

Real-Time Quantitative PCR (QPCR) and Reverse Transcription-QPCR for Detection and Enumeration of Total Yeasts in Wine[∇]

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Real-time PCR, or quantitative PCR (QPCR), has been developed to rapidly detect and quantify the total number of yeasts in wine without culturing. Universal yeast primers were designed from the variable D1/D2 domains of the 26S rRNA gene. These primers showed good specificity with all the wine yeasts tested, and they did not amplify the most representative wine species of acetic acid bacteria and lactic acid bacteria. Numerous standard curves were constructed with different strains and species grown in yeast extract-peptone-dextrose medium or incubated in wine. The small standard errors with these replicas proved that the assay is reproducible and highly robust. This technique was validated with artificially contaminated and natural wine samples. We also performed a reverse transcription-QPCR (RT-QPCR) assay from rRNA for total viable yeast quantification. This technique had a low detection limit and was more accurate than QPCR because the dead cells were not quantified. As far as we know, this is the first time that RT-QPCR has been performed to quantify viable yeasts from rRNA. RT-QPCR is a rapid and accurate technique for enumerating yeasts during industrial wine fermentation and controlling the risk of wine spoilage.

The wine industry needs rapid procedures for detecting and enumerating yeasts in order to monitor wine fermentation and detect contaminants. Traditional methods of yeast quantification rely on culturing (9). Although effective, these methods require several days and are therefore time-consuming. This is a problem for the winemaker because it means that decisions on wine processing are also delayed (21). Moreover, the presence of viable but nonculturable cells makes it still more difficult to detect all the metabolically active yeast cells in wine samples (18). In the last few years, researchers have used methods to directly identify yeasts from wine without the need for plating (5, 6, 14, 19, 21). Most of these methods rely on the direct amplification of yeast DNA from wine by PCR. Phister and Mills (21) highlighted two main advantages of the direct characterization of microorganisms over yeast enrichment and plating: first, regardless of their capacity to grow in a plate, all the yeast populations are detected, and, second, analysis is fast.

Because of its specificity and sensitivity, one of the most promising PCR techniques in food control is real-time PCR, or quantitative PCR (QPCR) (2). QPCR assays have been developed for detecting and enumerating various bacteria and fungi in food (1, 2, 10, 11). To date, this method has had limited application in the detection of microorganisms in wine (6, 17, 21, 22). With regard to yeasts, only the species *Dekkera bruxellensis* (or its anamorph *Brettanomyces bruxellensis*) and *Saccharomyces cerevisiae* have been enumerated by QPCR in wine samples. The growth of *Dekkera* during the aging of wine in barrels or bottles is an absolute nightmare for the enologist.

This yeast is involved in the production of volatile phenols (4-ethylphenol and 4-ethylguaiacol) and tetrahydropyridines, which are responsible for the unpleasant odors and tastes described as burnt plastic, smoky, horse sweat, leather, and mousy (4, 6, 12). *S. cerevisiae* is the species of yeast with the highest fermentative capacity and is therefore the most important agent of alcoholic fermentation.

In our opinion, this method should be used to quantify other species of wine yeast. However, the wine industry is mostly interested in quantifying contaminant yeast, regardless of the species, in the wine before bottling. For this reason we have developed a QPCR assay for detecting and quantifying the total yeast population in a sample of wine. The QPCR assays were first conducted on reference yeast strains from pure cultures to detect the specificity of the primers. We later validated this technique with artificially contaminated and natural samples of wine and, to determine effectiveness, compared the results with those obtained from plating.

A possible problem with the direct use of microbial DNA from wine is the quantification of dead cells. The detection of RNA by reverse transcription-PCR (RT-PCR) is considered to be a better indicator of cell viability than the detection of DNA (24). We used a primer that was homologous to the 26S rRNA (15) in order to obtain its reverse transcription. We compared total yeast quantification using DNA as the template with *Saccharomyces cerevisiae* quantification using RNA. As far as we know, this is the first time that RT-QPCR has been performed to quantify viable yeasts from rRNA.

MATERIALS AND METHODS

Yeast strains. The strains used in this study are listed in Table 1. All yeast strains were grown in YEPD medium (1% [wt/vol] yeast extract, 2% [wt/vol] peptone, and 2% [wt/vol] glucose) at 28°C. Yeast and bacterial strains were used to test the specificity of the primers (Table 1). Yeast strains *Candida stellata* 11046 and 11110, *Dekkera bruxellensis* 1451^T and 1009, *Hanseniaspora uvarum*

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TABLE 1. Reference strains used in this study

