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Brettanomyces bruxellensis prevalence in wines produced and marketed in Spain

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Abstract The sensory deterioration of wine caused by the formation of volatile phenols (4-ethylphenol and 4-ethylguaiacol) due to the growth of the spoilage yeast *Brettanomyces* during the winemaking process can cause serious financial loss and problems in export trade barriers. The presence of this microorganism is increasingly common in cellars. Fast, specific and early detection of this yeast during wine production enables the oenologist and producer to take preventive measures before the phenolic aspect appears. Several methods are available for the detection of this yeast species. A new, quick and easy-to-use kit based on RT-PCR to detect *Brettanomyces* has appeared recently on the market. To evaluate the prevalence of this microorganism in our country and the effectiveness of this new technique, we analysed 86 red wines produced and marketed in Spain (commercialised wines or just before being bottled). We compared the results with those obtained with traditional microbial counting on selective medium and with another molecular method based on the amplification of the internal transcribed spacers (ITS1 and ITS2) of the rRNA 5.8S. Fifteen samples (17.4%) were positive for the presence of *B. bruxellensis* by microbiological plate counting after 5–7 days of incubation and 14 (16.3%) also gave positive results with RT-PCR analysis, after only 7 h. Of 86 wines analysed, 16 (18.6%) were positive with at least one of the methods used.

Keywords *Brettanomyces* · Red wine · Prevalence · RT-PCR · Detection

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Introduction

Brettanomyces bruxellensis is generally considered a spoilage yeast in the wine industry. Moreover, it is one of the best adapted yeasts in wine, since it is resistant to high ethanol and sulphur dioxide concentrations and low nutrient requirements. Its presence causes sensorial defects, with the production of volatile phenols, occasionally combined with an increase in volatile acidity. These defects are characterised by aromas often described as "phenolic", "ink", "varnish", "horse sweat", "leather" or "stable smells" (Chatonnet et al. 1993; Rodrigues et al. 2001). These deviations from the olfactory optimum are most noticeable in premium wines that have been stored for long periods in wooden barrels where *Brettanomyces* sp. can remain in the pores of the wood after cleaning and sterilisation. The yeast can grow during the red wine aging (Froudiere and Larue 1988) and even after bottling. They are rarely found during alcoholic fermentation of the must, although they have been isolated alongside *Saccharomyces* (Froudiere and Larue 1988). A few studies have reported the rare presence of *Brettanomyces* on grape clusters (Pretorius 2000) and in wine storehouses (Peynaud and Domercq 1956; Chatonnet et al. 1999). However, they are more commonly found in vats and pumps, and on equipment that is difficult to sterilise (Fugelsang 1998).

Regular monitoring of *Brettanomyces* populations is recommended, and a sudden population increase should be treated by sulfiting, filtration or thermal inactivation

(Couto et al. 2005b; Divol and Lonvaud-Funel 2005; Barata et al. 2008). Traditional methods to identify this yeast in wine rely on culturing (Fugelsang 1998), using selective media containing ethanol as the sole carbon source, cycloheximide and *p*-coumaric acid to reveal the presence of phenolic off-flavour producers (POFs), and bromocresol to disclose the production of acetic acid (Rodrigues et al. 2001; Couto et al. 2005a). This technique sometimes lacks accuracy, since it cannot detect viable but non-culturable cells (VNC) and requires a long incubation time (7–10 days) to obtain results. In some cases, cell morphology can facilitate the presumptive discrimination of the yeast. However, methods to detect their morphological features or classical methods of identification are inadequate for routine oenological microbiological work, and do not identify viable populations (Millet and Lonvaud-Funel 2000). The need for a fast, specific, sensitive and reliable method in order to detect this yeast has increased in the last few years. Flow cytometry (Malacrino et al. 2001; Gerbaux 2007) or fluorescence microscopy with fluorescent probes specific for a fragment of *B. bruxellensis* 26S ribosomal RNA (Stender et al. 2001) are techniques that allow rapid and reliable identification of this species. However, when detection needs to be specific, molecular techniques based on PCR are preferred. *Brettanomyces* isolated from wine have been identified by the electrophoretic determination of their karyotypes and by the random amplification of polymorphic DNA (RAPD-PCR; Mitrakul et al. 1999). Specific sequences of internal transcribed spacer (ITS) regions between ribosomal RNA genes have been found that are sufficiently distinct to allow identification of four species of *Brettanomyces* (Egli and Henick-Kling 2001; Esteve-Zarzoso et al. 1999). Real-time or quantitative PCR (RT-PCR) assays have been developed for the detection and enumeration of a number of fungi and food-borne pathogens. One advantage of this method is that colony growth is not required. Several authors have designed primer sequences and amplification conditions for RT-PCR to detect and enumerate *B. bruxellensis* in wine (Phister and Mills 2003; Delaherche et al. 2004; Tessonnière et al. 2009). In this study, a new commercial kit for the detection of this spoilage microorganism directly in wine has been tested in order to develop a screening tool for the presence of *Brettanomyces* in samples of bottled wine or in the last stage of their production, just before being bottled.

