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An important step forward for the future development of an easy and fast procedure for identifying the most dangerous wine spoilage yeast, *Dekkera bruxellensis*, in wine environment

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Summary

Dekkera bruxellensis **is the main reason for spoilage in the wine industry. It renders the products unacceptable leading to large economic losses. Fluorescence *In Situ* Hybridization (FISH) technique has the potential for allowing its specific detection. Nevertheless, some experimental difficulties can be encountered when FISH technique is applied in the wine environment (*e.g.* matrix and cells' autofluorescence, fluorophore inadequate selection and probes' low specificity to the target organisms). An easy and fast in-suspension RNA-FISH procedure was applied for the first time for identifying *D. bruxellensis* in wine. A previously designed RNA-FISH probe to detect *D. bruxellensis* (26S D. brux.5.1) was used, and the matrix and cells' fluorescence interferences, the influence of three fluorophores in FISH performance and the probe specificity were evaluated. The results revealed that to apply RNA-FISH technique in the wine environment, a red-emitting fluorophore should be used. Good probe performance and specificity were achieved with 25% of formamide. The resulting RNA-FISH protocol was applied in wine samples artificially inoculated with *D. bruxellensis*. This spoilage microorganism was detected in wine at cell densities lower than those associated with phenolic off-flavours. Thus, the RNA-FISH procedure described in this work represents an advancement to facilitate early detection of the most dangerous wine spoilage yeast and, consequently, to reduce the economic losses caused by this yeast to the wine industry.**

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Introduction

Wine is a widely consumed and appreciated beverage all over the world. Beneficial and spoilage microorganisms can act during the winemaking process. *Dekkera bruxellensis* (or its anamorph *Brettanomyces bruxellensis*) is considered a major cause of wine spoilage worldwide (Fugelsang, 1997; Loureiro and Malfeito-Ferreira, 2003; Malfeito-Ferreira, 2018) since it confers undesirable phenolic flavours and odours to wine (Chatonnet *et al*., 1992, 1993, 1995, 1997; Fugelsang, 1997; Loureiro and Malfeito-Ferreira, 2003). Therefore, it is crucial for the wine industry to detect and identify *D. bruxellensis* before wine degradation in a fast and accurate way to prevent large economic losses.

Molecular biology has evolved considerably in the last years, with the development of many new methods including Polymerase Chain Reaction (PCR)-based technologies and Fluorescence *In Situ* Hybridization (FISH) (Bottari *et al*., 2006; Amann and Fuchs, 2008; Serpaggi *et al*., 2010; Sohier *et al*., 2014). These fast, specific, precise and sensitive methods allow not only to detect and identify the microorganisms present in a sample but also, if desired, to investigate the microbial population dynamics. FISH overcomes PCR-based methods (Amann *et al*., 1990; Friedrich and Lenke, 2006) since when combined with: (i) Epifluorescence Microscopy (EM) allows direct observation of targeted cells within their native environment (Amann *et al*., 1995; Bokulich and Mills, 2012); and (ii) Flow Cytometry (FC), termed Flow-FISH, enables rapid and specific enumeration of the cells and analysis of their properties in complex

environments such as that of wine (Moter and Gobel, 2000; Bottari *et al*., 2006; Amann and Fuchs, 2008; Serpaggi *et al*., 2010). Thus, FISH has been already applied to detect and identify microorganisms that can be detrimental for wine, including *D. bruxellensis* (Stender *et al*., 2001; Blasco *et al*., 2003; Xufre *et al*., 2006; Röder *et al*., 2007; Serpaggi *et al*., 2010; Andorra *et al*., 2011; Branco *et al*., 2012; Wang *et al*., 2014).