Microorganism	CECT designation ^a	Other designation ^b	Isolation source	Yeast-specific PCR result
Yeasts				
<i>Candida boidinii</i>	1014 ^T	CBS 2428	Tanning fluid	+
	10029	MCYC 113	Milk	+
	10035	MCYC 124	<i>Amygdalus communis</i>	+
<i>Candida sake</i>	1044	CBS 617	Lambic beer	+
<i>Candida stellata</i>	11046	CBS 2649	Grape juice	+
	11110	CBS 843	Wine grapes	+
<i>Dekkera bruxellensis</i>	1009	CBS 72	Lambic beer	+
	1451 ^T	CBS 74	Lambic beer	+
<i>Hanseniaspora uvarum</i>	1444 ^T	CBS 314	Muscat grape	+
	10389	MCYC 1857	Grape juice	+
	11105	CBS 2589	Grape must	+
	11106	CBS 5073	Wine grape	+
	11107	CBS 8130	Grapes	+
<i>Issatchenkia terricola</i>	11139	CBS 4715	Dregs of pressed grapes	+
	11176	CBS 2617	Soil	+
<i>Saccharomyces bayanus</i>	1941 ^T	CBS 300	Beer	+
	1969	CBS 395	Juice of <i>Ribes nigrum</i>	+
<i>Saccharomyces cerevisiae</i>	1171	CBS 1320		+
	1319	ATCC 26602	Sugar refinery	+
	1942 ^{NT}	CBS 1171	Beer	+
<i>Saccharomycodes ludwigii</i>	1371	IFI 979		+
	1382	IFI 982		+
<i>Schizosaccharomyces pombe</i> var. <i>pombe</i>	1378	ATCC 24751	Millet beer	+
	1379	ATCC 26760	Grape must	+
	10685 ^T	CBS 356		+
<i>Torulasporea delbrueckii</i>	1880		Wine	+
<i>Zygosaccharomyces bailii</i>	11042	CBS 3014	Wine	+
	11043	CBS 4688	Grape must	+
<i>Zygosaccharomyces rouxii</i>	1230	CBS 741	Honey	+
	1232 ^T	CBS 732	Must of black grape	+
Bacteria				
<i>Acetobacter aceti</i>	298 ^T	ATCC 15973	Wood of vinegar plant	-
<i>Gluconobacter oxydans</i>	360 ^T	LMG 1408 ^T	Beer	-
<i>Gluconobacter oxydans</i>		LMG 1414	Grapes	-
<i>Acetobacter pasteurianus</i>		LMG 1553	Spoiled beer	-
<i>Gluconacetobacter hansenii</i>		LMG 1527 ^T	Vinegar	-
<i>Oenococcus oeni</i>	217 ^T	ATCC 23279	Wine	-
<i>Lactobacillus plantarum</i>	220	ATCC 8014	Corn silage	-
<i>Lactobacillus brevis</i>	216	LMG 11401	Beer	-
<i>Lactobacillus hilgardii</i>	4786 ^T	ATCC 8290	Wine	-
<i>Pediococcus parvulus</i>	813 ^T	ATCC 19371	Silage	-
<i>Pediococcus pentosaceus</i>	4695 ^T	ATCC 33316	Beer	-

^a CECT, Spanish Type Culture Collection, Universidad de Valencia.

^b CBS, Centralalbureau voor Schimmelcultures, Delft, The Netherlands; ATCC, American Type Culture Collection, Manassas, VA; MCYC, Microbiology Collection of Yeast Cultures, Universidad Politécnica de Madrid; IFI, International Fabricare Institute; LMG, Laboratorium voor Microbiologie Universiteit Gent, Gent, Belgium.

1444^T and 11107, and *Saccharomyces cerevisiae* 1319 and 1942^{NT} were used to obtain the various standard curves.

Primer design. Primers were designed by aligning the variable D1/D2 domains of the 26S rRNA gene sequences from different yeast species. Sequences were obtained from the GenBank database, and alignment was performed with the

Clustal W multiple-sequence alignment (26). The final selection of the primers was performed using the ABI Primer Express program (Applied Biosystems, Foster City, CA). The BLAST search (Basic Alignment Search Tool, <http://www.ncbi.nlm.nih.gov/blastall/nucleotide.html>) was used to check the specificity of each primer. The universal yeast primers were YEASTF (5'-GAGTCGAGTTGTTT

GGGAATGC-3') and YEASTR (5'-TCTCTTTCCAAAGTTCTTTTCATCTT T-3'), which produce a 124-bp fragment.

Specificity of PCR assays. DNA samples from all yeasts were extracted as described by Querol et al. (23). PCRs were carried out in 25- μ l final volumes containing 5 μ l of DNA template (between 10 and 100 ng), 1 μ M of each respective primer, 100 μ M of each deoxynucleoside triphosphate, 2.5 mM MgCl₂, 10 \times buffer, and 2.5 U of *Taq* DNA polymerase (ARK Scientific, Darmstadt, Germany).

The PCR conditions were an initial denaturation at 95°C for 5 min; followed by 35 cycles of denaturing at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min; and with a final extension at 72°C for 5 min. All amplifications were performed in a GeneAmp PCR system 2700 (Applied Biosystems, Foster City, CA). The products of the PCR were analyzed by electrophoresis on a 3% (wt/vol) agarose gel in 1 \times Tris-borate-EDTA buffer stained with ethidium bromide and visualized under UV light. A 100-bp DNA ladder marker (Gibco BRL, Eggenstein, Germany) was used as the size standard.

