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Evaluation of three *Brettanomyces* qPCR commercial kits: results from an interlaboratory study

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Abstract

Aim: *Brettanomyces bruxellensis* is well adapted to high ethanol concentrations and low pH which allows it to develop in difficult environments, such as wine. *B. bruxellensis* is mainly found in red wine and is regarded as a spoilage yeast due to its production of ethylphenols and other compounds responsible for organoleptic defects. The detection and quantification of this yeast is essential to preventing wine spoilage. Several specific detection and quantification kits based on real time quantitative PCR (qPCR) are commercially available. Although these kits are frequently used by private enological and research laboratories, no scientific reports on the reliability and performance of these kits, including interlaboratory and interassay comparisons, have been published. The aim of this work was to compare commercially available kits for the quantification of *B. bruxellensis* in red wine to classical method (plate counting on selective medium) in an interlaboratory study.

Methods and results: Three different commercial kits were tested on three different wines from Bordeaux, Côtes du Rhône, and Burgundy inoculated with *B. bruxellensis* at four different concentrations. Five naturally contaminated wines from different French wine regions were also tested. Our results suggest that all the kits tested probably over or underestimate the quantity of *B. bruxellensis* in red wine and, under specific conditions, give false positives.

Conclusion: Quantification may be very heterogeneous depending on the wine, laboratory, or population level. Underestimations or false negative results may have serious consequences for winemakers. Overestimation may be partly due to the quantification of dead cells by qPCR.

Significance and impact of the study: This study highlights that quantification of *B. bruxellensis* in red wine using commercial kits requires a high level of expertise in molecular biology. We recommend that all users use a microbiological internal control to validate DNA extraction yield.

Keywords: *Brettanomyces bruxellensis*, commercial kits, DNA extraction, quantitative PCR, red wine

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Introduction

The yeast *Brettanomyces bruxellensis* is a source of wine spoilage, especially in red wines. Compounds naturally present in grape juice and wine that originate from the grapes are generally esterified (*p*-coutaric, fertaric and caftaric acids) (Dugelay *et al.*, 1993). Enzymes with an esterase activity can release the free form of the acids (*p*-coumaric, ferulic and caffeic acids) (Gerbaux *et al.*, 2002). These acids are decarboxylated to vinylphenols by yeast, fungi and lactic acid bacteria. *B. bruxellensis* is able to reduce these vinylphenols to ethylphenols leading to an unpleasant taste (Chatonnet *et al.*, 1997, 1995).

The detection and quantification of this yeast is necessary to prevent wine spoilage. The isolation of yeast on Wallerstein Laboratory Nutrient (WLN)-based medium is routinely used as a simple test in the wine industry (Rodrigues *et al.*, 2001) as recommended by the Oenological Codex 2016 Edition. In addition, several DNA-based techniques are used to detect or quantify this yeast by culture dependent or independent methods, such as RNA-FISH (Röder *et al.*, 2007; Serpaggi *et al.*, 2010; Stender *et al.*, 2001), PNA-FISH (Stender *et al.*, 2001), ITS PCR coupled to RFLP (Esteve-Zarzoso *et al.*, 1999), DGGE (Cocolin *et al.*, 2004; Prakitchaiwattana *et al.*, 2004; Renouf *et al.*, 2006), TGGE (Hernán-Gómez *et al.*, 2000), loop mediated isothermal amplification (Hayashi *et al.*, 2007), and quantitative PCR (qPCR) (Delaherche *et al.*, 2004; Phister and Mills, 2003; Tessonnière *et al.*, 2009; Willenburg and Divol, 2012). Several specific qPCR-based quantification kits have been developed based on previous studies reporting the efficiency of qPCR to specifically quantify *B. bruxellensis* and are commercially available.

In this study, we report on the variability of three qPCR kits designed to quantify *B. bruxellensis* in red wine based on data generated from three laboratories specialized in wine analyses. Each laboratory followed the commercial protocols to generate comparable data. The laboratories used standardized protocols and the same batch of DNA isolation and amplification reagents to limit variability.

