Use of a *Schizosaccharomyces pombe* Mutant to Reduce the Content in Gluconic Acid of Must Obtained from Rotten Grapes

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Schizosaccharomyces pombe YGS-5 and *Saccharomyces cerevisiae* G1 strains were used in order to develop an effective method for reducing the gluconic acid content of musts without altering the development of alcoholic fermentation or detracting from quality in the resulting wines. The best results in synthetic media were obtained by using a temperature of 24 °C and a sulfur dioxide rate below 100 mg/L under semiaerobic conditions. Sequential inoculation of the musts with YGS-5 first and fermentative G1 yeasts then reduced their gluconic acid content by 85% within 43 h; by contrast, simultaneous inoculation with YGS-5 and G1 provided a reduction of only 40%. The wines with the best sensory and analytical properties were obtained by sequentially inoculating the musts with YGS-5 and, once gluconic acid was removed, G1. The wine obtained by sequential inoculation without removing YGS-5 was that exhibiting the highest odorant activity value (OAV) for the volatile compounds in the floral odor series. A protocol for treating musts containing gluconic acid was developed and tested at the pilot plant scale. The treatment reduced the gluconic acid content by 70% within 46 h with no adverse effect on the analytical or sensory quality of the resulting wines.

KEYWORDS: Wine; rotten grapes; gluconic acid; *Schizosaccharomyces pombe*

INTRODUCTION

Common rot (also known as grey rot) is an endemic disease of grapevines caused by *Botrytis cinerea* and related fungi including *Penicillium*, *Aspergillus*, and *Mucor* or bacteria such as *Acetobacter* and *Gluconobacter*. Development of this disease is favored by some factors such as a high moisture or rainfall, and also by physiological factors including vine variety and bunch morphology (*1*). In warm areas, where rain during the ripening and harvesting periods is very scant, 5−10% of the overall weight of seemingly healthy grapes is in fact affected by common rot; also, the proportion rises above 50% at least once each decade.

Metabolic activity in *Botrytis* is known to produce gluconic acid. Also, according to Couto et al. (*2*), 1−2 g/L contents of this acid in grapes are suggestive of incipient infection by this fungus, whereas higher contents may be produced by other opportunistic microbes. Gluconic acid is used as an indicator of the rottenness level in harvested grapes; thus, musts with a content exceeding 0.5 g/L are usually discarded as useless for obtaining quality wines.

Rotten grapes cause a number of microbiological and chemical problems that hinder the suitable control of the fermentation process and detract from wine stability during storage and aging. In fact, wines from rotten grapes exhibit an altered color resulting from an increased activity of oxidase enzymes; an increased dry extract due to the formation of glycerine, polysaccharides, uronic acids, and aldonic acids; a decreased titratable acidity; and a substantially increased volatile acidity. Oenologists have fought against these problems by using different treatments; thus, unwanted enzyme activity and bacterial contamination have been fought by increasing the sulfur dioxide concentration, and the filtration problems, caused by the presence of polysaccharides, have been mitigated by using pectolytic enzymes. However, these treatments cannot reduce the gluconic acid content and have an adverse impact on the sensorial quality of wine.

Because gluconic acid cannot be metabolized by fermentation yeasts present in must, it remains in the wine after fermentation. However, the presence and growth of lactic bacteria or uncontrolled microorganisms capable of metabolizing this acid has two main effects. The first one is the increase of the volatile acidity of wine. The second effect is an increase in the population of these unwanted microorganisms growing at the expense of other substrates, which can severely detract from the analytical and sensorial quality of the resulting wine (*3*). In

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Table 1. Yeast Population (10^6 cell mL^{-1}) of *Schizosaccharomyces pombe* YGS-5 and Gluconic Acid Concentration (g L^{-1}) under Semiaerobic (S-ae) and Semianaerobic (S-an) Conditions at 24 and 28 °C

time (h)	28 °C S-ae		28 °C S-an		24 °C S-ae		24 °C S-an	
	cells	gluconic acid	cells	gluconic acid	cells	gluconic acid	cells	gluconic acid
0	3.0 ± 0.1	2.31 ± 0.01	3.0 ± 0.1	2.31 ± 0.01	3.0 ± 0.1	2.31 ± 0.01	3.0 ± 0.1	2.31 ± 0.01
14	54 ± 1	1.96 ± 0.01	45 ± 1	2.04 ± 0.01	32 ± 3	2.01 ± 0.01	20 ± 0.8	1.73 ± 0.06
36	220 ± 4	0.14 ± 0.01	201 ± 1	0.56 ± 0.01	197 ± 7	0.14 ± 0.01	108 ± 7.3	0.14 ± 0.06
40	260 ± 1	0.06 ± 0.01	233 ± 3	0.36 ± 0.01	244 ± 3	0.07 ± 0.01	134 ± 9.9	0.07 ± 0.06

Table 2. Rate of Gluconic Acid Removal (μg Acid/10^6 Cell/h) by *Schizosaccharomyces pombe* YGS-5 Yeasts under Semiaerobic (S-ae) and Semianaerobic (S-an) Conditions at 24 and 28 °C

time (h)	28 °C S-ae	28 °C S-an	24 °C S-ae	24 °C S-an
0−14	0.49	0.46	0.74	2.44
14−36	0.50	0.43	0.52	0.82
36−40	0.50	1.56	0.37	0.68

addition, gluconic acid can readily combine with sulfur dioxide (*4*) and reduce the amount of free SO$_2$, which is the active form of this additive, in the medium. Therefore, the presence of gluconic acid shows as another inconvenience the increase in the dose of SO$_2$ required to protect wine from oxidation and microbial attack during its storage and aging. Increasing volatile acidity and raising the dose of SO$_2$ detract from wine quality. Therefore, alleviating or avoiding the above-described problems entails reducing the gluconic acid content of must or wine.

Some *Schizosaccharomyces pombe* strains have proved effective in the biological deacidification of musts containing large amounts of malic acid (*5−12*); however, they have the disadvantage that they give off-odors when they ferment sugars in the must (*5, 13*).

An *S. pombe* strain and a *S. cerevisiae* (*capensis*) flor yeast strain were recently used to remove gluconic acid by 30−50% from wines to be subsequently aged biologically (*14−16*). Both yeasts produce large amounts of acetaldehyde and other carbonyl compounds characteristic of the biological aging process, but are undesirable in other types of wine. Also, as noted earlier, *S. pombe* gives off-odors, which has aroused some reluctance in their use among wine makers. A more efficient and widely applicable treatment capable of reducing the content in gluconic acid of must to acceptable levels in order to facilitate control during storage and aging of any type of wine and posing no microbial risks is therefore highly desirable.

Peinado et al. (*17, 18*) proposed various strategies to remove the gluconic acid content from synthetic media by using *S. pombe* YGS-5 yeast strain. The best results were obtained with sequential inoculation (i.e., by adding fermentative yeast after the gluconic acid was removed). In fact, the acid was completely removed after 30 h; however, the treatment diminished the fermentation rate of *S. cerevisiae* relative to a control treatment, as a result of a high amino acid uptake by YGS-5 while removing gluconic acid. Also, the treatment caused differences in the concentration of some fermentation byproducts.

The primary aim of this work was to develop an effective treatment protocol for reducing the gluconic acid content of must from rotten grapes by using the *S. pombe* YGS-5 yeast strain. The tests conducted to this end were performed in three different steps. In the first, different conditions were studied in synthetic media, in order to identify the one most rapidly decreasing the gluconic acid content and least altering the nitrogen content of the medium. In the second, the previously chosen conditions were used with the must obtained from rotten grapes, which

was inoculated with the YGS-5 deacidifying yeast and the *Saccharomyces cerevisiae* G1, a fermentative yeast, at different points in time in order to establish the most effective protocol. In the third, a pilot scale trial under typical winemaking conditions was performed and the resulting wine assessed in analytical and sensory terms.

MATERIAL AND METHODS

Yeasts Strain, Inocula, and Fermentation Medium. The yeasts used were *S. pombe* strain YGS-5 to remove gluconic acid and *S. cerevisiae* G1 to ferment the must. Tests were conducted both in a laboratory-made synthetic medium and in a natural medium obtained by pressing rotten grapes.