DNA extraction for the QPCR assay. Yeast cell suspensions were washed with sterile water, and the pellets were resuspended in 700 μ l of AP1 buffer (DNeasy Plant minikit; QIAGEN, Valencia, California) and transferred to a 2-ml conical-bottom microcentrifuge tube containing 1 g of 0.5-mm-diameter glass beads. The tubes were shaken in a mini-bead beater (Biospec Products Inc., Bartlesville, Okla.) for 3 min at the maximum rate and then centrifuged at 10,000 rpm for 1 min. The DNA in the supernatant was transferred to a sterile microcentrifuge tube and purified using the DNeasy Plant minikit (QIAGEN, Valencia, California) according to the manufacturer's instructions.

Standard curves. Standard curves were created by plotting the cycle threshold (C_T) values of the QPCRs performed on dilution series of DNA or yeast cells (10^6 to 1 CFU ml⁻¹) against the log input cells ml⁻¹. Eight different strains belonging to four yeast species (as mentioned above) were serially diluted and used for the construction of the standard curves in YEPD, white wine, and red wine.

A standard curve from RNA as the template was also created. Total RNA was isolated from pure culture of *S. cerevisiae* 1942^{NT} as described by Sierkstra et al. (25). RNA was purified using a High Pure RNA isolation kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions and quantified using a GeneQuant spectrophotometer (Pharmacia, Cambridge, United Kingdom). A 10-fold dilution series of isolated RNA was prepared. cDNA was synthesized from each RNA dilution by using primer NL-4 (5'-GG TCCGTGTTTCAAGACGG-3') (15) and Superscript II RNase H⁻ reverse transcriptase (Invitrogen, Carlsbad, CA) in a GenAmp PCR system 2700 (Applied Biosystems, Foster City, CA). The protocol provided by the manufacturer was used. cDNA from each dilution was used as the templates for QPCR assays in order to construct standard curves.

Artificially contaminated wine. Different mixed cultures with known populations of *S. cerevisiae*, *H. uvarum*, and *D. bruxellensis* were incubated for 24 h in macabeo and cabernet sauvignon wines previously sterilized by filtration. These cultures were then serially diluted, and their DNAs were isolated and used in the QPCRs. These dilutions were also plated in YEPD agar and incubated for 1 to 2 weeks to obtain the number of CFU per milliliter. A WASP spiral plater (AES Laboratoire, Combourg, France) was used for this CFU determination.

Natural fermentation samples. Sampling was taken from two different wine fermentations (malvasia for white fermentation and cabernet sauvignon for red fermentation) at different stages (must, middle fermentation [density, 1,050 to 1,040 g liter⁻¹], and final fermentation [density, 1,000 to 993 g liter⁻¹]). Wine fermentations were carried out in the experimental cellar of the Enology Faculty in Tarragona, Spain, during the 2005 vintage. Different samples of wine aging in oak barrels were also analyzed. Each sample was plated on YEPD agar, and DNA was isolated and used in the QPCRs. RNAs from wine samples were also isolated and used in the RT-QPCRs.

Quantitative PCR assays. PCR amplification was performed in 25- μ l final volumes containing 5 μ l of DNA or cDNA template, 0.2 μ M of each respective primer, and 12.5 μ l of SybrGreen Master Mix (Applied Biosystems). All the amplifications were carried out in optical-grade 96-well plates on an ABI Prism 5700 sequence detection system (Applied Biosystems) with an initial step at 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 60°C for 1 min, and 72°C for 30 s. The C_T was determined automatically by the instrument. All samples were analyzed in triplicate. The coefficients of efficiency (E) were calculated using the formula $E = (10^{-1/\text{slope}}) - 1$ (13). Confidence intervals were calculated by Student's t test with a significance level of 5% (7).

Heat inactivation of cultured *S. cerevisiae*. The experiment was very similar to that of Bleve et al. (2), with some modifications. An exponentially growing *S. cerevisiae* culture was diluted to a suspension containing 10^5 CFU ml⁻¹. Heat treatment involved incubation of the dilution tube (5 ml) at 60°C in a water bath

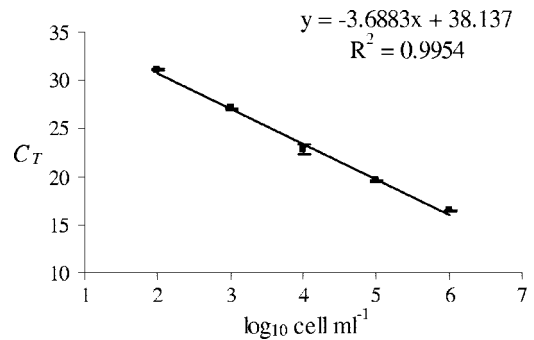


FIG. 1. Standard curve obtained from serially diluted *Saccharomyces cerevisiae* pure genomic DNA. C_T values are the average of three repetitions. Error bars represent standard errors.

for 20 min. Samples were taken during the treatment (after 10 min), at the end of the treatment, and during subsequent incubation at 25°C for 24 h (after 30 min and after 1, 12 and 24 h). Cell viability was analyzed both by plate count and by fluorescence microscopy.

For the latter method, we used the commercial kit LIVE/DEAD *BacLight* (Molecular Probes). The *BacLight* solution, which contained 100 μ M SYTO 9 and 600 μ M propidium iodide (PI), was mixed with an equal volume of the yeast solution. Sharp fluorescent images were produced with only 30 s of mixing. The intensities of SYTO 9 and PI were monitored at 480/500 nm and at 488 to 540/617 nm, respectively. Uptake of PI (orange/red fluorescence) indicated dead cells, while accumulation of only SYTO 9 (green fluorescence) indicated viable cells.