Materials and methods

Wine samples

Eighty six Spanish wines from different appellation regions were analysed for the presence of *Brettanomyces bruxellensis*. Forty four samples (51%) were already bottled and labelled wines that can be found in supermarkets: "marketed wines". Forty two samples (49%) were wines in the qualification process to obtain appellation and were collected from different cellars: "cellar wines". Because one of the main factors related to the presence of *Brettanomyces* is the process of wine aging in wooden barrels, we classified the samples according to this factor. Among the marketed wines, 37 had undergone oak aging and 7 had not. Among the cellar wines, 26 were with oak aging and 16 with no aging. The barrel aging period ranged between 7 and 14 months, depending on the samples.

Brettanomyces detection

To carry out an initial screening of the samples to be analysed, the presence of *B. bruxellensis* was determined by means of traditional microbiological count on selective medium. Two different commercial media were used: DBDM medium (Rodrigues et al. 2001) from Stabvida (Caparica, Portugal) and kit Brett from Vivelys (Villeneuve-lès-Maguelone, France; Barbin 2006; Gilis et al. 2008). A 100 ml sample of each wine was filtered through membranes with a pore size of 0.45 μm and incubated on the medium at 28±1°C for 10–12 days in the case of the DBDM medium or 5–6 days on Vivelys medium. Dilutions were performed, if necessary. Changes in colour and odour of the medium and the appearance of CFU (colony forming units) were checked over time. The characteristic morphology of *Brettanomyces* cells was also checked with optical microscope observations, enabling a presumptive identification but not confirmation.

Randomly selected colonies were analysed with a qualitative PCR-based method to confirm the species in some of wine samples that gave positive results on selective media. ITS1 and ITS2 of the rRNA 5.8S were amplified (Fig. 1). DNA from each colony was isolated according to Querol et al. 1992a, b. PCR amplification was carried out following conditions described by Guillamón et al. 1998 and Esteve-Zarzoso et al. 1999. PCR products were electrophoresed on 1.4% agarose (Roche Diagnostics, Barcelona, Spain), stained with ethidium bromide and

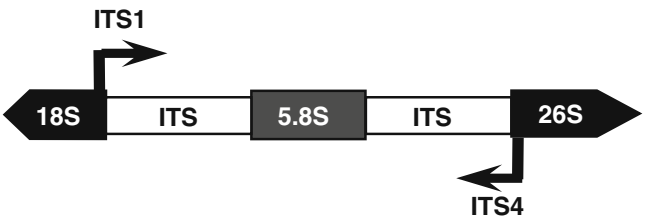

Fig. 1 Nuclear rRNA gene region amplified by PCR using primers ITS1 and ITS4. *ITS* Internal transcribed spacer

photographed. Later, a digestion of the amplicons with the restriction endonuclease *Hinf*I (New England Biolabs, http://www.neb.com/) was made following the supplier's instructions. In this case, restriction fragments were electrophoresed on 3% agarose. A 100-base pair (bp) ladder marker (Gibco BRL, http://www.invitrogen.com) served as the size standard. *Saccharomyces cerevisiae* strain P29 (Spanish Type Culture Collection – CECT 11770) was used as a negative control.

In order to obtain rapid results, a new kit based on RT-PCR developed by Bio-Rad and distributed by Sofralab Technologies (http://www.sofralabtechnologies.com/): Vineo™ *Brettanomy*test, was applied. Extraction of DNA was performed directly from the wine following manufacturer's instructions (Vineo™ Extract DNA Kit). Samples of 1.8 ml were processed in the case of "cellar" wines and samples of 45 ml in case of "marketed" wines. DNA amplification was performed with the VINEO™ *Brettanomy*test PCR kit consisting of fluorescent probes, amplification mix, negative PCR control and positive quality standard PCR control. Amplification was carried out in a MiniOpticon Bio-Rad thermocycler. The analysis software adapted to this test, IQ-Check™ Analysis, produced an automatic and quantitative result to measure the risk of *B. bruxellensis* presence in the wine sample.

Analysis of 4-ethylphenol in wines

The quantities of 4-ethylphenol in wines that were positive for the presence of *Brettanomyces* were analysed specifically by gas chromatography (Chatonnet et al. 1995).