The YGS-5 yeast strain, which is a glucose-transport-deficient and leucine-auxotrophic mutant of *S. pombe*, was obtained and supplied by Milbradt and Höfer (*19*). The yeast was grown on YNB medium with amino acids (Difco) supplemented with 100 mg/L L-leucine (Sigma) and 3% D-gluconate (potassium salt, Merck). *Saccharomyces cerevisiae* G1 (ATCC Number MYA-2451) was grown in YM medium (0.3% w/v yeast extract, 0.3% w/v malt extract, and 0.5% w/v peptone, pH 6.5) containing 5% w/v glucose as carbon source. Both yeast strains were incubated in 500 mL flasks at 27 ± 2 °C with shaking for 72 h, and cells collected by centrifugation at 3500*g* and used as starter cultures for inoculation.

The synthetic medium was prepared by adding glucose and fructose at a 200 g/L concentration in a 1:1 ratio, 3% yeast extract, 2.31 g/L gluconic acid, 1 g/L malic acid, 3 g/L tartaric acid, and 100 mg/L leucine in distilled water. This was followed by adjustment to pH 3.5 and sterilization by passage through Supra-EK filters from Pall-Seitz (Bad Kreuznach, Germany). Must from Pedro Ximénez grapes grown in the Montilla-Moriles winemaking region that had been attacked by the common rot was selected taking in account that must containing more than 2.5 g/L is usually rejected by winemakers. Must was sterilized through Supra EK filters for laboratory trials.

Experimental Design. Tests were conducted in three steps. The first involved optimizing the application conditions of YGS-5 (viz. temperature, aeration regime and SO$_2$ rate) in synthetic media. The selected condition was used with natural must (275 gL^{-1} sugar and 2.9 gL^{-1} gluconic acid) in the second step, in order to compare the influence of the point of addition of the inocula of each yeast and determine whether removing YGS-5 from the must prior to starting alcoholic fermentation with G1 was advisable. The aim of this second step was the selection of a treatment protocol for must from rotten grapes. The third, last step involved applying this protocol to musts from rotten grapes under typical winemaking conditions at a pilot plant.

Optimizing the Application Conditions for YGS-5 in a Synthetic Medium. In order to optimize the gluconic acid uptake rate, a synthetic medium at two different temperatures (24 and 28 °C) and under two different aeration regimes (semiaerobic and semianaerobic) was used. According to Valero et al. (*20*), a semiaerobic condition was established by allowing the fermenting grape must to stand in flasks stoppered with a cotton plug. In this case, a slow exchange of air through the cotton plug during the fermentation is allowed. The semianaerobic condition was established when the flasks were stoppered with a rubber plug crossed by a blue tip of the micropipette (1 mL) to obtain no exchange of air during the fermentation process. The influence of the sulfur dioxide rate on the yeast population was examined at 0, 50, 100, 150, 200, and 250 mg/L. All tests were performed in triplicate, using 500 mL flasks.

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Table 3. Amino Acid Concentration (mM) in the Initial Medium (IM) and after Gluconic Acid Removal by *Schizosaccharomyces pombe* YGS-5 under Semiaerobic (S-ae) and Semianaerobic (S-an) Conditions at 24 and 28 °C[a]

amino acid	IM	28 °C, S-ae	28 °C, S-an	24 °C, S-ae	24 °C, S-an
asparagine	0.47 ± 0.02 c	ND a	ND a	ND a	0.33 ± 0.05 b
serine	0.80 ± 0.01 c	ND a	ND a	ND a	0.61 ± 0.04b
aspartic acid	0.34 ± 0.02 b	ND a	ND a	ND a	ND a
glutamic acid	1.380 ± 0.001c	ND a	ND a	0.3 ± 0.1 b	1.48 ± 0.07 d
glycine	0.707 ± 0.004 d	ND a	ND a	0.199 ± 0.03 b	0.67 ± 0.01 c
arginine	1.324 ± 0.005 c	1.22 ± 0.06 b	0.667 ± 0.009 a	1.360 ± 0.004 c	1.87 ± 0.07 d
alanine	1.31 ± 0.01 c	ND a	ND a	ND a	0.92 ± 0.01 b
γ-aminobutyric acid	0.391 ± 0.008 c	ND a	0.075 ± 0.02b	ND a	ND a
α-aminobutyric acid	ND a	ND a	0.05 ± 0.01 b	ND a	ND a
proline	0.34 ± 0.02 d	0.22 ± 0.03 b	0.22 ± 0.02 b	0.16 ± 0.04 a	0.29 ± 0.02 c
methionine	0.263 ± 0.004 b	ND a	ND a	ND a	ND a
valine	1.03 ± 0.07 d	ND a	0.167 ± 0.003 b	0.19 ± 0.01 b	0.63 ± 0.03 c
tryptophan	0.127 ± 0.002 b	ND a	ND a	ND a	ND a
isoleucine + phenylalanine	0.790 ± 0.006 e	0.03 ± 0.01 b	ND a	0.19 ± 0.06 c	0.57 ± 0.06 d
leucine	1.72 ± 0.01 c	0.13 ± 0.06 a	0.41 ± 0.03 b	0.14 ± 0.06 a	0.37 ± 0.03 b
ornithine	0.136 ± 0.002 b	ND a	ND a	ND a	ND a
lysine	0.666 ± 0.003 c	0.04 ± 0.01a	0.033 ± 0.003 a	0.04 ± 0.02 a	0.20 ± 0.02 b
histidine	0.176 ± 0.01 b	ND a	ND a	ND a	ND a
tyrosine	0.18 ± 0.01 c	ND a	ND a	ND a	0.14 ± 0.01 b
Σ amino acids	12.14 ± 0.05 d	1.64 ± 0.08 a	1.63 ± 0.08 a	2.6 ± 0.3 b	8.1 ± 0.3 c

[a] ANOVA: different letters denote different homogeneous groups at the 95% confidence level. ND = not detected.
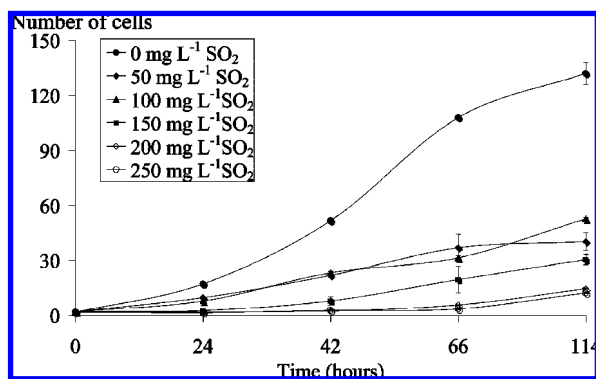
Figure 1. Population (10^6 cell mL^{-1}) of *Schizosaccharomyces pombe* YGS-5 in media containing variable sulfur dioxide concentrations. All data shown are the mean of 3 values with their respective deviation.

Development of a Treatment Protocol for Musts from Rotten Grapes. The protocol was established by adding starter cultures of each yeast to the musts previously sterilized in a sequential or simultaneous way. In the sequential inoculation procedure, inoculum of YGS-5 was added first in order to remove gluconic acid, and inoculum of G1 was then added to carry out the alcoholic fermentation. In the simultaneous procedure, both inocula were added at once to the initial must. The sequential inoculation procedure was performed in two different ways, namely, treatment 1 (T1), which involved the separation of YGS-5 by centrifuging the must after gluconic acid was removed but before G1 was added, and treatment 2 (T2), where YGS-5 was not separated from the medium. The simultaneous inoculation procedure was designated T3. A control test was also performed where the must was inoculated with the fermentation yeast alone. Therefore, the protocol was developed from three different treatments plus a control test. All tests were conducted at 24 °C by 1 L Erlenmeyer flasks in triplicate at the laboratory scale and finished after 9 days of treatment.