Statistical analysis. The results were statistically analyzed by one-way analysis of variance and the Scheffé test from the statistical software package SPSS 13.0. The statistical degree of significance was set at a P value of <0.05. All the C_T values are averages of at least three repetitions.

RESULTS

Primer design, specificity of PCR, and sensitivity of QPCR.

The universal yeast primers were designed from conserved sequences of the variable D1/D2 domains of the 26S rRNA gene. Sequences from virtually all known ascomycetous yeast species have been determined for this region (15), which facilitated the design and testing of the assays for species specificity.

We determined the primer specificity by amplifying the yeasts and bacteria listed in Table 1 with the designed primers. A DNA fragment of the expected size was found with all the yeast species tested. However, these primers were not able to amplify DNA from other wine microorganisms such as lactic acid bacteria and acetic acid bacteria.

To determine the sensitivity and detection limits of the QPCR, DNA obtained from an *S. cerevisiae* culture at a concentration of 10^6 CFU ml⁻¹ was serially diluted 10-fold. Each DNA dilution was used to construct a standard curve (Fig. 1). The assay was linear over 5 orders of magnitude, and the detection limit was approximately 10^2 CFU ml⁻¹. This result was lower than those of similar previous assays, in which linearity was over 6 orders of magnitude (2, 17, 21). The lack of linearity of the samples at low cell concentrations is due to the fact that the nontemplate samples used as controls in the QPCR assay showed positive signals (C_T values of approximately 36). This was probably due to an accumulation of dimers of oligonucleotide primers to which SYBR Green molecules bound. The melting curve obtained for this nonspecific

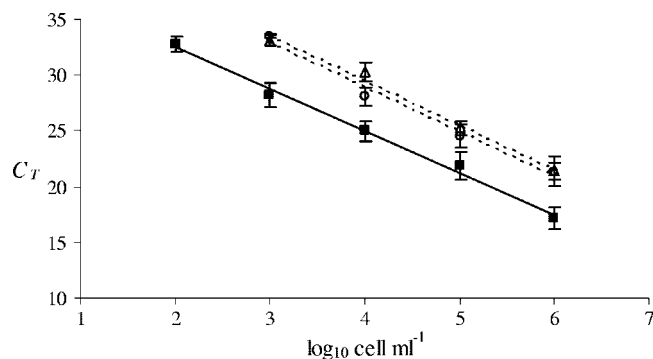


FIG. 2. Total-yeast standard curves obtained by QPCR from 10-fold serial dilutions of *Candida stellata*, *Dekkera bruxellensis*, *Hanseniaspora uvarum*, and *Saccharomyces cerevisiae* grown in YEPD medium (■, —) and incubated in white wine (○, ----) and red wine (△, ----). Correlation coefficients (r^2), slopes, and efficiencies are shown in Table 2. Detection limits were 10^2 and 10^3 cells ml^{-1} for YEPD medium and wine, respectively. C_T values of standard curves from YEPD medium, white wine, and red wine are the averages of eight, four, and four individual standard curves, respectively. Error bars represent standard errors.

product was different from that of the correct amplicon (results not shown). These C_T values of the nontemplate controls overlapped with the C_T value obtained for cell number concentrations of below 10^2 CFU ml^{-1} .

Quantification of total yeasts by QPCR. A total of eight individual standard curves were constructed with different strains and species grown in YEPD medium in independent experiments. Likewise, four individual standard curves were constructed with different strains and species incubated in white wine and the same number in red wine. From these individual curves, we constructed a general standard curve for each matrix used (Fig. 2). The small standard errors of these curves proved that all the strains were similarly useful for generating a standard curve. The correlation coefficients, slopes, and efficiencies of the amplification for the standard curves are shown in Table 2. The presence of the cells in a complex matrix such as wine influenced the amplification efficiency. Some major compounds of wine, such as polyphenols, are known to have an inhibitory effect on the PCR. However, these compounds did not seem to be the main inhibitors of the amplification process, because the efficiency in red wine, which has a much higher proportion of polyphenols, was very similar to that in white wine. Another possibility is that the wine matrix interfered with the process of DNA extraction and purification. As a result of this decrease in the efficiency of amplification

TABLE 2. Correlation coefficients, slopes, and efficiencies of standard curves obtained from serial dilutions of yeast cells grown in YEPD medium and of yeast cells incubated in white and red wines^a

Yeast cell assay	r^2	Slope	Efficiency ^b (%)
YEPD	0.9651 ± 0.04	-3.374 ± 0.11*	98.01 ± 4.34*
White wine	0.9710 ± 0.02	-4.003 ± 0.29	78.27 ± 7.46
Red wine	0.9742 ± 0.01	-3.984 ± 0.35	79.02 ± 9.11

^a All values are means and standard errors. Asterisks indicate statistically significant differences ($P < 0.05$).

^b Efficiency was estimated by the formula $10^{-1/\text{slope}} - 1$.