Wine (10 ml) was extracted three times with successively 5 ml, 2 ml and 2 ml dichloromethane. The combined organic extracts were slowly concentrated to 1 ml at room temperature by evaporation under nitrogen gas flow.

Gas chromatography was performed with a HP5890 series II instrument by injecting 1 μl of the concentrate extract by means of a splitless injector (splitless time 30 s; split ratio 1/50; temperature 250°C) into a capillary column (Suprawax 280, 30 m, 0.53 mm internal diameter), programmed from 45°C to 230°C at 3°C/min, final isotherm 30 min, with hydrogen as carrier gas (1 ml/min). Detection was performed with a flame ionization detector (FID) at 260°C. Quantification was carried out by reference to a standard range prepared under the same conditions.

Sensorial description

In order to evaluate the effect of the growth of *Brettanomyces* sp. and detect the off-odour caused by this yeast, a descriptive tasting of wines was carried out by ten expert panellists. The most representative descriptors of each evaluated wine were registered.

Results and discussion

To test the efficiency of the Vineo™ *Brettanomy*test with regard to DNA extraction from wine and amplification conditions, a correlation between plate count (CFU/ml) and quantitative results given by the RT-PCR analysis (genomic units: GU/ml) was established in 11 positive wines for the presence of *B. bruxellensis*. An acceptable correlation (R^2= 0.837) was obtained between both techniques (Fig. 2). A common problem of real-time PCR is failure of DNA amplification due to the presence of inhibitory substances in the wine. Many wine components, such as polysaccharides, tannins and pigments, can lead to poor DNA isolation and/or amplification efficiency, thus producing false-negative results (Rossen et al. 1992; Wilson 1997). No problems were observed in the wines analysed: both positive and negative controls of the kit gave the expected results.

In order to ensure the specificity of the selective media used to detect *Brettanomyces* sp., amplification of ITS1 and ITS2 of the rRNA 5.8S was performed in some randomly selected colonies of positive wine samples. Figure 3 shows electrophoretic patterns obtained from the isolates. PCR products (Fig. 3a) showed a 485 bp band for the strains isolated as *Brettanomyces* and a band of 880 bp for the *Saccharomyces cerevisiae* strain. These PCR products were digested with the restriction endonuclease *Hinf*I and the restriction fragments are showed in Fig. 3b. The lengths corresponding to *B. bruxellensis* (265 bp and 215 bp) and *S. cerevisiae* (365 bp and 155 bp) were in agreement with those obtained by Guillamón et al. 1998, thus confirming the identity at species level.

Once the methodology was established, the prevalence of this spoilage yeast was determined in 44 marketed and 42 cellar wines. Table 1 summarises the number of samples that were positive or negative for the presence of *Brettanomyces* in each group using selective media and RT-PCR. Aging and non-aging results, in each group of wines (marketed and cellar wines, plate count or RT-PCR), are shown in Fig. 4. It is well known that these yeasts

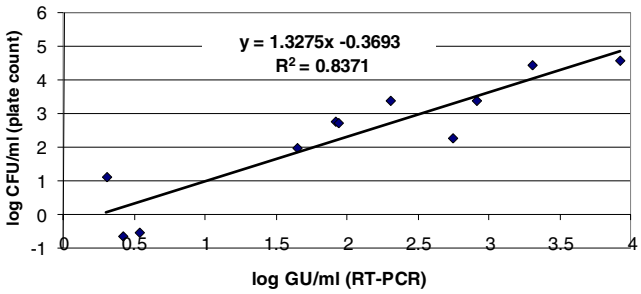

Fig. 2 Correlation of quantification of *Brettanomyces* in 11 wines by classical culture on selective media (colony forming units: CFU/ml) and by RT-PCR (genomic units: GU/ml)