Materials & Methods

1. Participants

Three laboratories were selected for participation: the VAlMiS lab (Dijon, FR), the Microflora-ISVV lab (Bordeaux, FR) and the Inter-Rhône lab (Orange, FR). The names of the laboratories were randomly codified (lab 1, lab 2 and lab 3).

2. Conditions

Each laboratory used a different strain of *B. bruxellensis* to artificially contaminate red wine. The Inter-Rhône, Microflora, and VAlMiS labs used the strains GSLEV17, CRBO LO417 (Centre de Ressources Biologiques Oenologiques, ISVV, Villenave d'Ornon, France), and LO2E6, respectively, and red wine of each region was inoculated at four different population levels: 10^2, 10^3, 10^4, and 10^5 cells/mL, referred to as levels 1, 2, 3, and 4, respectively. The cells were adapted to ethanol by growing them on YPD agar (10 g/L yeast extract, 20 g/L bacto-peptone, 20 g/L glucose, 20 g/L agar) supplemented with 5% (*v/v*) ethanol at 28°C for 5 days. Stationary phase cells were used to inoculate diluted red wine (50% red wine: 50% physiological saline water) and incubated for one week at 28°C. The adapted cells were then used to inoculate filter sterilized red wine from each region.

Moreover, five naturally contaminated wines from different wineries were also analyzed by the three laboratories.

3. Methods for the enumeration of *B. bruxellensis*

a. Reference method

The reference method consisted of counting *B. bruxellensis* colonies on nutritive media plates. Each sample was enumerated in Petri dishes on ITV selective medium: 10 g/L yeast extract, 20 g/L bacto-peptone, 20 g/L glucose, 0.1 g/L *p*-coumaric acid, 0.1 g/L ferulic acid, 0.03 g/L bromocresol green, 0.2 g/L chloramphenicol, 0.006% (*w/v*) cycloheximide, 20 g/L agar; pH adjusted to 5 (Gerbaux *et al.*, 2000).

b. Alternative method

The alternative method consisted of qPCR performed with commercial kits. Three different commercial kits (arbitrarily named Kit 1, Kit 2, and Kit 3) were tested for *B. bruxellensis* DNA extraction and amplification. DNA extraction and amplification protocols were performed according to the manufacturers' instructions. Standard curves were used in two kits, whereas one kit allowed direct quantification based on the amplification of reference DNA. Each laboratory used two kits, i.e. each sample was analyzed by the same kit in triplicate by two different laboratories. Amplification reactions were performed on a CFX96 real-time PCR system (Bio-Rad) for two laboratories and on an iCycler IQ5 system (Bio-Rad) for the third. Results were analyzed using Bio-Rad CFX Manager® software. The PCR cycle where

fluorescence first occurred (quantification cycle: C_q) was determined automatically after setting the regression method.

Red wines from Côtes du Rhône, Burgundy, and Bordeaux were supplemented with a high level of molecular sulfite (approximately 2 mg/L mSO_2) to determine whether the kits quantify dead *B. bruxellensis*. Cell quantifications using the three kits were performed after two weeks. The total *B. bruxellensis* population and culturable cells present in the red wines were determined by flow cytometry (FCM) or microscopy methods and plate counting, respectively. Viability was determined by FCM. Solutions containing cells used to determine total populations were stained with dyes (propidium iodide and fluorescein diacetate) according to the protocol described in the study of Salma *et al.* (2013).

4. Construction of the accuracy profiles and statistical processing

The construction of the accuracy profile was performed as described by Boubetra *et al.* (2011). The acceptability criterion was defined as ± 0.3 log units/mL for the alternative method in our study. Target values, based on the median values obtained using the reference method, were determined. The results were generated using the alternative method, and the reproducibility standard deviation (SD) (s_R), the limits of acceptability (λ), the proportion of β-expectation tolerance interval (β), and the difference between the level determined by qPCR and the target value (*Bias*) were determined for each inoculation concentration. The accuracy profiles were constructed using these results.