Pilot-Plant Winemaking Tests. The above-described protocol for application of YGS-5 at 24 °C and subsequent fermentation with indigenous wild yeasts was tested on a volume of 100 L of must from rotten grapes (2.3 g L^{-1} gluconic acid and 234 g L^{-1} sugars), using the conditions, standards, and prefermentation treatments typically employed by the collaborating winemaker. A control test was also performed on untreated must. Tests were finished after 22 days of treatment.

Analytical Methods. *Analyses of Winemaking Variables.* The number of total and viable cells was determined by counting under a light microscope in a Thoma chamber, following staining with methylene blue, while ethanol, titratable acidity, pH, and volatile acidity were determined in accordance with the European Community's official methods (*21*). Gluconic acid was determined by using specific enzyme kits from Boehringer (Mannheim, Germany).

Amino Acids. Amino acids were quantified from the absorbance at 254 nm of their dansyl derivatives (*22*), which were previously isolated by high-performance liquid chromatography on a Spectra-Physics P200 HPLC instrument (Darmstadt, Germany), equipped with an SP 8450 UV−V detector and a 15 × 0.4 cm reversed-phased column packed with Spherisorb ODS2 resin of 5 μm particle size obtained from Tracer Analítica (Barcelona, Spain) and thermostatted at 25 °C. A volume of 20 μL of 5 mmol L^{-1} L-norleucine was used as the internal standard.

Volatile Compounds and Polyols. Major volatile compounds and polyols were quantified on a Model 6890 gas chromatograph from Agilent Technologies (Palo Alto, CA), using the method described by Peinado et al. (*23*). A CP-WAX 57 CB capillary column (60 m long × 0.25 mm i.d., 0.4 μm film thickness) from Varian (Palo Alto, CA) was used, and 0.5 μL aliquots from 10 mL samples previously supplied with 1 mL of 1 g/L 4-methyl-2-pentanol as internal standard were injected into the instrument. Tartaric acid in the wine was removed by precipitation with 0.2 g of calcium carbonate and centrifugation at 3500 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 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 (16 min) and followed by a 0.2 mL/min ramp to the final value (1.1 mL/min), which was held for 52 min.

Minor volatile compounds were determined by solid-phase microextraction of the head space of wines and capillary-column gas chromatography−mass spectrometry (HS-SPME-GC-EM), using CAR/DVB/PDMS 50/30 μm fibers from Supelco (Bellefonte, PA) in accordance with the manufacturer's instructions and the recommendations of specialized literature (*24, 25*). For SPME analyses, both must samples and model solutions were analyzed in 40 mL glass vials filled with 25 mL of each sample, to which 0.1 mL of internal standard (22.32 mg/L ethyl nonanoate in 99.9% ethanol), 6 g of Na_2SO_4, and a 1 cm long magnetic stirring bar were added. The vial was placed over a magnetic stirrer equipped with an electronic thermostatting system. Solid-phase microextraction was performed at 40 °C with agitation at 500 rpm for 30 min and immediately followed by desorption into the GC injector for 5 min. The injector was used in the pulsed mode (20 psi for 5 min) and a septum purge of 10 mL was employed after which the injector pressure was reduced to 14.6 psi.

Table 4. Concentrations of Selected Compounds of Oenological Interest in Musts from Rotten Grapes Subjected to Treatments 1 and 2, and in the Wines Provided by the Different Treatments[a]

compound	musts with treatments 1 and 2			wines			
	0 h	43 h	CL	T1	T2	T3	control
gluconic acid (g L^{-1})	2.9 ± 0.2	0.4 ± 0.1	***	0.4 ± 0.1 b	0.26 ± 0.03 a	1.7 ± 0.2 b	2.7 ± 0.2 c
glucose (g L^{-1})	141.9 ± 0.7	141 ± 3	NS	0.18 ± 0.07 a	0.7 ± 0.3 b	0.61 ± 0.01 b	0.7 ± 0.2 b
fructose (g L^{-1})	133 ± 7	133 ± 2	NS	6 ± 2 a	9 ± 5 ab	17.9 ± 0.6 bc	23 ± 4 c
ethanol (% v/v)	0	0	NS	15.8 ± 0.3 b	15.6 ± 0.1 b	15.1 ± 0.2 a	14.7 ± 0.3 a
acetic acid (g L^{-1})	0.16 ± 0.05	0.24 ± 0.05	NS	0.32 ± 0.01 a	0.54 ± 0.07 b	0.39 ± 0.02 ab	0.49 ± 0.02 b
titratable acidity (g tartaric acid L^{-1})	5.3 ± 0.2	4.4 ± 0.2	***	4.9 ± 0.2 a	4.94 ± 0.05 a	5.6 ± 0.2 b	6.85 ± 0.06 c
pH	3.51 ± 0.02	3.53 ± 0.03	NS	3.76 ± 0.04 c	3.80 ± 0.02 c	3.64 ± 0.02 b	3.43 ± 0.04 a
urea (mgL^{-1})	3.8 ± 1	6 ± 3	NS	14 ± 1 a	18 ± 7 a	11 ± 0.7 a	11 ± 2 a
ammonium (mg L^{-1})	48.1 ± 0.6	42.3 ± 0.5	***	1.5 ± 0.5 a	2.1 ± 0.5 a	1.5 ± 0.1 a	1.8 ± 0.1 a

[a] CL, ANOVA statistical confidence level between musts; NS, not significant; * 95%; ** 99%; *** 99.9%. Homogeneous group for wines: different letters denote different homogeneous groups at the 95% confidence level. T1 = treatment 1: sequential inoculation of the must with *Schizosaccharomyces pombe* YGS-5 and *Saccharomyces cerevisiae* G1 after YGS-5 was removed. T2 = treatment 2: sequential inoculation of the must with *YGS-5* and *G1* without removing YGS-5. T3 = treatment 3: simultaneous inoculation of the must with YGS-5 and G1 yeasts. Control = the must was inoculated with G1 only.

Table 5. Aminoacid Contents (mM) in Musts from Rotten Grapes Subjected to Treatments 1 and 2, and in the Wines Provided by the Different Treatments[a]

amino acid	musts with treatments 1 and 2			wines			
	0 h	43 h	CL	T1	T2	T3	control
Gln	0.25 ± 0.04	0.22 ± 0.05	NS	ND a	ND a	ND a	ND a
Ser	0.44 ± 0.04	0.28 ± 0.04	*	ND a	ND a	ND a	ND a
Gly	0.00	0.26 ± 0.03	**	ND a	0.30 ± 0.04 b	ND a	ND a
Thr	0.61 ± 0.02	0.88 ± 0.00	**	0.56 ± 0.04 ab	0.56 ± 0.06 ab	0.46 ± 0.05 a	0.63 ± 0.05 b
Arg	4.71 ± 0.02	4.0 ± 0.2	*	ND a	0.20 ± 0.2 b	0.18 ± 0.02 b	0.17 ± 0.03 b
Ala	0.60 ± 0.09	0.39 ± 0.05	***	ND a	0.21 ± 0.00 c	0.14 ± 0.03 b	ND a
Gaba	0.90 ± 0.05	1.36 ± 0.03	**	0.15 ± 0.02 a	1.0 ± 0.1 b	ND a	ND a
Pro	7.7 ± 0.4	6.8 ± 0.2	NS	8.9 ± 0.1 a	8.5 ± 0.2 a	10.3 ± 0.1 a	13 ± 2 b
Val	0.45 ± 0.01	0.23 ± 0.02	**	ND a	ND a	ND a	ND a
Trp	0.22 ± 0.02	0.07 ± 0.01	**	ND a	ND a	ND a	ND a
Leu	0.91 ± 0.07	0.14 ± 0.02	**	1.40 ± 0.08 a	1.3 ± 0.1 a	1.31 ± 0.02 a	1.4 ± 0.1 a
Cys	0.00	0.00	NS	4.30 ± 0.06 ab	4.17 ± 0.06 a	4.31 ± 0.01 b	4.18 ± 0.06 ab
Orn	0.24 ± 0.01	0.22 ± 0.02	NS	ND a	ND a	ND a	ND a
Lys	0.15 ± 0.02	0.06 ± 0.01	*	0.07 ± 0.00 a	0.22 ± 0.00 d	0.11 ± 0.00 b	0.15 ± 0.01 c
His	0.40 ± 0.03	0.30 ± 0.03	NS	0.08 ± 0.01 a	0.09 ± 0.01 a	0.09 ± 0.00 a	0.09 ± 0.01 a
Tyr	0.10 ± 0.01	0.09 ± 0.01	NS	ND a	ND a	ND a	ND a
Σ amino acids	17.7 ± 0.6	15.3 ± 0.5	*	15.5 ± 0.2 a	16.5 ± 0.3 a	16.87 ± 0.04 a	20 ± 2 b