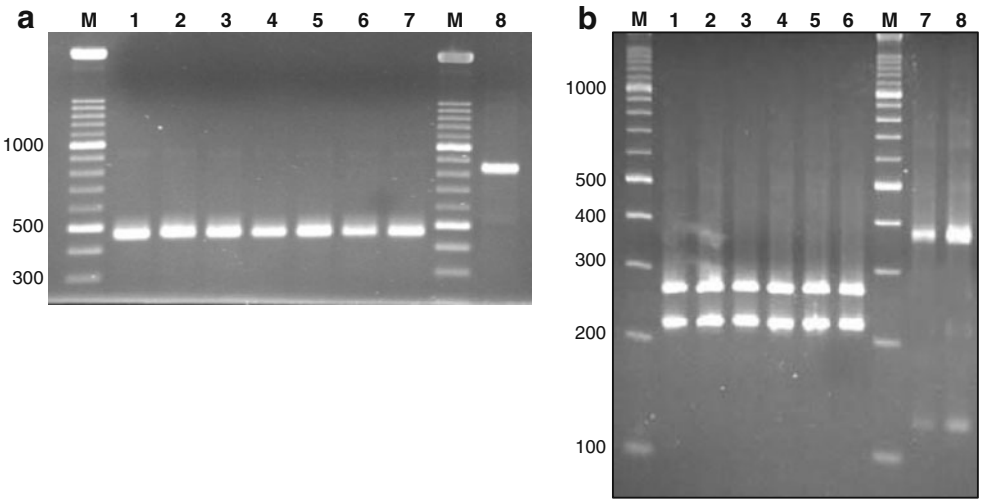

Fig. 3 Size of the PCR-amplified rRNA 5.8 S region of wine yeast isolations (**a**) and restriction analysis with the endonuclease *Hinf* I (**b**). Lanes: *M* Molecular size standards (100 bp DNA ladder); **a** *1–7 Brettanomyces bruxellensis* isolated from selective medium; *8*: *Saccharomyces cerevisiae* strain P29; **b** *1–6 B. bruxellensis* isolates; *7*, *8 S. cerevisiae* strain P29

generally proliferate after alcoholic and/or malolactic fermentations, during wine aging and after the wine has been bottled (Gerbaux et al. 2000; Renouf et al. 2007; Suárez et al. 2007). In this study, the three marketed samples in which *Brettanomyces* was detected (Table 2, Fig. 4a, b) were wines with oak aging. However, this result was not seen in cellar samples, where *Brettanomyces* predominated in non-aging wines (see Fig. 4c,d). This fact should be taken into account by winemakers in order to take preventive measures, even in non-aging wines, before wines are bottled if they are not fined or filtered. Suárez et al. 2007 summarised some of these optional methods for the control of *Brettanomyces* during winemaking.

To determine statistically significant differences between marketed and cellar wines when Brett detection had been positive, Fisher's exact tests were conducted. Fisher's exact test was used because the sample size was small and, consequently, the Chi-Square test is not appropriate. Using either the plate count method or the RT-PCR method, cellar wines showed a significantly higher proportion of Brett's positive samples compared to marketed wines ($P<0.0012$ and $P<0.013$ for plate count and RT-PCR methods, respectively). Conclusive results regarding aging versus non-aging effects over Brett's positive proportion were not reached because of the sample size limited the power of the statistic test applied.

In marketed wines, only 3 out of 44 wines gave positive results by RT-PCR (A,B,C), ranging from 5.5×10^2 GU/ml to 8.3×10^3 GU/ml (Table 2). Two of these wines (A and B) were also positive using the plate count. In sample C (see Table 2), plate count was performed with the DBDM and Vivelys media but neither of them could detect *Brettanomyces*. This could be a case of VNC detection by RT-PCR. Yeasts are unable to grow at this metabolic state and are not detected by classical microbiological analysis (Millet and Lonvaud-Funel 2000; Renouf et al. 2007). At this state yeast cells still remain metabolically active and can produce 4-ethylphenol. This could explain the fact that a wine free of *Brettanomyces* (by plate count) during winemaking may cause alterations in the bottle. At this stage of the process, a method like RT-PCR, capable of detecting VNC, is of great interest. Millet and Lonvaud-Funel (2000) reported that micro-organisms that survive in wine long after wine-making and sulfiting could exhibit a reduction in size and form microcolonies that were not visible within the usual incubation time. Also, the reduction in cell size may cause them to pass through a 0.45 μm membrane. These could be some of the reasons why some wines may be considered "Brett sterile", but are found to be spoiled by analysing with RT-PCR methods. Moreover, the DNA purification kit is optimized for nucleic acid extraction only from living cells (due to centrifugation speed steps and differential density between live and dead cells; manufacturer's personal communication). This fact would exclude the possibility that dead cells were detected as it could be hypothesised that a sample could be Brett negative by traditional plate count and positive when analysed by RT-PCR (e.g. sample C, Table 2).