Dekkera bruxellensis identification has already been done by different FISH variants, all of them based on specific fluorescent labelling of the target microorganism by hybridization of synthetic fluorescently labelled oligonucleotide probes to specific regions of the DNA or RNA. It has been achieved by labelling oligonucleotide sequences specific for *D. bruxellensis* using: (i) uncharged Peptide Nucleic Acid (PNA) probes on glass slides (Stender *et al*., 2001) or (ii) traditional DNA probes which is a much cheaper alternative. DNA probes targeting RNA sequences (RNA-FISH) have already been applied: (i) on glass slides with EM detection (Röder *et al*., 2007) to isolate wine microorganisms; or (ii) in suspension with FC analysis (Serpaggi *et al*., 2010) in wine artificially inoculated. Even though RNA-FISH in suspension represents a promising alternative to analyze and monitor *D. bruxellensis* by EM and FC, it has not yet been routinely used. This is because, among other reasons, the existing protocol is time-consuming (it takes more than 40 h, Serpaggi *et al*., 2010) and RNA-FISH application in the wine environment can involve some experimental difficulties associated with the technique such as the occurrence of: (i) false positives due to the matrix (grape must or wine) or cells' autofluorescence; (ii) false negatives associated with low photostability and quantum yield of the fluorophores; or (iii) both (negative and positive) owed to low specificity of the oligonucleotide probe.

Thus, the aim of the present study was to overcome these difficulties while simplifying and shortening the in-suspension RNA-FISH procedure in order to facilitate the future development of an easy and fast tool to identify the main spoilage yeast in winemaking: *D. bruxellensis*. To our knowledge, this is the first study: (i) to investigate the way of overcoming the limitations of RNA-Fluorescence *In Situ* Hybridization (RNA-FISH) for detecting and identifying *D. bruxellensis* in the wine environment; and (ii) to apply an easy and fast in-suspension RNA-FISH procedure for identifying *D. bruxellensis.*

Results and discussion

Autofluorescence tests

Various fluorescent compounds are commonly found in wine such as polyphenols, flavonoids and tannins (Dufour *et al*., 2006). Thus, even before bottling, wines are filtered to remove hazes and precipitates (Bisson, 2004), thereby reducing the concentration of fluorescent compounds. However, the remaining background autofluorescence could hamper microbial detection/identification in wine by RNA-FISH. Also, natural autofluorescence or fixative-induced autofluorescence of the microbial cells could lead to false positives in RNA-FISH analysis. Considering that, background (matrix) autofluorescence and natural or fixative-induced fluorescence of the microbial cells were investigated before applying RNA-FISH.

The autofluorescence of (i) grape must and wine (white and red), (ii) the cells without treatment and (iii) the cells after fixation with absolute ethanol was investigated by observing the samples under an epifluorescence microscope with Cy3, FITC and Cy5 filter sets. The microphotographs taken showed that the white and red grape musts presented autofluorescence under Cy3 (Fig. 1A and D) and FITC filters (Fig. 1B and E) but none under the Cy5 filter set (Fig. 1C and F). However, observations of the red (Fig. 1G–I) and white wine (data not shown) revealed that only red wine presented autofluorescence using the Cy3 filter (Fig. 1G). The results of the autofluorescence tests are in agreement with the polyphenol content of grape musts and wines: (i) red wine is richer in polyphenols (2207 $\pm$ 11 mg GAE/l) and tannins (fluorescent compounds present in skins of grapes, seeds and stems) than white wine (254 $\pm$ 0.2 mg GAE/l), and (ii) the grape musts have a higher polyphenol content (4683 $\pm$ 114 and 1556 $\pm$ 95 mg GAE/l for the red and white grape musts, respectively) than the corresponding wines (Fig. 1G–I). The latter confirms that the clarification performed before bottling contributes to eliminate precipitates as well as to reduce the polyphenol content (Ribéreau-Gayon *et al*., 2006), and consequently the autofluorescence. On the other hand, the evaluation of the natural and fixative-induced fluorescence of yeast cells belonging to 12 wine yeast strain species (Table 1) revealed that only one yeast species, *Z. bailli*, showed natural autofluorescence under Cy3 and FITC filter sets (data not shown). This autofluorescence is maintained after fixation as can be observed in Fig. 1J and K for Cy3 and FITC filter sets respectively.