[a] CL = ANOVA statistical confidence level between musts. NS, not significant; * 95%; ** 99%; *** 99.9%. Wines ANOVA: different letters denote different homogeneous groups at the 95% confidence level. T1 = treatment 1: sequential inoculation of the must with *Schizosaccharomyces pombe* YGS-5 and *Saccharomyces cerevisiae* G1 after YGS-5 was removed. T2 = treatment 2: sequential inoculation of the must with *YGS-5* and *G1* without removing YGS-5. T3 = treatment 3: simultaneous inoculation of the must with YGS-5 and G1 yeasts. Control = the must was inoculated with G1 only. ND = not detected.

Table 6. Concentrations of Major Volatile Compounds and Polyols in Musts from Rotten Grapes Subjected to Treatments 1 and 2, and in the Wines Provided by the Different Treatments[a]

compound	musts with treatment 1 and 2			wines			
	0 h	43 h	CL	T1	T2	T3	control
acetaldehyde (mg L^{-1})	124 ± 24	279 ± 16	***	96 ± 2 b	67 ± 6 a	73 ± 4 a	72 ± 4 a
acetoin (mg L^{-1})	72 ± 4	152 ± 4	***	45.2 ± 1.2 b	29 ± 4 a	45 ± 11 b	39 ± 4 ab
methanol (mg L^{-1})	77 ± 14	91.5 ± 16.5	NS	375 ± 163 b	147 ± 17 a	102 ± 8a	104 ± 2 a
1- propanol (mg L^{-1})	30 ± 6	36 ± 5	NS	96.9 ± 0.2 b	54 ± 9 a	54 ± 2 a	56.0 ± 0.6a
isobutyl alcohol (mg L^{-1})	ND	ND	NS	34 ± 2 a	32.5 ± 2.5 a	34.7 ± 0.7 ab	37.35 ± 0.05b
isoamyl alcohols (mg L^{-1})	3.8 ± 0.8	17.5 ± 2.5	***	221 ± 9 b	186 ± 11 a	254 ± 2 c	283 ± 3 d
2-phenylethanol (mg L^{-1})	ND	19 ± 1	***	26 ± 2 b	15 ± 2 a	25.7 ± 0.3 b	38 ± 2 c
ethyl acetate (mg L^{-1})	ND	ND	NS	71 ± 3 b	108 ± 6 c	68 ± 6 b	55 ± 2 a
diethyl succinate (mg L^{-1})	ND	ND	NS	38 ± 3 a	46 ± 15 a	42 ± 5 a	45 ± 2 a
glycerine (g L^{-1})	2.36 ± 0.04	3.9 ± 0.5	***	10.6 ± 2 c	8.1 ± 0.4 b	4.8 ± 0.1 a	5.2 ± 0.3 a
2,3-butanediol (*levo*) (g L^{-1})	0.09 ± 0.01	0.5 ± 0.1	***	1.3 ± 0.1 d	0.90 ± 0.01 c	0.53 ± 0.02 a	0.63 ± 0.01 b
2,3-butanediol (*meso*) (g L^{-1})	0.31 ± 0.01	0.4 ± 0.1	NS	0.52 ± 0.02 b	0.39 ± 0.07 b	0.28 ± 0.03 a	0.24 ± 0.02 a

[a] CL, ANOVA statistical confidence level between musts; NS, not significant; * 95%; ** 99%; *** 99.9%. Wines ANOVA: different letters denote different homogeneous groups at the 95% confidence level. T1 = treatment 1: sequential inoculation of the must with *Schizosaccharomyces pombe* YGS-5 and *Saccharomyces cerevisiae* G1 after YGS-5 was removed. T2 = treatment 2: sequential inoculation of the must with *YGS-5* and *G1* without removing YGS-5. T3 = treatment 3: simultaneous inoculation of the must with YGS-5 and G1 yeasts. Control = the must was inoculated with G1 only. ND = not detected.

GC-EM analyses were done with an HP-6890 gas chromatograph equipped with a CP-WAX 57 CB capillary column (60 m long × 0.25 mm i.d., 0.4 μm film thickness) from Varian (Palo Alto, CA) and an HP MS 5972A mass detector (Agilent Technologies, Palo Alto, CA). The temperature program was as follows: initial temperature 40 °C, held for 10 min, and 1 °C/min ramp to 180 °C, held for 35 min. Helium

Table 7. Concentrations of Minor Volatile Compounds (mg L^{-1}) in Musts from Rotten Grapes Subjected to Treatments 1 and 2, and in the Wines Provided by the Different Treatments[a]

compound	must with treatment 1 and 2			wines			
	0 h	43 h	CL	T1	T2	T3	Control
ethyl butanoate	0.05 ± 0.01	0.09 ± 0.02	*	1.2 ± 0.2 a	1.6 ± 0.3 a	1.5 ± 0.3 a	1.2 ± 0.1 a
butyl acetate	ND	0.09 ± 0.02	***	0.03 ± 0.00 b	0.03 ± 0.01 b	0.03 ± 0.01 b	ND a
hexanal	0.26 ± 0.05	0.06 ± 0.01	**	ND a	ND a	ND a	ND a
isoamyl acetate	0.12 ± 0.01	0.4 ± 0.1	*	8 ± 1 a	16 ± 4 b	17 ± 1 b	14 ± 2 a
2-hexenal	0.44 ± 0.09	0.6 ± 0.2	NS	0.80 ± 0.02 b	ND a	ND a	ND a
ethyl hexanoate	0.07 ± 0.01	0.15 ± 0.09	NS	1.03 ± 0.03 a	2.3 ± 0.3 c	1.46 ± 0.01 b	1.19 ± 0.01 a
hexyl acetate	0.01 ± 0.00	0.02 ± 0.00	**	0.11 ± 0.01 a	0.27 ± 0.02 d	0.21 ± 0.01 c	0.16 ± 0.01 b
1-hexanol	15 ± 2	7.76 ± 0.05	**	1.92 ± 0.09 c	1.9 ± 0.1 c	1.01 ± 0.07 a	1.5 ± 0.2 b
E-3-hexen-1-ol	0.21 ± 0.04	0.14 ± 0.08	NS	ND a	0.27 ± 0.06 b	ND a	ND a
Z-3-hexen-1-ol	0.57 ± 0.09	0.4 ± 0.2	NS	ND a	ND a	ND a	ND a
E-2-hexen-1-ol	0.42 ± 0.12	0.58 ± 0.06	NS	0.32 ± 0.02 c	0.36 ± 0.04 c	ND a	0.24 ± 0.03 b
ethyl octanoate	0.02 ± 0.00	0.15 ± 0.07	*	0.68 ± 0.02 a	1.2 ± 0.2 b	1.02 ± 0.03 b	0.77 ± 0.05a
furaldehyde	0.31 ± 0.05	0.2 ± 0.2	NS	1.08 ± 0.01 a	1.3 ± 0.2 a	2.4 ± 0.3 b	2.2 ± 0.4 b
benzaldehyde	0.04 ± 0.01	0.38 ± 0.06	***	ND a	0.003 ± 0.001 a	0.01 ± 0.00 b	0.01 ± 0.00 b
linalool	0.01 ± 0.00	0.02 ± 0.01	NS	ND a	ND a	ND a	ND a
1-octanol	0.05 ± 0.02	0.05 ± 0.01	NS	ND a	0.02 ± 0.00 b	ND a	ND a
5-methylfurfural	0.15 ± 0.07	ND	*	0.35 ± 0.04 a	0.35 ± 0.07 a	0.9 ± 0.1 b	0.8 ± 0.2 b
ethyl decanoate	0.01 ± 0.01	0.17 ± 0.02	***	0.10 ± 0.01 a	0.19 ± 0.05 b	0.31 ± 0.02 b	0.21 ± 0.02 b
E-citral	ND	ND	NS	1.01 ± 0.16 c	0.39 ± 0.07 b	0.07 ± 0.01 a	0.10 ± 0.01a
α-terpineol	0.01 ± 0.00	0.03 ± 0.00	***	ND a	ND a	ND a	ND a
nerol	0.04 ± 0.04	ND	NS	ND a	ND a	ND a	ND a
2-phenethyl acetate	0.02 ± 0.01	0.01 ± 0.01	NS	0.33 ± 0.02 a	0.6 ± 0.1 b	1.3 ± 0.1 c	1.20 ± 0.08 c
ethyl dodecanoate	0.00 ± 0.00	0.11 ± 0.03	**	0.03 ± 0.01 a	0.05 ± 0.01 a	0.32 ± 0.03 c	0.24 ± 0.03 b
geraniol	0.05 ± 0.01	0.06 ± 0.03	NS	ND a	ND a	ND a	ND a
hexanoic acid	1.7 ± 0.2	0.7 ± 0.1	**	0.10 ± 0.01 a	0.44 ± 0.06 b	0.57 ± 0.07 c	0.43 ± 0.04 a
ethyl tetradecanoate	ND	0.04 ± 0.02	*	0.02 ± 0.00 a	0.03 ± 0.01 ab	0.057 ± 0.003 c	0.04 ± 0.01 b
octanoic acid	0.6 ± 0.1	0.4 ± 0.1	NS	0.01 ± 0.00 a	1.5 ± 0.3 b	1.82 ± 0.03 c	1.7 ± 0.1 bc
γ-decalactone	0.02 ± 0.00	0.01 ± 0.00	**	ND a	ND a	ND a	ND a
ethyl hexadecanoate	0.05 ± 0.01	0.04 ± 0.01	*	0.95 ± 0.01 a	0.12 ± 0.03 a	1.4 ± 0.2 c	0.56 ± 0.05 b
decanoic acid	0.55 ± 0.05	ND	***	ND a	0.19 ± 0.01 b	0.37 ± 0.02 c	1.19 ± 0.07 d