Plate count results also depend on the medium used, as in the case of sample B (Table 2), where there was a difference of 1 log. For detection of *Brettanomyces / Dekkera* it is

Table 1 Detection of *Brettanomyces bruxellensis* in marketed and cellar wines using culture on selective media (DBDM or Vivelys medium) or by means of RT-PCR. Results are expressed as number of samples. Data as percentage of the total number of samples ($n=86$) are shown in parenthesis

	Plate count		RT-PCR	
	Bret +	Bret -	Bret +	Bret -
Total	15 (17.4)	71 (82.6)	14 (16.3)	72 (83.7)
Marketed wines	2 (2.3)	42 (48.8)	3 (3.5)	41 (47.7)
Cellar wines	13 (15.1)	29 (33.7)	11 (12.8)	31 (36.0)

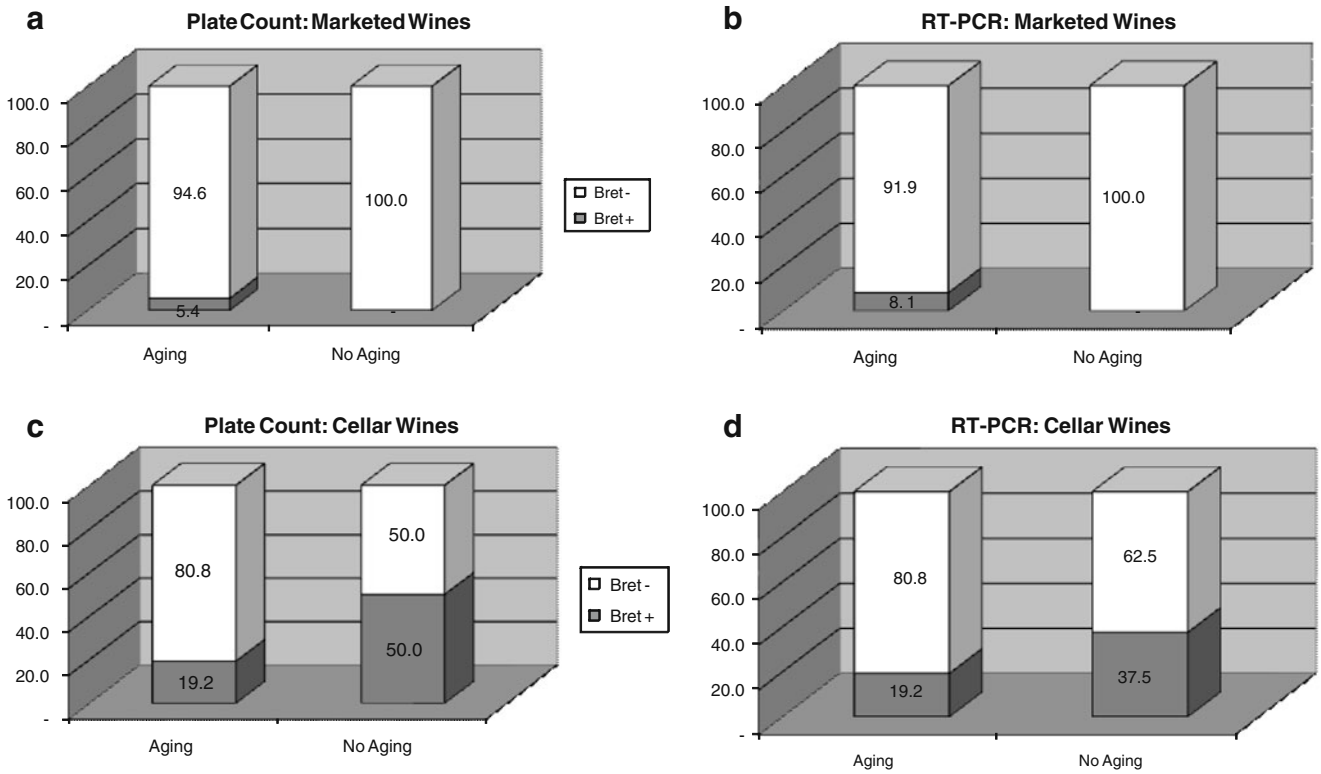

Fig. 4 Distribution of *B. bruxellensis* as percentage of positive and negative samples in aging and non aging wines

Table 2 Summary of evaluated parameters in *B. bruxellensis* positive samples by either method. *CFU* Colony forming units, *GU* genomic units