In summary, the autofluorescence tests performed indicated that to obtain accurate results in the detection of microorganisms in the wine environment it is crucial to minimize or avoid background fluorescence interference (matrix and cells' autofluorescence). For reaching this, the results suggest that the use of RNA-FISH probes labelled with fluorophores with maximal emission in the red region (e.g. Cy5, AF647 and ATTO 647N) can be a good alternative. However, in our knowledge, until now RNA-FISH has been applied to identify wine yeasts using probes labelled with green- or orange-emitting fluorophores (Röder *et al.* 2007; Xufre *et al*., 2006; Serpaggi *et al*., 2010; Andorra *et al*., 2011; Branco *et al*., 2012; Wang *et al*., 2014).

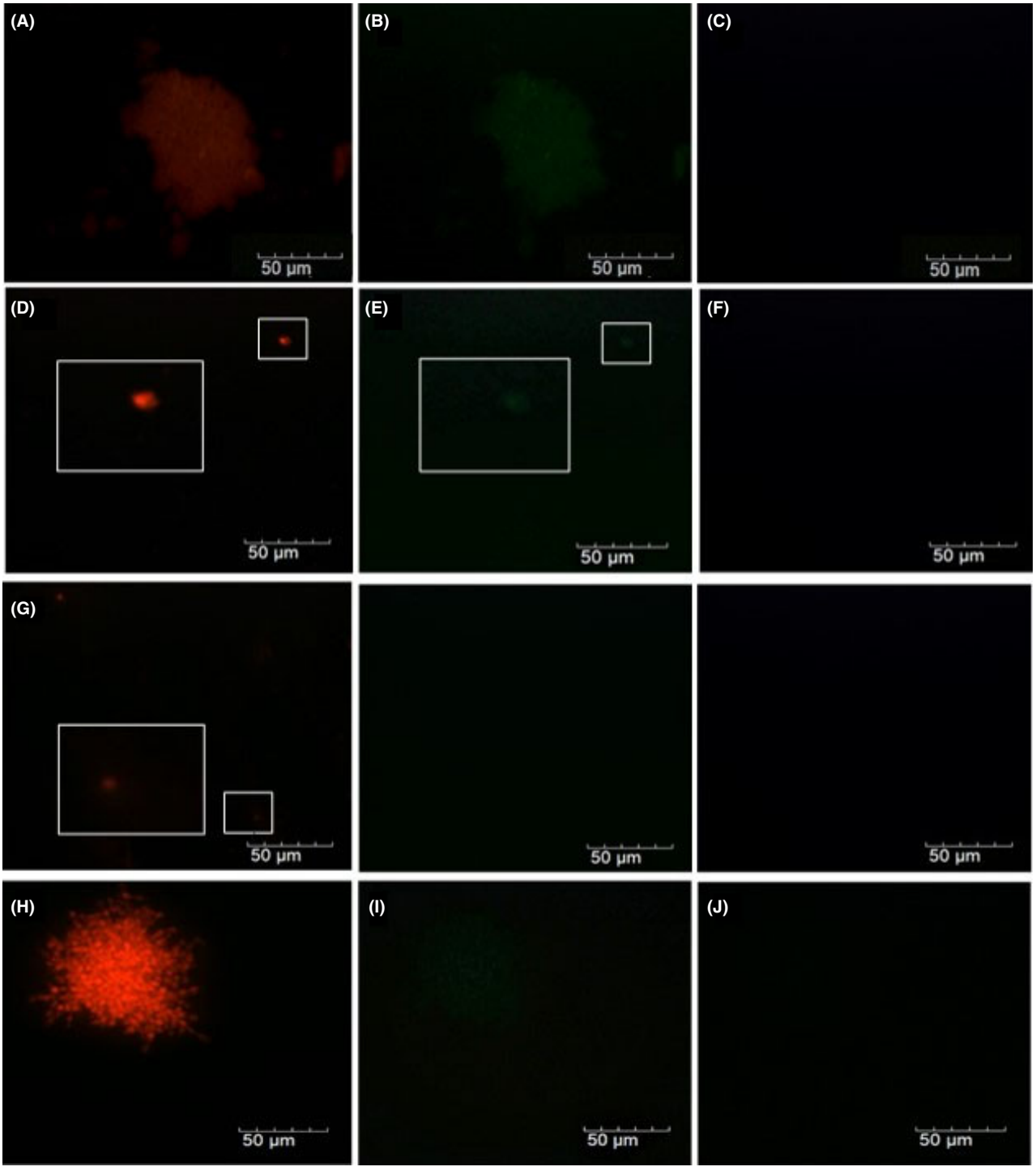

Fig. 1. Microphotographs captured under the epifluorescence microscope in objective amplification of 40× of white (A, B, C) and red (D, E, F) grape must, red wine (G, H, I) and *Z. bailli* ATCC 58445 fixed cells (J, K, L) using the Cy3 (A, D, G, J), FITC (B, E, H, K) and Cy5 (C, F, I, L) filter sets.

Efficiency of three red-emitting fluorophores: AF647, ATTO 647N and Cy5

It is well known that selecting a fluorophore with strong emission properties (high extinction coefficient, quantum yield and stability) is critical for a reliable FISH signal. The evaluation of background and yeast cells' autofluorescence performed indicated that a red-emitting fluorophore could be a reliable possibility to avoid the autofluorescence interferences. Therefore, among the

Table 1. Yeast strains used in the present study.

Yeast specie	Strain	Culture collection
Candida krusei	CCLBH-YW101	Culture Collection Laboratory of Biodegradation HERCULES, Évora, Portugal
Dekkera bruxellensis	CBS 2797	Centraalbureau voor Schimmelcultures, Utrecht, Netherlands
	ISA 2101	Instituto Superior de Agronomia, Lisboa, Portugal
Hanseniaspora guilliermondii	CCLBH-YW701	Culture Collection Laboratory of Biodegradation HERCULES, Évora, Portugal