[a] CL, ANOVA statistical confidence level between musts; NS, not significant; * 95%; ** 99%; *** 99.9%. Wines ANOVA: different letters denote different homogeneous groups at 95% confidence level. T1 = treatment 1: sequential inoculation of the must with *Schizosaccharomyces pombe YGS-5* and *Saccharomyces cerevisiae G1* after YGS-5 was removed. T2 = treatment 2: sequential inoculation of the must with *YGS-5* and *G1* without removing YGS-5. T3 = treatment 3: simultaneous inoculation of the must with YGS-5 and G1 yeasts. Control = the must was inoculated with G1 only. ND = not detected.

at a constant flow-rate of 0.9 mL/min was used as carrier gas. The mass detector was used at a voltage of 1612 V to scan the mass range from 39 to 300 amu.

Retention times, spectral libraries supplied by Wiley and pure chemical compounds obtained from Merck, Sigma-Aldrich, Riedel de Haën, and Fluka were used for identification, confirmation, and preparation of standard solutions of the volatile compounds quantified. Each compound was quantified from its response factor, which was obtained by using standard solutions of known concentration previously subjected to the same treatment as the samples in conjunction with the target and qualifier ions selected for each compound by the Hewlett-Packard Chemstation (Palo Alto, CA).

Statistical processing. The results reported here are the averages of the analyses carried out on three separate experiments. The statistical software package Statgraphics Plus v. 2, from STSC, Inc. (Rockville, MD), was used for the one-way analysis of variance (ANOVA) and for the discrimination analysis of the means, according to Fisher's least significant difference (LSD) procedure at 95% confidence level.

Sensory Evaluation. The sensory evaluation of wines was carried out by 23 expert tasters chosen among oenologist and winemaker from the Montilla-Moriles viticultural area. Wines were presented in clear tulip-shaped glasses marked with three-digit numbers and covered with Petri-dishes, in a randomized order of presentation for each session. Tasters were asked to score each wine (in a scale from 0 to 10 points) by comparing it with the standard used in their winery for color, aroma, and taste.

RESULTS AND DISCUSSION

As was described by Peinado et al. (*17, 18*), the sequential inoculation of the fermentation yeast after the gluconic acid was completely removed by YGS-5, diminished the fermentation

rate of *S. cerevisiae* relative to a control treatment, as a result of a high amino acid uptake by YGS-5 while removing gluconic acid. Therefore, the first aim of this work was to optimize the use of YGS-5 by minimizing changes in amino acid contents.

Optimization of the Application Conditions for *Schizosaccharomyces pombe* (YGS-5) in a Synthetic Medium. The fermentation medium, containing 2.3 g/L gluconic acid, was inoculated with 3 10^6 cell/mL of YGS-5 yeast in the four treatments. The variation of the yeast population and gluconic acid concentration are shown in **Table 1**. As can be seen, the number of yeast cells at the end of the tests was greater under semiaerobic conditions and independent of the temperature used. Also, the gluconic acid concentration fell below 0.1 g/L within 40 h with all treatments except under semianaerobic conditions at 28 °C. Such conditions resulted in the highest yeast concentrations and semianaerobic conditions at 24 °C in the lowest.

The gluconic acid uptake rate is an important influential factor here because the musts obtained from rotten grapes contain bacteria and yeasts in a substantial population (*3*), and these wild microorganisms could grow jointly with *S. pombe* YGS-5 during the first few hours of the deacidification treatment and detract from its efficiency as a result. The deacidifying efficiency of YGS-5 was calculated as the ratio of the amount of gluconic acid removed, in μg mL^{-1}, by 10^6 cell mL^{-1}, in 1 h. On the basis of the results, the most efficient application conditions in

Table 8. Volatile Compounds Quantified: Odor Descriptor, Odor Perception Threshold (OPT) and Assignment to Odorant Series

volatile compound	odor descriptor	OPT (mg L^{-1})	odorant series[a]
acetaldehyde	pungent, overripe apple	110	1
2,3-butanediol (*l+m*)	fruity	150	1
1-propanol	ripe fruit, alcohol	306	1,3
ethyl acetate	pineapple, varnish	12	1,3
butyl acetate	fruity, banana	1.83	1
isoamyl acetate	banana, fruity	0.16	1
hexyl acetate	apple, pear, cherry	0.67	1
2-phenylethyl acetate	rose, fruity	0.25	1,2
ethyl butanoate	banana, pineapple, strawberry	0.4	1
ethyl hexanoate	apple, floral	0.08	1,2
ethyl octanoate	banana, pineapple, floral	0.58	1,2
ethyl decanoate	grape, fruity, floral	0.51	1,2
ethyl dodecanoate	fruity, floral	0.5	1,2
ethyl tetradecanoate	delicate, cocoa	0.5	1
ethyl hexadecanoate	delicate, cocoa, wax	0.1	1
E-citral	fruity, floral	0.03	1,2
γ-decalactone	peach, roasted	0.01	1,6
diethyl succinate	wine-like, grape	1250	1
1-propanol	ripe fruit, solvent	306	1,3
isobutyl alcohol	alcohol, nail polish, solvent	75	3
isoamyl alcohol	alcohol, nail polish, solvent	60	3
1-octanol	grass, solvent	0.8	3,4
2-phenyl ethanol	rose, honey	200	2
furaldehyde	fragrant, floral, roasted	150	2,6
linalool	floral, spice	0.015	2,7
5-methylfurfural	floral, candy, roasted	20	2,6
geraniol	floral, geranium, grass	0.03	2,4
α-terpineol	floral, lilac	5	2
nerol	rose	0.5	2
1-hexanol	herbaceous, grass	1.1	4
hexanal	grass, roasted almond	0.35	4,6
2-hexenal	herbaceous	0.6	4
E-3-hexen-1-ol	herbaceous, grass	1	4
Z-3-hexen-1-ol	herbaceous, grass	1	4
E-2-hexen-1-ol	herbaceous, grass	15	4
hexanoic acid	fatty, rancid, cheese	3	5
octanoic acid	fatty, rancid	10	5
decanoic acid	fatty, rancid	6	5
benzaldehyde	roasted, burnt	2	6
acetoin	buttery, cream	150	8

[a] Odorant series: fruity (1); floral (2); solvent (3); herbaceous (4); fatty (5); roasted (6), spicy (7), and milky (8).

this respect were provided by a semianaerobic regime at 24 °C (see **Table 2**). Also, the highest gluconic acid uptake rate was obtained at the beginning of the treatment.