The reproducibility standard deviation (SD) (s_R) is calculated based on the SD between triplicates (s_r) and SD between labs (s_L):

$$s_R = \sqrt{s_r^2 + s_L^2}$$

A β expectation tolerance interval (β-ETI) is defined as an interval that covers an average percentage of a variable distribution. For example, a β-ETI can claim to contain 80% of future measurements, on average. A β-ETI can be expressed as:

$$\overline{x} \pm k_M \times s_r$$

where k_M is the coverage factor, given by the equation:

$$k_M = Qt\left(v, \frac{1+\beta}{2}\right)\sqrt{1+\frac{1}{I \times J \times G^2}}$$

where s_r is the repeatability standard deviation, Qt the percentile of a Student t test distribution, β the chosen probability (80% in this study), I the number of laboratories, J the number of replicates, v the number of degrees of freedom, and G given by the equation:

$$G = \sqrt{\frac{H+1}{J \times H+1}}$$

where $H = s_L^2 / s_r^2 = s_R^2 / s_r^2 - 1$, s_R^2 is the reproducibility variance, and s_r^2 the repeatability variance. The number of degrees of freedom, v, is given by the equation:

$$v = \frac{(H-1)^2}{\frac{H+\frac{1}{J}}{I-1} + \frac{1-\frac{i}{J}}{I \times J}}$$

where i is the number of laboratories performing the analysis (i < I). In our study, i was equal to 2.

Results

1. Reference results

We calculated the reference values, also called target values, for each level of contamination from the median values obtained using the reference method (plate counting). Table 1 shows the theoretical values (10^2, 10^3, 10^4, and 10^5 cells/mL). *B. bruxellensis* populations counted by plating were very close to the expected cell population except for one wine. The Bordeaux wine had a population that was lower than

Table 1 - Mean ± standard deviation (log_{10} CFU/mL) of culturable populations determined by plate counting on selective medium of red wines artificially contaminated at four population levels analyzed by the three laboratories, in triplicate.

Theoretical values		Levels(log_{10} CFU/mL)			
		2	3	4	5
Values found	Burgundy wine	2.3 ± 0.6	3.7 ± 0.3	4.8 ± 0.4	5.8 ± 0.4
	Bordeaux wine	0.4 ± 0.3	1.1 ± 0.6	2.5 ± 0.5	3.7 ± 0.1
	Côtes du Rhône wine	2.6 ± 0.6	3.4 ± 0.9	4.5 ± 0.6	5.4 ± 0.6

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Table 2 - r^2 values according to the kit, lab and wine analyzed at the four population levels, in triplicate.

	r^2 values									
	Burgundy Wine			Bordeaux wine			Côtes du Rhône wine			Means
Kits	Lab 1	Lab 2	Lab 3	Lab 1	Lab 2	Lab 3	Lab 1	Lab 2	Lab 3	
Kit 1	nd	0.9960	0.9867	nd	0.9584	0.9715	nd	0.8648	0.9574	0.9558
Kit 2	0.9560	nd	0.8684	0.9105	nd	0.7551	0.9908	nd	0.8797	0.8934
Kit 3	0.9557	0.9670	nd	0.7985	0.9655	nd	0.8723	0.8974	nd	0.9094

nd: not determined

the theoretical values due to the inability to obtain a high cell concentration for this *B. bruxellensis* strain in this wine and a likely decrease in viability after inoculation.

2. Linearity

Linearity of the results for each wine and each kit was determined by plotting the logarithmic results obtained by plate counting (mean of the three labs) against the values determined using the qPCR commercial kits. The correlation coefficient (r^2) values are shown in Table 2. The mean r^2 values were 0.9558 ± 0.0471 for Kit 1, 0.8934 ± 0.0820 for Kit 2, and 0.9094 ± 0.0670 for Kit 3. With four population levels, the degree of freedom is equal to 2 for this statistical analysis. With a risk of error (α) of 10%, the critical r^2 value is 0.9. Eight of 18 results were not valid ($r^2 < 0.9$) (Table 2).