Sample[a]	Aging	Plate count (CFU/ml)	RT-PCR (GU/ml)	ITS	4-ethylphenol (μg/l)	Sensory description
A (m)	Yes	3.4×10^4	8.3×10^3	(+)	>2,000	(+) "Phenolic"
B (m)	Yes	1.2×10^1 / 1.7×10^2 [b]	5.5×10^2	(+)	2,400	(+) "Phenolic"
C (m)	Yes	0 / 0 [(a)]	7.8×10^2	nd [c]	2,500	(+) "Phenolic"
D (c)	No	1.4×10^3	1.3×10^2	(+)	Not determined	(+) "SH_2"
E (c)	No	5.3×10^2	8.2×10^1	(+)	900	(-)
F (c)	No	2.2×10^3	2.0×10^2	(+)	800	(+) "Mould, humidity"
G (c)	No	4.8×10^2	8.6×10^1	(+)	800	(-)
H (c)	No	8.7×10^1	4.4×10^1	(+)	800	(-)
I (c)	No	0.28×10^0	Not detected	(+)	2,152	(+) "Phenolic"
J (c)	Yes	0.27×10^0	3.4×10^0	(+)	Not determined	(-)
K (c)	Yes	0.21×10^0	2.6×10^0	(+)	1,021	(+) "Leather, match"
L (c)	No	1.6×10^0	Not detected	(-)	Not detected	(-)
M (c)	Yes	1.2×10^1	2.0×10^0	(+)	186	(-)
N (c)	No	2.2×10^3	8.1×10^2	(+)	609	(+) "SH_2, humidity"
O (c)	Yes	0.01×10^0	2.2×10^1	(+)	785	(-)
P (c)	Yes	2.5×10^4	2.0×10^3	(+)	859	(+) "Phenolic"

[a] (m) and (c) indicate marketed and cellar wines, respectively

[b] Two different selective media were used

[c] Not done

necessary to use selective-differential factors, such as antibiotics, antifungals (cycloheximide) or antibacterials (chloramphenicol), sorbic acid, selective carbon sources such as ethanol or maltose, trehalose and sucrose, and ethanol as an antimicrobial agent (Chatonnet et al. 1992; Mitrakul et al. 1999; Rodrigues et al. 2001) since their detection by conventional isolation media is difficult due to the slower rate of population growth and low presence with respect to other species of yeasts and fungi. The main drawbacks of the currently used media are false positives produced by yeasts resistant to cycloheximide, time-consuming colony growth (from 1 to several weeks) and opportunistic fungal contamination, especially on solid media. In the incubation period other yeast genera of faster growth (*Saccharomyces*, *Kloeckera*, *Metschnikowia*, *Pichia* or *Candida*) may lead to erroneous detections even in selective and/or differential media (Benito et al. 2006). This could explain the difference of CFU/ml between the two media.

The other parameters—PCR-ITS, 4-ethylphenol concentration and sensorial description—analysed in the three positive marketed wines clearly indicated the presence of *Brettanomyces*. Although the detection limit of the kit used in RT-PCR is 1–10 GU/ml (manufacturer's personal communication), when the volume of a sample is lower than recommended in the DNA extraction protocol, as in the case of sample I (see Table 2), RT-PCR was not able to detect *Brettanomyces*, although all the other indicators used demonstrated their presence. In sample L, when 100 ml wine was processed and analysed on selective medium, 160 CFU appeared on the filter. However, neither RT-PCR, ITS-PCR, 4-ethylphenol nor sensory perception evaluations detected the presence of *Brettanomyces*. Although selectivity of the medium was based on the effect of cycloheximide, a known inhibitor of *Saccharomyces*, wine yeasts other than *Brettanomyces* can grow and give false positive results. Benito et al. (2006) showed the resistance to 10 and 100 mg/l cycloheximide of strains of *Dekkera anomala* and *Dekkera bruxellensis* but also strains of *Kloeckera apiculata*, *Hanseniaspora uvarum*, *Pichia guilliermondi*, *Schizosaccharomyces pombe* and *Candida parapsilosis*. Generally, the *Brettanomyces* population was detected up to 3.4×10^4 CFU/ml using plate count and up to 8.3×10^3 GU/ml by quantification with RT-PCR. Results given by plate count were higher than those of RT-PCR in many samples. Therefore, this fact agrees with the theory of false positive results, in some cases, in the selective media used in this study. Although there were no apparent differences in colonial morphology, Benito et al. (2006) described the growth of *Pichia guilliermondi in* DBDM medium since this yeast is able to assimilate ethanol as sole carbon source, as does *Brettanomyces bruxellensis*.