	NCYC 2380	National Collection of Yeast Cultures, Norwich, United Kingdom
Kluyveromyces marxianus	PYCC 2671	Portuguese Yeast Culture Collection, FCT/UNL, Caparica, Portugal
Lachancea thermotolerans	CBS 2908	Centraalbureau voor Schimmelcultures, Utrecht, Netherlands
Torulaspora delbrueckii	CCLBH-YW301	Culture Collection Laboratory of Biodegradation HERCULES, Évora, Portugal
	PYCC 4478	Portuguese Yeast Culture Collection, FCT/UNL, Caparica, Portugal
Saccharomyces cerevisiae	CCMI 396	Culture Collection of Industrial Microorganisms, INETI, Lisboa, Portugal
Rhodotorula mucilaginosa	CCLBH-YW501	Culture Collection Laboratory of Biodegradation HERCULES, Évora, Portugal
Zygosacharomyces bailii	ATCC 58445	American Type Culture Collection, Virginia, USA

large range of existing red-emitting fluorophores with appropriate photophysical and photochemical properties [Cy5, Cy5.5, ATTO 647N, ATTO 655 and AF647 among others (Hohng *et al*., 2004; Roy *et al*., 2008; Vogelsang *et al*., 2009)], we selected three (AF647, ATTO 647N and Cy5). They were used for evaluating their influence on the performance of a universal eukaryotic probe and a species-specific probe for *D. bruxellensis* (EUK516 and 26S D. brux.5.1 respectively). In addition to each test assay, a negative control (EUB338 probe) and a blank (without probe) were carried out. The blanks and the negative controls gave the expected results both by EM and by FC, showing no fluorescence (data not shown). Cells hybridized with EUK516-AF647 and EUK516-Cy5 were detectable but not photographable by epifluorescence microscopy (data not shown), while cells tagged with EUK516-ATTO 647N were both detectable and photographable (Fig. 2A and C). As expected, microscopic observations of the test assays revealed that EUK516-ATTO 647N probe conferred higher fluorescence photostability to the hybridized cells (Zheng *et al*., 2014) than those labelled with the other fluorophores tested (AF647 and Cy5). Likewise, by FC a higher percentage of