3. Validation criteria and statistical results

Counts obtained using the alternative method are presented in $\log_{10}$ units. The validation criteria and statistical results for the Côtes du Rhône, Burgundy, and Bordeaux wines are shown in Supplementary data 1, Supplementary data 2 and Supplementary data 3, respectively.

Repeatability was calculated for each wine according to population level and kit. The mean repeatability was 0.257, 0.183, and 0.390 $\log_{10}$ cells/mL for Kits 1, 2, and 3, respectively.

Kit 1 underestimated the four population levels in Burgundy wine by a mean of 1.2 $\log_{10}$ cells/mL. The population levels determined by Kit 1 for the Côtes du Rhône and Bordeaux red wines were overestimated by 0.5 and 1.1 $\log_{10}$ cells/mL relative to the reference method.

Kit 2 underestimated all population levels in the Burgundy and Côtes du Rhône wines (bias of -2.3 and -0.9 $\log_{10}$ cells/mL, respectively). This kit also underestimated the lowest three population levels in the Bordeaux wine, with a bias of -0.9 $\log_{10}$ cells/mL, whereas the highest population level had a bias of approximately 0.6 $\log_{10}$ cells/mL.

Kit 3 also led to an underestimation of all population levels in the Burgundy wine (mean bias of 0.8 $\log_{10}$ cells/mL). This kit also underestimated two population levels in the Bordeaux and Côtes du Rhône wines, with a bias of -0.2 and -1.8 $\log_{10}$ cells/mL, respectively, whereas two others were overestimated by a mean of 1.1 and 0.9 $\log_{10}$ cells/mL.

In summary, we could not establish any relation between the population level and the reproducibility or bias.

We compared the reproducibility standard deviation (SD) and absolute bias between the kits (Table 3). All absolute values for the reproducibility SD were high (from 0.4 to 1.1 $\log_{10}$ cells/mL). The best bias was 0.5 and the highest was 2.3 $\log_{10}$ cells/mL. Based on these results, no kit precisely quantified *B. bruxellensis* levels in red wine because the reproducibility SD and bias exceeded the acceptability limits. *B. bruxellensis* levels were frequently underestimated, highlighting the imprecision of this contamination measure and the risk of obtaining false negative results.

4. Accuracy profiles

We generated accuracy profiles to visualize the level of imprecision in quantifying *B. bruxellensis* levels in red wine. Examples of the accuracy profiles for the Côtes du Rhône wine calculated from the results obtained using Kits 1, 2, and 3 are presented in Fig. 1a, 1b, and 1c, respectively. The acceptability limit for this study was ± 0.3 $\log_{10}$ cells/mL. This value was the maximum acceptable limit and the performance of each kit was tested to determine whether the alternative method is at least as good as the reference method. For this wine, only two values obtained using Kit 1 (level 1) and Kit 2 (level 4) were within the acceptable limits. However, the β-ETIs