TABLE 3. Determination of C_T values for a dilution series of *S. cerevisiae* cells without or with 10^6 cells of lactic acid bacteria ml^{-1} or 10^6 cells of acetic acid bacteria ml^{-1}

Log ₁₀ yeast cells ml^{-1}	C_T (mean ± SE)		
	<i>S. cerevisiae</i>	<i>S. cerevisiae</i> plus lactic acid bacteria	<i>S. cerevisiae</i> plus acetic acid bacteria
6	19.4 ± 0.7	19.5 ± 0.5	18.4 ± 0.3
5	22.3 ± 0.1	22.5 ± 0.2	21.5 ± 0.4
4	25.3 ± 0.4	25.2 ± 0.1	24.5 ± 0.2
3	29.4 ± 0.1	29.1 ± 0.1	29.1 ± 0.3
2	31.3 ± 0.3	31.5 ± 0.3	30.6 ± 0.5

from cells incubated in wine, the C_T values were higher for the same cell concentration and the detection limit in wine was 1 log unit higher than that obtained for cells grown in YEPD.

We also tested the impact of a large amount of nontarget DNA coming from other wine microorganisms (lactic acid bacteria and acetic acid bacteria) on the QPCR assay. For this purpose, *S. cerevisiae* cells (target microorganism) were serially diluted and supplemented with 10^6 cells of lactic acid bacteria ml^{-1} or with 10^6 cells of acetic acid bacteria ml^{-1} (nontarget microorganisms). The C_T values obtained for each dilution (with or without contaminating DNA) are shown in Table 3. Statistical treatment by analysis of variance did not show significant differences in the C_T values obtained for the same dilutions with or without contaminating DNA. These results suggest that the presence of other, nontarget wine microorganisms in the samples did not significantly affect the QPCR assay.

The same white and red wines used for the standard curves were artificially contaminated with different mixtures of *S. cerevisiae*, *H. uvarum*, and *D. bruxellensis* ranging from 10^4 to 10^7 CFU ml^{-1} . After 24 h of incubation, the samples were analyzed by QPCR. Results obtained from mixed cultures were compared to the standard curves generated from wine. The effectiveness of the quantification by the method was tested by correlating the cell concentrations estimated by the plate count in YEPD and by QPCR (Fig. 3). The results showed that the numbers obtained by QPCR did not differ significantly from those measured by plating.

We also analyzed natural samples during and after alcoholic fermentation. Samples were taken from two different wine fermentations (malvasia for white wine fermentation and ca-

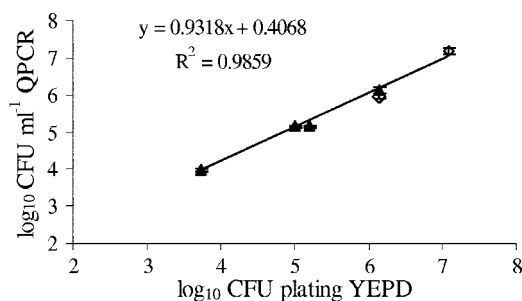


FIG. 3. Relationship between results of yeast quantification by QPCR and by plating of artificially contaminated white wine samples (◇) and red wine samples (▲). C_T values are averages of results from three replicates. Error bars represent standard errors.

bernet sauvignon for red wine fermentation) at different stages (must, middle fermentation, and final fermentation), and samples were also taken from wine aging in an oak barrel. Each sample was plated on YEPD agar, and DNA was isolated and quantified by QPCR. The correlation between the enumerations of the fermentation samples by QPCR and by plating was good (Table 4). Conversely, the enumeration of the oak barrel sample by QPCR was higher than the enumeration by plating as a result of viable but nonculturable yeasts or, more likely, due to the presence of dead cells in the sample.

Quantification of *Saccharomyces cerevisiae* yeasts by RT-QPCR. To obtain more accurate quantification of viable yeasts, an RT-QPCR assay was developed. To construct the standard curves, RNA isolated from an *S. cerevisiae* YEPD culture at a concentration of 10^6 CFU ml⁻¹ was serially diluted 10-fold. cDNA from each RNA dilution was synthesized using primer NL-4, as described in Material and Methods. The synthesized cDNA was subjected to RT-QPCR assay. The same procedure was carried out with a culture of *S. cerevisiae* incubated for 24 h in cabernet sauvignon wine. These standard curves are shown in Fig. 4. To verify that traces of DNA did not exist in RNA samples, a non-reverse-transcribed control was used. No amplification was obtained in any non-reverse-transcribed control sample. The detection limit of the RT-QPCR assay was of 10 CFU ml⁻¹, and the coefficient correlations were 0.9975 and 0.9777 in the standard curves obtained from YEPD and red wine, respectively. The efficiencies were 92.67% and 93.53%, respectively. These efficiency values were better than those obtained using DNA as the template. Wine did not interfere with RT-QPCR assays for quantifying total viable yeasts (Fig. 4).