There was no correlation between concentration of *Brettanomyces* cells and levels of 4-ethylphenol. Detection of ethylphenols in wine is a sign of Brett activity. However, this method has the drawback that by the time yeasts are detected it may be too late to do anything about it (Suárez et al. 2007). In addition, the absence of these yeasts from "phenolic" wines may be explained by cell death after production of volatile phenols (Fugelsang 1997; Malfeito-Ferreira et al. 2000). Although it has not been demonstrated in the present work, Rodrigues et al. (2001) suggested that the remaining microbial flora, especially bacteria, in "phenolic" wines could also play a minor role in the production of 4-ethylphenol. Significant activity of vinylphenol reductase (the enzyme that transform 4-vinylphenol in 4-ethylphenol) has been described, apart from *Dekkera bruxellensis* and *Dekkera anomala*, in *Pichia guillermondi*, *Candida halophila*, *Candida mannitofaciens* and *Lactobacillus plantarum* (Chatonnet et al. 1995; Edlin et al. 1995). Although LAB were originally believed to be responsible for the formation of ethylphenols, their capacity to generate them under oenological conditions is not comparable with that of *Dekkera/Brettanomyces* (Benito et al. 2009). Nevertheless, in samples analysed in this study there was a clear relationship between high ethylphenol concentration and sensory description (phenolic, leather,...) given by ten expert wine tasters. Chatonnet et al. 1990, cited the sensorial threshold of this compound as 425 μg/l, but this trait is very subjective. Licker et al. 1999, reported that wines with high, medium and no *Brettanomyces* character had average 4-ethylphenol concentrations of 3,000, 1,740 and 680 μg/l, respectively. In this study, with the exception of sample E, wines with 850 μg/l of this compound or higher were clearly described as "phenolic".

This study has shown that *Brettanomyces* is prevalent in bulk and labelled wines. For this reason, early detection of this spoilage yeast during winemaking process is advisable in order to take preventive action before the phenolic odour appears. RT-PCR could be a good tool for both quick and reliable detection and as a method to quantify *Brettanomyces* in the early steps of wine production and also just before bottling.

References

Barata A, Caldeira J, Botelheiro R, Pagliara D, Malfeito-Ferreira M, Loureiro V (2008) Survival patterns of *Dekkera bruxellensis* in wines and inhibitory effect of sulphur dioxide. Int J Food Microbiol 121:201–207

Barbin P (2006) Maîtrise de la contamination des vins rouges par *Brettanomyces* au cours des procédés de vinification. PhD Thesis. INP Toulouse, France

Benito S, Palomero F, Morata A, Calderón F, Suárez-Lepe JA (2006) Detección de *Brettanomyces*/*Dekkera* en vinos tintos mediante el uso de medios selectivo-diferenciales. Tecnol Vino 32:27–31

Benito S, Palomero F, Morata A, Calderón F, Suárez-Lepe JA (2009) A method for estimating *Dekkera/Brettanomyces* populations in wines. J Appl Microbiol 106:1743–1751

Chatonnet P, Boidron J, Pons M (1990) Elevage des vins rouges en fûts de chêne: évolution de certains composés volatils et de leur impact arômatique. Sci Alim 10:587–656

Chatonnet P, Dubourdieu D, Boidron JN, Pons M (1992) The origin of ethylphenols in wines. J Sci Food Agric 60:165–178

Chatonnet P, Dubourdieu D, Boidron J, Lavigne V (1993) Synthesis of volatile phenols by *Saccharomyces cerevisiae* in wines. J Sci Food Agric 10:101–202

Chatonnet P, Dubourdieu D, Boidron JN (1995) The influence of *Brettanomyces/Dekkera* sp. yeasts and lactic acid bacteria on the ethylphenol content of red wines. Am J Enol Vitic 46:463–468

Chatonnet P, Masneuf I, Gubbiotti M-C, Dubourdieu D (1999) Prévention et détection des contaminants par *Brettanomyces* au cours de la vinification et de l'elevage des vins. Rev Fr Oenol 179:20–24

Couto JA, Barbosa A, Hogg T (2005a) A simple cultural method for the presumptive detection of the yeasts *Brettanomyces/Dekkera* in wines. Lett Appl Microbiol 41:505–510

Couto JA, Neves F, Campos F, Hogg T (2005b) Thermal inactivation of the wine spoilage yeasts *Dekkera/Brettanomyces*. Int J Food Microbiol 104:337–344

Delaherche A, Claisse O, Lonvaud-Funel A (2004) Detection and quantification of *Brettanomyces bruxellensis* and « ropy » *Pediococcus damnosus* strains in wine by real-time polymerase chain reaction. J Appl Microbiol 97:910–915

Divol B, Lonvaud-Funel A (2005) Evidence for viable but non culturable yeasts in botrytis-affected wine. J Appl Microbiol 99:85–93

Edlin DAN, Narbad A, Dickinson JR, Lloyd D (1995) The biotransformation of simple phenolic compounds by *Brettanomyces anomalus*. FEMS Microbiol Lett 125:311–316

Egli CM, Henick-Kling T (2001) Identification of *Brettanomyces/Dekkera* species based on polymorphism in the rRNA internal transcribed spacer region. Am J Enol Vitic 52:241–247

Esteve-Zarzoso B, Belloch C, Uruburu F, Querol A (1999) Identification of yeasts by RFLP analysis of the 5, 8S rRNA gene and the two ribosomal internal transcriber spacers. Int J Syst Bacteriol 49:329–337