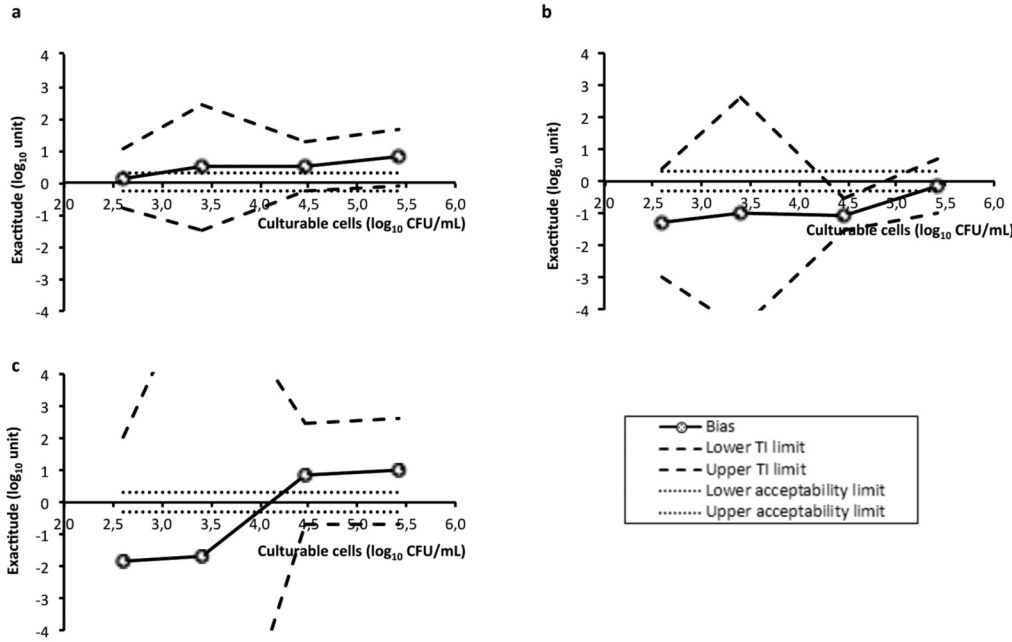

Figure 1 - Accuracy profiles of the alternative method based on the results of Kit 1 (a), Kit 2 (b), and Kit 3 (c) for the Côtes du Rhône red wine with a β equal to 80% and a λ of ± 0.3 log_{10} unit.
The X axis shows the results from the reference method (log_{10} CFU/mL)
and the Y axis the differences between the reference method and the qPCR kits.

were not within these limits, meaning that there is an 80% probability that future analyses will be outside these limits.

The accuracy profiles for the Burgundy red wine are presented in Supplementary data 4. Only two values were within the acceptable limits (levels 3 and 4 with Kit 3) but the bias (β-ETIs) was outside the acceptable limits.

The accuracy profiles for the Bordeaux wine are presented in Supplementary data 5. Two values obtained with Kit 2 were within acceptable limits (levels 1 and 2) as were two values obtained with Kit 3 (levels 2 and 3), but, as above, the β-ETIs were not within these limits.

5. Analysis of dead yeast

We performed trials with the commercial kits on cells subjected to sulfite treatment to test whether overestimation of *B. bruxellensis* populations may be due to the quantification of dead yeast. The results are shown in Table 4. None of the three wines contained culturable *B. bruxellensis*. Only the Côtes du Rhône wine contained a viable population of *B. bruxellensis*, whereas the red wines from Burgundy and Bordeaux did not, validating the cell death caused by the sulfite treatment.

The quantification results using the kits were precise for the same kit and sample (repeatability) for the three red wines.

The viable population in the Côtes du Rhône wine was higher than the culturable population, probably due to viable but non-culturable (VBNC) cells. Kits 1 and 3 led to an overestimation of 1.7 and 0.9 log_{10} cells/mL, respectively, relative to the viable population determined by FCM combined with viability staining. Such overestimation may come from the quantification of dead cells.

The population levels determined for the Burgundy wine from Kits 2 and 3 were approximately identical to the total population (dead cells).

The quantification of *B. bruxellensis* in the Bordeaux wine by Kit 1 largely underestimated the population (-3.7 log_{10} cells/mL), whereas Kit 2 led to an overestimation of the population (+0.6 log_{10} cells/mL).

6. Quantification of *B. bruxellensis* in five potentially contaminated red wines

B. bruxellensis populations present in five red wines (Wine 1, Wine 2, Wine 3, Wine 4, and Wine 5) from different wineries were determined by plate counting and quantification using the commercial kits. The results are shown in Table 5.

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Table 3 - Absolute mean standard deviations of reproducibility and bias according to the wine tested and the kit used, independent of the contamination levels.