Samples from the cabernet sauvignon fermentation and from wine aging in oak barrels were analyzed using RNA as the template. The results obtained by RT-QPCR were compared with those obtained by QPCR and by plating (Table 5). During alcoholic fermentation, although with significant differences, the counts obtained by the three methods were similar. However, in some oak barrel samples, the QPCR method detected up to 10-fold more cells than the plating method. Instead, enumeration by RT-QPCR showed counts similar to those obtained by plating (B05 and B11) or by QPCR (B04 and B13),

TABLE 4. Quantification of yeast populations in samples during and after alcoholic fermentation by plating and QPCR^a

Wine sample	CFU ml ⁻¹ (plating on YEPD agar)	Cells ml ⁻¹ (QPCR for total yeasts)
Malvasia		
Must	$(7.1 \pm 0.6) \times 10^6$	$(5.0 \pm 0.3) \times 10^6$
MF ^b	$(9.8 \pm 1.4) \times 10^6$	$(3.5 \pm 0.3) \times 10^{7*}$
FF ^c	$(5.0 \pm 0.8) \times 10^6$	$(6.3 \pm 0.7) \times 10^6$
Cabernet sauvignon		
Must	$(1.5 \pm 0.1) \times 10^6$	$(2.2 \pm 0.4) \times 10^6$
MF	$(9.4 \pm 1.9) \times 10^6$	$(1.9 \pm 0.1) \times 10^{7*}$
FF	$(5.4 \pm 0.5) \times 10^6$	$(2.5 \pm 0.1) \times 10^{7*}$
Oak barrel	$(2.1 \pm 0.8) \times 10^1$	$(1.7 \pm 0.2) \times 10^{2*}$

^a All values are means and standard errors. Asterisks indicate statistically significant differences compared to plating results ($P < 0.05$).

^b MF, middle fermentation (density, 1,050 to 1,040 g liter⁻¹).

^c FF, final fermentation (density, 1,000 to 993 g liter⁻¹).

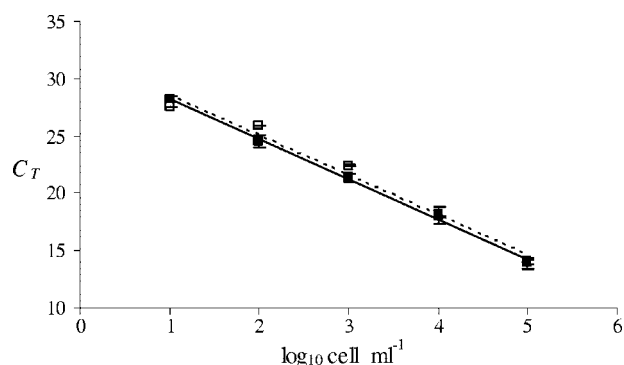


FIG. 4. Standard curves obtained from serially diluted RNA isolated from *Saccharomyces cerevisiae* grown in YEPD medium (■, —) and incubated in red wine (□, ----). Correlation coefficients (r^2) are 0.9975 for YEPD medium and 0.9777 for red wine. C_T values are the averages from three repetitions.

depending on the samples. Assuming that the RT-QPCR technique may have detected only viable cells whereas the QPCR technique detected both viable and dead cells, differences in the enumerations by RT-QPCR and plating should be interpreted as due to the presence of viable but nonculturable yeasts, whereas differences between RT-QPCR and QPCR should be due to the presence of dead cells in the sample.

Stability of 26S rRNA and rDNA in heat-killed cells. The detection of RNA by RT-QPCR is considered a better indicator of cell viability than the detection of DNA. However, this is mostly accepted with regard to mRNA, while the stability of rRNA in yeasts has not been documented. Therefore, we reproduced an experiment similar to that of Bleve et al. (2) in which the suitability of actin gene mRNA, in comparison with its DNA, was evaluated as an indicator of fungal cell viability. The stability of rRNA and ribosomal DNA (rDNA) was assessed by the C_T values obtained for samples taken at various times during and after heat treatment at 60°C for 20 min of a culture of 10^5 CFU ml⁻¹ of *S. cerevisiae* (Fig. 5). Cell viability was simultaneously evaluated during and after the heat treatment by standard plate count and by microscopy fluorescence. After 10 min of heat treatment, no colonies were detected and 100% of the cells were stained red, indicating lost of viability. C_T values from rRNA and rDNA did not change after 12 h of heat treatment, so both molecules were stable during this period. However, the C_T obtained from rRNA increased approximately from a value of 16 to a value of 21 at 1 day after the loss of cell viability. A 99% decrease in rRNA detection was therefore observed, since 3 units of C_T is approximately equivalent to 1 log unit. On the other hand, 100% of the DNA was detected during the same period. These results suggest that 26S rRNA was less stable than rDNA but more stable than mRNA (which was undetectable by RT-QPCR amplification after 20 min of heat treatment [2]).

