXIV Conferences of Viticulture and Enology Tierra de Barros, Almendralejo (Badajoz), Spain, 2002.
(21) Van Aelst, L.; Hohmann, S.; Zimmermann, F. K.; Jans, A. W. H.; Thevelein, J. M. A yeast homologue of the bovine lens fibre MIP gene family complements the growth defect of a *Saccharomyces cerevisae* mutant on fermentable sugars but not its defect in glucose-induced RAS-mediated cAMP signalling. *EMBO J.* **1991**, *10*, 2095−2104.
(22) Luyten, K.; Albertyn, J.; Skibbe, W. F.; Prior, B. A.; Ramos, J.; Thevelein, J. M.; Hohmann, S. *Fps1*, a yeast member of the MIP family of channel proteins, is a facilitator for glycerol uptake and efflux and is inactive under osmotic stress. *EMBO J.* **1995**, *14*, 1360−1371.
(23) Sutherland, F. C. W.; Lages, F.; Lucas, C.; Luyten, K.; Albertyn, J.; Hohmann, S.; Prior, B. A.; Kilian, S. G. Characteristics of *Fps1*-dependent and -independent glycerol transport in *Saccharomyces cerevisiae*. *J. Bacteriol.* **1997**, *179*, 7790−7795.
(24) Tamas, M. J.; Luyten, K.; Sutherland, F. C. W.; Hernandez, A.; Albertyn, J.; Valadi, H.; Li, H.; Prior, B. A.; Kilian, S. G.; Ramos, J.; Gustafsson, L.; Thevelein, J. M.; Hohmann, S. *Fps1p* controls the accumulation and release of the compatible solute glycerol in yeast osmoregulation. *Mol. Microbiol.* **1999**, *31*, 1087−1104.
(25) Lages, F.; Lucas, C. Contribution to the physiological characterization of glycerol active uptake in *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* **1997**, *1322*, 8−18.
(26) Holst, B.; Lunde, C.; Lages, F.; Oliveira, R.; Lucas, C.; Kielland-Brandt, M. C. *GUP1* and its close homologue *GUP2*, encoding multimembrane-spanning proteins involved in active glycerol uptake in *Saccharomyces cerevisiae*. *Mol. Microbiol.* **2000**, *37*, 108−124.
(27) Cortés, M. B. Application of pure cultures of *Saccharomyces cerevisae* to the biological aging of sherry wines in the D.O. Montilla-Moriles. Ph.D. Thesis, University of Cordoba, 2002.
(28) Casas, J. F. Summarized description of the technical enological in sherry wines. In *Proceedings, III University Conferences on the Sherry*; Service of Publications, Cadiz University: Cádiz, Spain, 1985; pp 333−361.
(29) Vaseghi, S.; Baumeister, A.; Rizzi, M.; Reuss, M. *In vivo* dynamics of the pentose phosphate pathway in *Saccharomyces cerevisiae*. *Metab. Eng.* **1999**, *1*, 128−140.
(30) Radler, F.; Brohl, K. The metabolism of several carboxylic acids by lactic acid bacteria. *Z. Lebensm. Unters. Forsch*. **1984**, *179*, 228−231.
(31) Caspari, T.; Urlinger, S. The activity of the gluconate-H^+ symporter of *Schizosaccharomyces pombe* cells is down-regulated by D-glucose and exogenous cAMP. *FEBS Lett.* **1996**, *395*, 272−276.
(32) Mehta, S.; Velmurugan, S.; Lobo, Z. Repression of enzymes of the pentose phosphate pathway by glucose in fission yeast. *FEBS Lett.* **1998**, *440*, 430−433.
(33) Sinha, A.; Maitra, P. K. Induction of specific enzymes of the oxidative pentose phosphate pathway by glucono-δ-lactone in *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* **1992**, *138*, 1865−1873.
(34) Mauricio, J. C.; Moreno, J.; Valero, E.; Zea, L.; Medina, M.; Ortega, J. M. Ester formation and specific activities of *in vitro* alcohol acetyltransferase and esterase by *Saccharomyces cerevisiae* during grape must fermentation. *J. Agric. Food Chem.* **1993**, *41*, 2086−2091.
(35) Zea, L.; Moreno, J.; Ortega, J. M.; Medina, M. Content of free terpenic compounds in cell and must during vinification with three *Saccharomyces cerevisiae* races. *J. Agric. Food Chem.* **1995**, *43*, 1110−1114.

Received for review May 19, 2003. Revised manuscript received July 22, 2003. Accepted August 3, 2003. This research was supported by a grant from the Government of Spain. Project VIN 00-039-C2-01.

JF034512J