	Absolute means (log_{10} cells/mL)								
	Burgundy wine			Bordeaux wine			Côtes du Rhône wine		
	Kit 1	Kit 2	Kit 3	Kit 1	Kit 2	Kit 3	Kit 1	Kit 2	Kit 3
Reproducibility SD	0.624 ± 0.119	0.710 ± 0.226	1.090 ± 0.516	0.946 ± 0.415	0.391 ± 0.208	0.913 ± 0.556	0.362 ± 0.127	0.462 ± 0.366	1.123 ± 0.935
Bias	1.168 ± 0.275	2.283 ± 0.537	0.804 ± 0.750	1.060 ± 0.350	0.747 ± 0.843	0.672 ± 0.898	0.465 ± 0.273	0.867 ± 0.501	1.345 ± 1.555

Table 4 - Analyses of red wines containing *B. bruxellensis* killed by a high sulfite dose (2 mg/L molecular SO_2).

The experiments were performed on Côtes du Rhône, Burgundy and Bordeaux red wines.
The results are expressed in log_{10}. Total *B. bruxellensis* populations were determined by flow cytometry or microscopy, the culturable populations by plate counting (reference method), and quantification by commercial kits (Kit 1, Kit 2 and Kit 3; alternative method), in triplicate.

	Absolute means (log_{10} cells/mL)								
	Burgundy wine			Bordeaux wine			Côtes du Rhône wine		
	Kit 1	Kit 2	Kit 3	Kit 1	Kit 2	Kit 3	Kit 1	Kit 2	Kit 3
Reproducibility SD	0.624 ± 0.119	0.710 ± 0.226	1.090 ± 0.516	0.946 ± 0.415	0.391 ± 0.208	0.913 ± 0.556	0.362 ± 0.127	0.462 ± 0.366	1.123 ± 0.935
Bias	1.168 ± 0.275	2.283 ± 0.537	0.804 ± 0.750	1.060 ± 0.350	0.747 ± 0.843	0.672 ± 0.898	0.465 ± 0.273	0.867 ± 0.501	1.345 ± 1.555

Table 5 - Quantification (log_{10} cells/mL) of *B. bruxellensis* in five naturally contaminated red wines (Wine 1, Wine 2, Wine 3, Wine 4, and Wine 5) using the commercial kits.

The standard deviations were calculated from three independent triplicates. Asterisks indicate data significantly different from plate counting results (ANOVA with a Dunnett test; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

	Kit 1		Kit 2		Kit 3		
	Lab 2	Lab 3	Lab 1	Lab 3	Lab 1	Lab 2	Petri dish
Wine 1	2.3 ± 1.0**	3.4 ± 0.1	1.8 ± 0.2***	2.4 ± 0.0**	3.3 ± 0.8	0.0 ± 0.0***	3.6 ± 0.3
Wine 2	0.0 ± 0.0	-0.1 ± 0.2*	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.0***	0.0 ± 0.0	0.0 ± 0.0
Wine 3	0.2 ± 0.3***	1.9 ± 0.1	0.0 ± 0.0***	0.4 ± 0.5***	2.2 ± 0.2	0.0 ± 0.0***	2.1 ± 0.5
Wine 4	1.4 ± 1.0*	3.0 ± 0.0	1.0 ± 0.1**	1.8 ± 0.2	2.3 ± 1.0	1.5 ± 1.4	2.8 ± 0.4
Wine 5	0.0 ± 0.0	2.3 ± 0.2***	1.1 ± 0.1***	1.4 ± 0.1***	1.5 ± 0.6***	0.0 ± 0.0	0.0 ± 0.0

Only two results for Wine 1 were not significantly different from the enumeration results by plate counting (from Kit 1 performed by lab 3 and Kit 3 performed by lab 1) (Table 5). No culturable cells were detected in Wine 2, whereas there were two positive results from the kit quantifications. The results for Wine 3 were significantly identical to plate counting when the quantifications were performed by lab 3 with Kit 1 and lab 1 with Kit 3. Two quantifications were significantly different from the populations determined by plate counting for Wine 4 (Table 5). Wine 5 did not contain culturable *B. bruxellensis*, whereas four kit-based quantifications were positive.