DISCUSSION

Rapid and sensitive methods for detecting and enumerating yeasts are needed in the wine industry to enable winemakers to make decisions to control and avoid spoilage of their products. Real-time PCR (QPCR) is a suitable technique that has been

TABLE 5. Enumeration by plating, QPCR, and RT-QPCR of yeasts in samples from cabernet sauvignon alcoholic fermentation and barrel aging^a

Sample	CFU ml ⁻¹ (plating on YEPD agar)	Cells ml ⁻¹ (QPCR for total yeasts)	CFU ml ⁻¹ (RT-QPCR for total yeasts)
Cabernet sauvignon			
Must	$(1.5 \pm 0.1) \times 10^6$	$(2.2 \pm 0.4) \times 10^6$	$(1.1 \pm 0.05) \times 10^6$
MF ^b	$(9.4 \pm 1.9) \times 10^6$	$(1.9 \pm 0.1) \times 10^{7*}$	$(1.1 \pm 0.1) \times 10^{7*}$
FF ^c	$(5.4 \pm 0.5) \times 10^6$	$(2.5 \pm 0.1) \times 10^{7*}$	$(6.6 \pm 0.2) \times 10^{6*}$
Oak barrel			
B04	$(2.1 \pm 0.8) \times 10^1$	$(1.7 \pm 0.2) \times 10^{2*}$	$(1.3 \pm 0.2) \times 10^{2*}$
B05	$(8.8 \pm 1.0) \times 10^2$	$(4.1 \pm 0.8) \times 10^{3*}$	$(9.0 \pm 0.3) \times 10^2$
B06	$(2.1 \pm 0.3) \times 10^2$	$(4.7 \pm 0.5) \times 10^{2*}$	$(3.6 \pm 0.1) \times 10^2$
B11	$(7.9 \pm 2.3) \times 10^1$	$(3.0 \pm 0.2) \times 10^{2*}$	$(6.3 \pm 0.5) \times 10^1$
B13	$(5.4 \pm 0.4) \times 10^1$	$(5.1 \pm 0.5) \times 10^{2*}$	$(4.8 \pm 0.1) \times 10^{2*}$

^a All values are means and standard errors. Asterisks indicate statistically significant differences compared to plating results ($P < 0.05$).

^b MF, middle fermentation (density, 1,050 to 1,040 g liter⁻¹).

^c FF, final fermentation (density, 1,000 to 993 g liter⁻¹).

applied in the last few years to detect and quantify microorganisms associated with food. Studies on the quantification of *D. bruxellensis* and *S. cerevisiae* in wine by real-time PCR have been developed (6, 17, 21), but we have used the technique to detect and enumerate the total number of yeasts in a wine sample. Controlling contamination by species that are particularly dangerous for wine spoilage (e.g., *D. bruxellensis*) is important. However, the most widespread microbiological analysis in the wine industry is the enumeration of total yeast in the final product (after bottling), regardless of the species of contaminant.

To achieve this, universal yeast primers were designed from the variable D1/D2 domains of the 26S rRNA gene. This is one of the few gene sequences available for all known ascomycetous yeast species, and the region has been used successfully in the past to develop QPCR methods for other yeasts (3, 21). These QPCR primers showed good specificity with all the wine yeasts tested and did not amplify the most representative wine species of acetic acid bacteria and lactic acid bacteria. Moreover, standard curves constructed with different yeast strains and species showed similar efficiencies of amplification, which

reinforced the specificity of the primers for yeast enumeration. It should be mentioned that a BLAST analysis of the sequences of the designed primers against a nucleotide database showed many filamentous fungi with an identity of 100% with the sequences of both primers. For example, *Botrytis cinerea*, a typical grape spoilage fungi, showed a mismatch only with the sequence of the reverse primer. Therefore, in the case of grapes contaminated with filamentous fungi, we should expect amplification of fungal DNA present in must samples along with yeast DNA. However, the selective pressure (anaerobic conditions and high sugar and ethanol concentrations) exerted during alcoholic fermentation makes the presence of fungi during and after this process impossible.

We also determined the sensitivity, or detection limit, of the QPCR assay with the designed primers. Our QPCR analysis efficiently enumerated cells at concentrations of as low as 10^2 CFU ml⁻¹ when the standard curve was constructed from cells in YEPD medium and at concentrations of as low as 10^3 CFU ml⁻¹ when the standard curve was constructed from cells in wine. These differences in sensitivity between the two systems should be attributed to the interference produced by the matrix wine on the PCR amplification or on the process of DNA extraction and purification. All authors who have used the QPCR technique for wine have reported problems of amplification with DNA directly isolated from wine (6, 17, 21). Wine is a complex matrix that is known to possess various PCR inhibitors (21, 27), such as polyphenols, tannins and polysaccharides. This is why we also constructed standard curves by the dilution of cells incubated in wine. Polyphenols and tannins are mostly responsible for this PCR inhibition, because the inhibitory effect is stronger in red wines (which have higher polyphenol concentrations) than in white wines (6, 17). However, this was not true in our case, because we did not detect any significant differences between the two main types of wines. A common matrix interference must therefore be taken into account for wines, and a standard wine-specific curve must be generated.