Changes in Amino Acid Contents. S. pombe YGS-5 uses large amounts of amino acids while it consumes gluconic acid; this was found to affect subsequent fermentation of the must by a *Saccharomyces cerevisiae* yeast strain (*18*). It is therefore desirable to minimize the impact of YGS-5 on amino acid contents. As can be seen from **Table 3**, aspartic acid, methionine, tryptophan, ornithine, and histidine were all depleted by *S. pombe* YGS-5 in the four treatments and so was *γ*-aminobutyric except under semianaerobic conditions at 28 °C. Asparagine, serine, glutamic acid, glycine, alanine, and tyrosine were consumed preferentially at 28 °C under both aeration regimes, but less markedly so under semianaerobic conditions at 24 °C. A similar behavior was observed for lysine, which exhibited its highest final contents under semianaerobic conditions at 24 °C; the content in this amino acid was reduced by 70% with respect to the initial value under these conditions and by as much as 94% under the others.

Arginine and proline were scarcely consumed or released to the medium by YGS-5 while gluconic acid was depleted, its reduction pattern varying depending on the particular conditions.

Worth special note was the high uptake of arginine under semianaerobic conditions at 28 °C; by contrast, this amino acid exhibited an increase of ca. 40% under semianaerobic conditions at 24 °C. Proline contents decreased by about 50% under semiaerobic conditions at 24 °C, and only by 15−35% with the other treatments.

Because *Saccharomyces pombe* YGS-5 is a leucine-dependent yeast (*19*) its growing medium should contain a high initial concentration of this amino acid for the yeast to be efficient. In our tests, the leucine uptake was dependent on the aeration regime and higher under semiaerobic conditions than under a semianaerobic regime (92% vs 75%). All media contained some residual leucine (0.13−0.41 mM); therefore, this aminoacid constituted no limiting factor for yeast growth in the tests.

Glutamic acid, arginine, alanine, and valine were the amino acids exhibiting the highest initial contents in the medium. Such contents decreased to a lesser extent under semianaerobic conditions at 24 °C, where some arginine was released into the medium.

The initial total concentration of amino acids decreased more markedly at 28 °C than it did at 24 °C. The smallest reduction (33%) was observed under semianaerobic conditions at 24 °C.

Effect of the Sulfur Dioxide Concentration on the Cell Population of YGS-5. As is well know, winemakers supply the musts with SO$_2$ at concentrations between 50−100 mg L^{-1} in order to prevent the growth of spoilage microorganism as acetic acid bacteria and the binding phenomena of SO$_2$ with gluconic acid (*4*). However, too high levels of sulfur dioxide can also alter the growth of YGS-5 and have an adverse impact on gluconic acid depletion as a result. **Figure 1** illustrates the influence of SO$_2$ concentration on the YGS-5 population. As can be seen, using 50 or 100 mg L^{-1} SO$_2$ concentration diminished the number of cells relative to that when not using this additive, without significant differences between the two levels. By contrast, using a SO$_2$ rate above 100 mg L^{-1} had a strong impact on the cell population. Thus, gluconic acid was depleted by YGS-5 within 41 h in the presence of SO$_2$ at concentrations below 100 mg L^{-1}. By contrast, depleting the acid with a 150 mg L^{-1} of SO$_2$ took 112 h; in addition, after this time an unpleasant odor typical of sulfides was perceived at rates above 100 mg L^{-1}.

In summary, on the basis of the final amino acid concentrations, gluconic acid removal efficiency, and effect of sulfur dioxide on the cell population, the most effective treatment for removing gluconic acid was that involving semianaerobic conditions, a temperature of 24 °C, and the addition of SO$_2$ at 50−100 mg L^{-1} prior to inoculation of the must with YGS-5 yeasts.

Development of a Treatment Protocol for Must from Rotten Grapes. The must from rotten grapes subjected to the three above-described treatments and control test contained an initial gluconic acid concentration of 2.9 g mL^{-1}, and the starter cultures used supplied the must with a deacidifying (YSG-5) and fermentation (G1) yeast population of 3·10^6 and 1·10^6 cell mL^{-1}, respectively. An amount of 50 mgL^{-1} of SO$_2$ was added to the must also.

As can be seen from **Table 4**, the gluconic acid content of the musts remained constant throughout the control test and was decreased only 41% with the simultaneous inoculation treatment (T3). However, it was decreased by about 86% with the sequential inoculation treatments (T1 and T2) 43 h after inoculation with YGS-5. Therefore, the presence of the fermentation yeast decreased the efficiency of the gluconic acid

Table 9. Odorant Series in Musts from Rotten Grapes Subjected to Treatments 1 and 2, and in the Wines Provided by the Different Treatments[a]

odorant series (OAV)	must with treatment 1 and 2			wines			
	0 h	43 h	CL	T1	T2	T3	control
fruity	8.0 ± 0.3	16 ± 1	***	94 ± 7 a	161 ± 29 bc	165 ± 10 c	131 ± 14 b
floral	3.3 ± 0.7	6 ± 3	NS	49 ± 5 b	46.4 ± 4.1 b	28.5 ± 0.7 a	24.8 ± 0.2 a
solvent	0.22 ± 0.01	0.47 ± 0.02	***	10.39 ± 0.08 ab	12.7 ± 0.2 c	10.6 ± 0.5 b	10.0 ± 0.1 a
herbaceous	17 ± 2	11 ± 2	*	3.10 ± 0.05 d	2.0 ± 0.2 c	0.9 ± 0.1 a	1.4 ± 0.2 b
milky	0.48 ± 0.03	1.01 ± 0.03	***	0.30 ± 0.01 b	0.19 ± 0.03 a	0.3 ± 0.1 b	0.26 ± 0.03 ab
fatty	0.71 ± 0.03	0.27 ± 0.06	***	0.034 ± 0.003 a	0.33 ± 0.04 b	0.43 ± 0.02 c	0.52 ± 0.04 d
roasted	2.5 ± 0.4	1.37 ± 0.06	**	0.025 ± 0.002 a	0.03 ± 0.01 a	0.07 ± 0.01 b	0.06 ± 0.01 b
spicy	0.60 ± 0.07	1.2 ± 0.7	NS	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a

[a] CL, ANOVA statistical confidence level between musts; NS, not significant; * 95%; ** 99%; *** 99.9%. Homogeneous group for wines: different letters denote different homogeneous groups at the 95% confidence level. T1 = treatment 1: sequential inoculation of the must with *Schizosaccharomyces pombe YGS-5* and *Saccharomyces cerevisiae G1* after YGS-5 was removed. T2 = treatment 2: sequential inoculation of the must with *YGS-5* and *G1* without removing YGS-5. T3 = treatment 3: simultaneous inoculation of the must with YGS-5 and G1 yeasts. Control = the must was inoculated with G1 only.
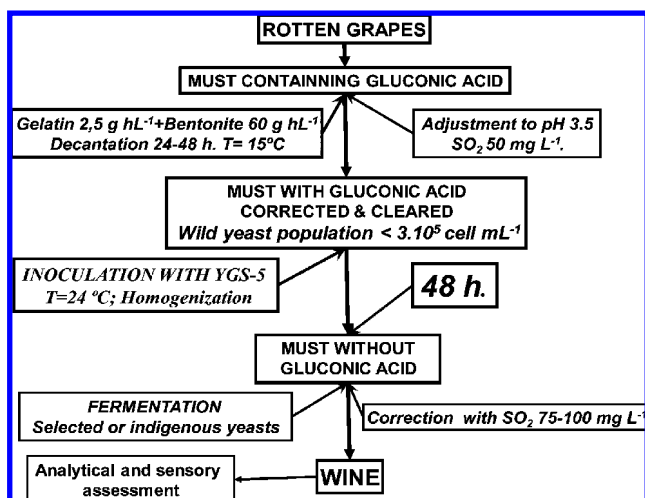
Figure 2. Treatment protocol developed to remove gluconic acid from grape must.

removal treatment, in accordance with the results obtained in synthetic media (*18*). Similar behavior was also reported in experiments with *Hansenula anomala* and *S. cerevisiae* inoculated at the same time because no malic acid consumption was detected, probably as a consequence of inhibition in acid transport produced by ethanol (*26*).