The results of *B. bruxellensis* quantification of naturally contaminated red wines validate the previous results performed in artificially contaminated red wines. The results were similar or the population was underestimated when the yeast was detected by plate counting. No significant overestimation was made for these red wines.

Discussion

Accuracy profiling was applied to analyze an alternative method against the reference method. In our study, the reference method chosen was plate counting on selective medium as this approach is the most widely used by enological laboratories to study the culturability of this yeast. Three commercial kits that quantify *B. bruxellensis* in red wine were used as the alternative method.

Using a β of 80% and a λ equal to ± 0.3 log_{10} cells/mL, none of the kits were validated because the level of *B. bruxellensis* determined by these kits was under or overestimated with a bias that was generally higher than the acceptable limit. Moreover, the predicted results resulted in a large discrepancy, leading to a large incertitude of future quantifications. However, the quantification results were precise for the same kit and sample (repeatability).

Using the results of the accuracy profile, a correction factor can be applied if, for example, all results are

slightly and repeatedly overestimated according to the population levels. However, in our study, the kits sometimes overestimated the population at one level and underestimated it at another. We observed no continuous error between the alternative and reference methods, making it impossible to apply a correction factor to the results. Moreover, as the tolerance intervals were higher than the acceptability limits, no quantification limit could be determined.

These results highlight the poor quantification by the commercial kits, given the experiments were performed by three laboratories specialized in the wine field and in the use of qPCR technics. It is necessary for all winemakers to use the same quantification methods to monitor *B. bruxellensis* populations. The reference method based on plate counts provides reliable results. Nutritive media have different selectivity and it is essential to always use the same nutritive media to monitor yeast from the same tank throughout vinification and aging. It is also essential to have knowledge and know how in molecular biology and qPCR analysis, because of the sensitivity of the method.

Overestimation of *B. bruxellensis* using the qPCR kits may be due to the presence of VBNC cells in the wine which may not be detectable by plate counting (Du Toit *et al.*, 2005; Millet and Lonvaud-Funel, 2000; Serpaggi *et al.*, 2012). As we show here, it may also be due to the fact that the kits do not discriminate live from dead or VBNC cells, confirming previous studies (Andorrà *et al.*, 2010; Vendrame *et al.*, 2014; Willenburg and Divol, 2012). Propidium monoazide (PMA) and ethidium monoazide bromide can discriminate between live and dead microorganisms (Andorrà *et al.*, 2010; Rizzotti *et al.*, 2015; Vendrame *et al.*, 2014) and could be used in this context.

The best solution to prevent underestimation is the use of an internal control. The internal control is often a microorganism not found in wine (Longin *et al.*, 2016; Tessonnière *et al.*, 2009) and added to the samples at a known concentration before DNA extraction. If, for example, *Yarrowia lipolytica* is added as an internal control (Tessonnière *et al.*, 2009), a first amplification of this yeast must be performed to validate the quantification. Similar values for the quantification of the internal control and the initial added population that are not significantly different indicate that the DNA extraction yield is acceptable. *B. bruxellensis* quantification is feasible under these conditions.

To conclude, our study highlights that commercial kits for the quantification of *B. bruxellensis* have different extraction yields leading to different quantification results. The drawbacks of the methods described above could negatively affect a winemaker's decision and lead to wine spoilage due to over or underestimation. It is thus necessary to add a standardized qPCR protocol for *B. bruxellensis* quantification in wines. One such standardized protocol based on the work of Tessonnière *et al.* (2009) which includes a microbial internal control is already available in the OIV methods (OIV-OENO 414-2011). Although longer than commercial kit protocols, it has been shown to be sensitive and efficient. However, in this protocol, the target DNA corresponds to the RAD4 gene. Thus, the amplification of this gene after cell death needs to be assessed to prevent overestimation. Alternatively, the commercial kits could be improved by using both a microbiological internal control and PMA.

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