Froudiere I, Larue F (1988) Condition de survie de *Brettanomyces* (*Dekkera*) dans le moût de raisin et le vin. Connaiss Vigne Vin 2:296–303

Fugelsang K (1997) Wine microbiology. Chapman & Hall Enology Library, New York

Fugelsang K (1998) *Brettanomyces*: Dr Jekyll ou Mr. Hyde des vins ? Biofutur 182:22–23

Gerbaux V (2007) Dénombrement rapide de *Brettanomyces* dans un vin rouge par cytométrie de flux. Rev Oenolog 123:21–24

Gerbaux V, Jeudy S, Monamy C (2000) Study of phenol volatiles in Pinot noir wines in Burgundy. Bulletin de l'OIV 73:581–599

Gilis JF, Barbin P, Strehaiano P, Taillandier P (2008) Presencia de la levadura *Brettanomyces* en bayas de uva: influencia de los factores de la viña. Rev Enol 1:1–8

Guillamón JM, Sabaté J, Barrio E, Cano J, Querol A (1998) Rapid identification of wine yeast species based on RFLP analysis of the ribosomal internal transcribed spacer (ITS) region. Arch Microbiol 169:387–392

Licker JL, Acree TE, Henick-Kling T (1999) What is « Brett » (*Brettanomyces*) flavour ? A preliminary investigation. In: Waterhouse AL, Ebeler SE (eds) Chemistry of wine flavour. ACS symposium series, vol. 714:96–115. American Chemical Society, Washington

Malacrino P, Zapparoli G, Torriani S, Dellaglio F (2001) Rapid detection of viable yeasts and bacteria in wine by flow cytometry. J Microbiol Methods 45:127–134

Malfeito-Ferreira M, Rodrigues N, Loureiro V (2000) The influence of oxygen on the "horse sweat taint" in red wines. In: Proceedings of the 7th International Enology Symposium, 1–2 June. Pavia, Italy. Chiriotti, Pinerolo, Italy

Millet V, Lonvaud-Funel A (2000) The viable but non-culturable state of wine microorganisms during storage. Lett Appl Microbiol 30:136–141

Mitrakul CM, Henick-Kling T, Egli CM (1999) Discrimination of *Brettanomyces/Dekkera* yeast isolates from wine by using various DNA fingerprinting methods. Food Microbiol 16:3–14

Peynaud E, Domercq S (1956) Sur les *Brettanomyces* isolés de raisins et des vins. Arch Mikrobiol 24:266–280

Phister TG, Mills DA (2003) Real-time PCR assay for detection and enumeration of *Dekkera bruxellensis* in wine. Appl Environ Microbiol 69:7430–7434

Pretorius IS (2000) Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. Yeast 16:675–729

Querol A, Barrio E, Ramón D (1992a) A comparative study of different methods of yeast strain characterization. Syst Appl Microbiol 15:439–446

Querol A, Barrio E, Huerta T, Ramón D (1992b) Molecular monitoring of wine fermentations conducted by dry yeast Straits. Appl Environ Microbiol 58:2948–2953

Renouf V, Claisse O, Lonvaud-Funel A (2007) Inventory and monitoring of wine microbial consortia. Appl Microbiol Biotechnol 75:149–164

Rodrigues N, Gonçalves G, Pereira-da-Silva S, Malfeito-Ferreira M, Loureiro V (2001) Development and use of a new medium to detect yeast of the genera *Dekkera*/*Brettanomyces* sp. J Appl Microbiol 90:588–599

Rossen L, Norskov P, Holmstrom K, Rasmussen OF (1992) Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. Int J Food Microbiol 17:37–45

Stender H, Kurtzman C, Hyldig-Nielsen JJ, Sorensen D, Broomer A, Oliveira K (2001) Identification of *Dekkera bruxellensis* (*Brettanomyces*) from wine by fluorescence in situ hybridization using peptide nucleic acid probes. Appl Environ Microbiol 67:938–941

Suárez R, Suárez-Lepe JA, Morata A, Calderon F (2007) The production of ethylphenols in wine by yeasts of the genera *Brettanomyces* and *Dekkera*: a review. Food Chem 102:10–21

Tessonnière H, Vidal S, Barnavon L, Alexandre H, Remize F (2009) Design and performance testing of real-time PCR assay for sensitive and reliable direct quantification of *Brettanomyces* in wine. Int J Food Microbiol 129:237–243

Wilson IG (1997) Inhibition and facilitation of nucleic acid amplification. Appl Environ Microbiol 63:3741–3751