There are no legal limits for the content of yeast in wine; there are only recommendations by the International Organization of Vine and Wine (OIV). The OIV recommends that the microbial load should be less than 10^4 to 10^5 CFU ml⁻¹ for

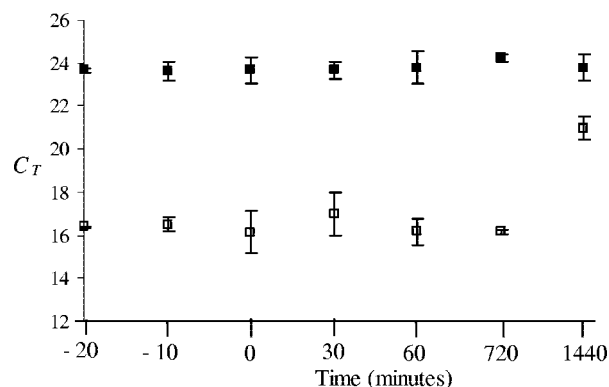


FIG. 5. Comparison of mean C_T values obtained by QPCR with rDNA as the template (■) and by RT-QPCR with rRNA as the template (□) at different times during and after heat treatment (60°C, 20 min) of an *S. cerevisiae* suspension. Time zero is the end of the heat treatment. C_T values are averages of results from three replicates. Error bars represent standard errors.

microorganisms that produce powdery sediments and less than 10^2 to 10^3 CFU ml⁻¹ for microorganisms that produce flocculent sediments. Below these levels, bottled wine is clear and therefore acceptable (16, 17). If we take these numbers into account, our primers are useful for detecting the minimum cell number. However, the acceptable levels of microorganisms for the wine industry are much lower than those recommended by the OIV. Contamination with a small number of cells is a potential cause of spoilage during storage and before commercialization. The simplest way to decrease the detection limit is to concentrate the sample by centrifugation or filtration, which is not a problem for the volumes involved in the wine industry. As we explain below, these detection limits decreased when RNA was used as template.

The numerous replicas for obtaining the standard curves and the small standard errors among these replicas proved that the assay is reproducible and highly robust, even with DNA isolated from wine. In both YEPD medium and wine, we obtained a very good correlation between the predicted number of CFU per milliliter, as determined by QPCR, and the number of CFU per milliliter, as determined by plating. The QPCR assay also effectively enumerated the yeast population in the true wine samples, and there was a good correlation with enumeration by plating. Only for the wine in the oak barrel was the yeast population determined by plating much lower than that determined by QPCR. This may be due to the presence of viable but nonculturable cells, which are amplified by the QPCR. Millet and Lonvaud-Funel (18) have already reported the presence of microorganisms in this state during wine storage. However, this lack of correlation between plating and the QPCR assay may also be explained by DNA amplification of dead cells.

Since RNA is less stable than DNA after cellular death, the detection of this molecule is considered to be a better indicator of cell viability than the detection of DNA (24). However, QPCR systems based on reverse transcription have mostly used mRNA as the template, whereas our primers were designed to amplify rDNA. Our template for the reverse transcription reaction was therefore the 26S rRNA. Fontaine and Guillot (8) studied the stability of another rRNA (18S rRNA) by RT-QPCR as a possible marker of viability of *Cryptosporidium parvum* and concluded that 18S rRNA was more stable than mRNA but less stable than its encoding rDNA after a thermal shock. Similar conclusions are obtained from our study of the stability of the 26S rRNA in *S. cerevisiae*. We detected degradation equivalent to 99% of the 26S rRNA molecules at 24 h after cell death, while the DNA molecule remained stable. Therefore, 26S rRNA may be correlated with cell viability, but with the restraint that the loss of viability did not produce immediate degradation of this molecule. We should also point out that the instability of the mRNA should be considered advantageous for the rapid detection of cellular death but also that it involves problems of degradation during the extraction and manipulation of the samples, which may lead to an underestimation of the quantification of the samples. Furthermore, the durations of the aging, bottling, and storing processes in the wine industry are long enough to discard as irrelevant yeast death in a 1-day period, which would be the method's error. This underestimation would be more

critical during the alcoholic fermentation, where the changes are quicker.

However, in contrast to the case for DNA molecules, the use of a specific RNA (mRNA or rRNA) for microbial enumeration may have the problem of variation of gene expression in response to various factors or growth phases. Yeasts in wines are found in different growth stages (lag, exponential, and stationary phases), and variable rRNA copy number might influence the accuracy of the quantification. In order to check the impact of the growth stage on rRNA copy number, we collected 10^5 cells in lag, exponential, and stationary phases and analyzed them by RT-QPCR. We detected differences of up to $2 C_T$ lower in the stationary-phase samples than in the lag and exponential phases (data not shown). Despite this fluctuation, the differences are not dramatic, and therefore we propose the use of rRNA for yeast quantification.

A promising and easy-to-use alternative to the RNA-based quantification methods has been recently published (20). This method uses the DNA-intercalating dye ethidium monoazide bromide, which penetrates only into dead cells. Subsequent photoinduced cross-linking was reported to inhibit PCR amplification of DNA from dead cells. So far this selective removal of DNA from dead cells has been assayed with bacterial cells, and it should be tested with other microorganisms.

In conclusion, QPCR is a fast, direct (without culture), sensitive, and reliable technique for quantifying the total yeast population in wine. QPCR can be used to enumerate yeasts during industrial wine fermentation and to rapidly control the risks of wine spoilage. A drawback of the quantification by using DNA as the template is that live and dead cells are not differentiated. RT-QPCR using primer NL-4 may be useful for enumerating total viable yeast because it has a low detection limit and because only viable cells are quantified. The quantification by using RNA may have the problem of variation of gene expression, depending on the physiological state of the cell. As far as we know, this is the first time that RT-QPCR has been performed from rRNA to quantify viable yeasts.

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