An ANOVA of the contents found in the initial must and after 43 h of treatment with YGS-5 involving sequential inoculation revealed that this yeast had little effect on the sugar, ethanol, acetic, and urea contents of the must. However, YGS-5 substantially reduced the gluconic acid content and hence titratable acidity. The ammonium ion concentration was diminished by 12% too; although the decrease was statistically significant, it was insubstantial since amino acids present in the must constitute an efficient source of easily assimilable nitrogen for fermentation yeasts to grow after YGS-5 had removed gluconic acid from the must.

The analysis of means for the content in wines of the most important winemaking variables (**Table 4**) allowed homogeneous groups to be established at the 95% LSD level. It revealed that the wines obtained with T1 and T2 had slightly increased ethanol content relative to the others (15.5 vs 15.0% v/v). Reducing sugars were found at concentrations of 6, 9, 18, and 23 g/L in the musts treated with T1, T2, T3, and the control procedure, respectively. This suggests that sugars were more strongly depleted and that the fermentation process was more efficient as a result with the sequential inoculation treatments. Titratable acidity was higher with the control treatment and T3 (simultaneous inoculation) than it was with T1 and T2 by effect

of the presence of gluconic acid. pH was also lower with T3 and the control treatment than it was with T1 and T2. The acetic acid content was below 0.5 g L^{-1} with all treatments and lowest when the deacidifying yeast was withdrawn from the must prior to fermentation (T1).

The amino acid contents 43 h after YGS-5 was added (T1 and T2) exhibited a significant decrease for serine, arginine, alanine, valine, tryptophan, and lysine. In any case, the total amino acid content was decreased by only 13.5% since the previous losses were partly offset by an increase in the contents of glycine, threonine, and γ-aminobutyric acid under the action of YGS-5 (**Table 5**).

The wines provided by T2, where YGS-5 was not withdrawn from the medium prior to alcoholic fermentation, exhibited higher glycine, γ-aminobutyric, and lysine contents than the others. However, the contents in threonine, leucine, cysteine, and histidine were similar in the four wines; and the highest concentration of proline, which is the major amino acid in wines and musts, was obtained in the control test. The contents in glutamine, serine, valine, tryptophan, ornithine, and tyrosine of the wines were undetectable; also, glycine was found at levels of 0.3 mM at most (T2 wine). The total amino acid content of the control wine was substantially higher than those of the treated wines, which were similar to one another and to those of the musts after the sequential treatments with YGS-5.

The increased reduction of the gluconic acid content of the must, in addition to the little effect on nitrogen-containing compounds and decreased volatile acidity obtained, indicate that the most effective treatment was that where the deacidifying yeast (YGS-5) was withdrawn from the medium after gluconic acid was removed. However, removing the yeast entails using expensive equipment. Also, centrifuging the must to this end can cause it to absorb large amounts of atmospheric oxygen and introduce uncontrolled changes in composition and sensory quality in the resulting wine. We therefore expanded the initial study by examining changes in major and minor volatile compounds with a view to establishing the impact of the treatment on the aroma of the wines.

Volatile compounds in the wines were classified as major (**Table 6**) or minor (**Table 7**) depending on whether or not their contents exceeded 10 mgL^{-1}. An ANOVA of the contents in the major volatile compound of the musts revealed that those of 2,3-butanediol (*meso*), methanol, propanol, ethyl acetate, and diethyl succinate do not change during the 43 h of treatment with YGS-5 and that those of acetaldehyde, acetoin, glycerine, and 2,3-butanediol (*levo*), isoamyl alcohols and 2-phenylethanol change markedly over the same period. All of these results are consistent with those obtained in previous studies on synthetic

Table 10. Analysis of Must and Wines Obtained According to the Established Protocol for Removing Gluconic Acid[a]

compound or fraction	must	treated wines	control wines	CL
gluconic acid (g L^{-1})	2.33 ± 0.05	0.6 ± 0.1	2.4 ± 0.2	***
glucose + fructose (g L^{-1})	234 ± 4	0.69 ± 0.01	0.09 ± 0.01	***
ethanol, % v/v	0.0 ± 0.0	12.2 ± 0.2	12.3 ± 0.2	NS
acetic acid (g L^{-1})	0.14 ± 0.06	0.41 ± 0.02	0.27 ± 0.01	***
titratable acidity (g L^{-1})	4.40 ± 0.05	6.6 ± 0.1	6.5 ± 0.1	NS
pH	3.53 ± 0.02	3.41 ± 0.03b	3.45 ± 0.03	NS
urea (mg L^{-1})	0.8 ± 0.2	ND	ND	NS
ammonium (mg L^{-1})	68.4 ± 0.2	1.3 ± 0.3	3.26 ± 0.05	***

[a] Tests were conducted at the pilot plant scale and under winemaking conditions. The initial yeast population of YGS-5 added was 15×10^6 cell mL^{-1} in the presence of a 3×10^5 cell mL^{-1} population of indigenous fermentation yeast. CL, ANOVA statistical confidence level between wines; NS, not significant; * 95%; ** 99%; *** 99.9%. ND = not detected.
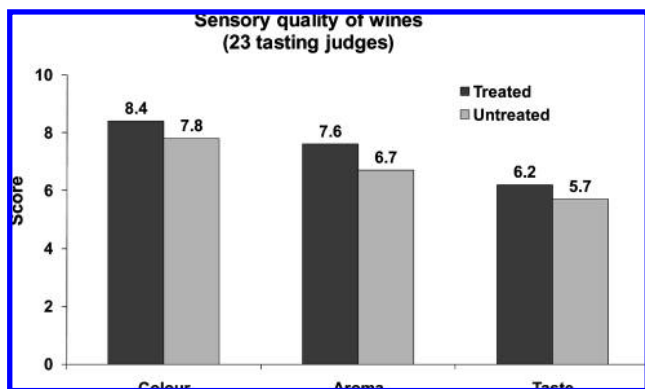
Figure 3. Sensory scores for the wines obtained with the proposed treatment for reducing the gluconic acid content of musts by using *Schizosaccharomyces pombe* YGS-5 and for control wines without treatment.

media (*17, 18*). Also, an analysis of homogeneous groups revealed that the wines provided by T1 had significantly higher contents in acetaldehyde, acetoin, methanol, and propanol than the others; only the contents in methanol and propanol, however, were high relative to the usual levels for wine (*27, 28*) The contents in isobutyl, isoamyl and 2-phenethyl alcohols, and diethyl succinate, in the wine provided by T2 were similar to or lower than those of the other wines. The greatest differences among treatments were those in polyol contents; thus, glycerine and 2,3-butanediol (*levo* and *meso*) had significantly different contents in the wines obtained with T1 and T2 and were higher when YGSA-5 was withdrawn from the fermentation medium (T1). As a result of the withdrawing process, the content in dissolved oxygen of the must increases, allowing an activation of the glyceropyruvic fermentation (*18*). The contents in major volatile compounds of T2 wine were similar to those of the control wine, the sole substantial difference being increased ethyl acetate content and decreased isoamyl alcohol contents in the former.

Especially high among the contents in minor volatile compounds in the musts were those of 1-hexanol and other alcohols and aldehydes of six carbon atoms, which are responsible for herbaceous odors. Only hexanol and hexanal exhibited a significant decrease after 43 h of treatment with YGS-5 in T1 and T2. Also worth special note was the presence in the musts of very small amounts of some terpene compounds (responsible for floral aroma) including linalool, α-terpineol, nerol, and geraniol; only α-terpineol increased in content, however, the others remaining essentially unchanged during the treatments T1 and T2. The wines exhibited contents around 30 mg L^{-1} in the minor volatile compounds quantified; only the wines provided by T1 contained lower concentration (18 ± 2 mg L^{-1}). Ethyl esters and acetates of the higher alcohols have fruity odors

and were the most abundant compounds in this group of volatiles. In qualitative terms, none of the wines exhibited off-odors or off-flavors.

In order to relate the results of the chemical (qualitative and quantitative) and sensory analyses (perception threshold and aroma descriptors), the odorant activity value (OAV) for each studied volatile compound was calculated. As is known, the volatile compounds are the major contributors to the sensory quality of fermented beverages. The odor of a volatile compound can be described in terms of one or several descriptors agreed upon by experts (*29−31*). Some authors have used the odorant series to describe the aroma of wine and alcoholic beverages (*32, 33*). By grouping volatile compounds with a similar descriptor into the odorant series, one can establish an odorant profile and determine the contribution of each individual compound to a particular series. Although this procedure cannot be interpreted as an arithmetic addition of odor sensations, it has the advantage over other existing alternatives that it reduces the number of variables to be analyzed. In addition, it is simpler, uses more objective criteria, and facilitates comparison of the results as the odorant series always comprises the same compounds (*18, 34−36*). The OAV for a volatile compound in wine can be obtained as the ratio of its concentration in the wine to its perception threshold. For this purpose, volatile compounds were classified into the odorant series on the basis of the aroma descriptor for each compound (see **Table 8**). This allowed 8 odorant series to be established and the OAV for each to be calculated as the combination of those of its individual members (**Table 9**). The results thus obtained for musts were subjected to ANOVA and those for the wines to LSD analysis in order to discriminate homogeneous groups at the 95% significance level.

Following the removal of gluconic acid by YGS-5, the musts exhibited a significant increase in OAV for the fruity, solvent, and milky series, which doubled their initial values, and a decreased OAV for the herbaceous, fatty, and roasted series, the spicy and floral series exhibiting no appreciable variation in this respect. These changes resulted in improved sensory properties relative to the starting must (especially, by the decreased OAV for the herbaceous series and increased OAV for the fruity series).

The OAVs for the fruity series in the wines were highest in those obtained by simultaneous inoculation of YGS-5 and G1 (T3) or sequential inoculation without removal of the former yeast (T2). By contrast, the lowest OAVs were those for T1. The OAVs for the floral series were maximal for the two sequential inoculation treatments and so were those for the herbaceous and solvent series. All other series had OAVS < 1 in all wines; therefore, their odorant impact can be deemed small. The OAV for the fatty series was significantly lower in T1 wine than in the others, and the opposite was true of the

milky series. Finally, the roasted series exhibited higher OAVs in T1 and T2 wine, and the spicy series was undetectable in all wines. In summary, the wine obtained by sequential inoculation without removing the deacidifying yeast from the must prior to fermentation was that exhibiting the greatest OAVs in the odorant series representing pleasant descriptors.

The sensory tests performed by the expert tasters of the collaborating cellars showed no off-flavors in the wines. Also, the tasters gave the wine obtained with the sequential treatment involving no removal of the deacidifying yeast (T2) the highest sensory scores. On the basis of the analytical and sensory results, the treatment involving sequential inoculation of the deacidifying yeast and allowing it to stay in the medium after gluconic acid is removed was the best choice for tests at the pilot plant scale.

The selected condition differs from those proposed by other authors. In this way, Taillander et al. (*10*) establish that the delayed inoculation of *Saccharomyces* after *S. pombe* was not a good solution to obtain a partial deacidification of must. By contrast, Kim et al. (*37*) proposed the cofermentation with the simultaneous addition of *S. cerevisiae* W-3 and *Issatchenkia orientalis* (malic acid-degrading yeasts) in a 1:1 (v/v) inoculum ratio as the best option.

In summary, all laboratory tests pointed to temperature, aeration regime, SO_2 rate, and initial concentration of fermentative yeasts as the most influential factors to decrease the gluconic acid content of the musts. **Figure 2** shows the treatment protocol developed on the basis of these critical factors for use under typical winemaking conditions at a pilot plant.

Tests under Typical Winemaking Conditions and Analytical and Sensory Assessment of the Resulting Wines. The must used in the tests performed at the pilot plant scale was obtained from rotten grapes of the 2006 harvest and contained 2.3 g L^{-1} gluconic acid. Prior to inoculating YGS-5, the must was subjected to the prefermentative treatment usually employed by the collaborating winemaker, which involves supplying the must with 2.5 g hL^{-1} gelatine and 60 g hL^{-1} bentonite as clarifying agents, tartaric acid to pH 3.5, and SO_2 to a concentration of 50 mg L^{-1}.

After decantation for 48 h at 15 °C, the must was transferred to another vessel thermostatted at 24 °C in order to count indigenous wild yeasts and to add the YGS-5 starter culture. Under the experimental conditions used, a concentration of indigenous fermentation yeasts of 3×10^5 cell mL^{-1} and one of YGS-5 of $1.5 \ 10^7$ cell/mL allowed the initial gluconic acid to be reduced by 70% (from 2.33 to 0.6 g L^{-1}) within 46 h. Because of the initial population of wild yeast, this procedure should be considered like a simultaneous inoculation of YGS-5 and the wild yeasts remaining after must clarification. This is a common situation in the winemaking process because the complete elimination of wild fermenting yeast is not affordable for the industrial wineries. This result confirms those obtained in the laboratory trials, showing that the efficiency of the treatment with YGS5 is very dependent on the initial population size of the fermentative yeast.

As can be seen from **Table 10**, treated and untreated wines exhibited no significant differences in ethanol or residual sugar contents, titratable acidity, or pH. Only volatile acidity was slightly higher in the treated wines than in the control test (0.4 vs 0.2 g L^{-1}); in any case, it did not exceed the recommended threshold value for this type of wine: 0.5 g L^{-1}, discarding bacterial spoilage.

A panel consisting of 23 expert tasters for the typical wine of the viticultural region found the treated wines to have a better aroma, flavor, and color than the untreated wine (**Figure 3**);

also, they detected no off-flavors or undesirable odors in the former. Two-thirds of the tasters found the wines suitable for aging under the flor-velum yeasts, used typically for the elaboration of fino wine in Jerez and Montilla-Moriles (Spain).

In summary, the best results as regards removing gluconic acid from synthetic media without substantially altering their amino acid contents were obtained by using *S. pombe* (YGS-5) under semianaerobic conditions at 24 °C in the presence of an SO_2 concentration below 100 mg L^{-1}. These conditions allowed the initial gluconic acid content of the sterilized musts to be reduced by 85% within 43 h in the absence of fermentation yeasts. Simultaneously inoculating YGS-5 deacidifying yeasts and *S. cerevisiae* (G1) fermentation yeasts only reduced such a content by 40%. The wines judged to possess the best analytical and sensory properties were those obtained with sequential inoculation of the starter cultures (the deacidifying yeast first and, once gluconic acid was removed, the fermentation yeast next). Of the two sequential inoculation procedures studied, that involving no withdrawal of YGS-5 prior to inoculating G1 provided the highest odorant activity for the fruity series. These results allowed a treatment protocol for musts with a high content in gluconic acid to be developed that was tested under winemaking conditions at a pilot plant. On the basis of the results, the treatment reduced the initial gluconic acid content of the musts by 70% within 46 h without detracting from analytical and sensory quality in the resulting wine.

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