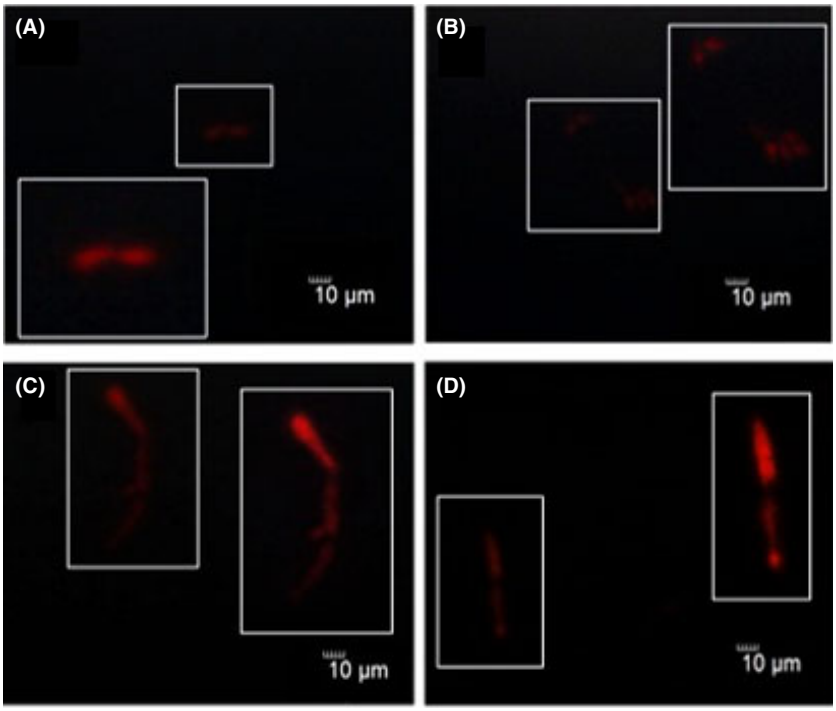

Fig. 2. Microphotographs captured under the epifluorescence microscope with objective amplification of 100× of *D. bruxellensis* ISA 2101 (A, B) and CBS 2797 (C, D) hybridized with EUK516-ATTO 647N (A, C) and 26S D. brux.5.1-ATTO 647N (B, D) probes.

fluorescent cells with stronger Fluorescence Intensity (FI) were detected for the cells hybridized with EUK516-ATTO 647N than for those hybridized with EUK516-AF647 or EUK516-Cy5 (Figs 3 and 4 A, C). In addition, FC results revealed that cells stained with Cy5 fluorophore showed the lowest FI (Fig. 4A and C). Considering that only EUK516-ATTO 647N and EUK516-AF647 showed good RNA-FISH performance, thus, 26S D. brux.5.1-ATTO 647N and 26S D. brux.5.1-AF647 were the only species-specific probes tested for specific detection of *D. bruxellensis*. The analysis of the cells hybridized with 26S D. brux.5.1-AF647 by FC allowed to detect 10.0% of *D. bruxellensis* ISA 2101 and 30.5% *D. bruxellensis* CBS 2797 fluorescent cells (Fig. 3B and D) with low fluorescence intensity (Fig. 4B and D). However, no fluorescence was detected by EM (data not shown). This indicates that 26S D. brux.5.1 probe labelled with AF647 allowed the specific detection of *D. bruxellensis* by FC but not by EM. Conversely, when the target cells (*D. bruxellensis* CBS 2797 and ISA 2101) were hybridized with the same RNA-FISH probe (26S D. brux.5.1) but labelled with ATTO 647N, a high percentage (95.0% and 84.5%, respectively) of intense fluorescent cells were detected both by EM and by FC (Figs 2, 3 and 4 B, D).

Our results are in accordance with the previous studies. They also refer that ATTO 647N is the red-emitting fluorophore that meets all the emission requirements (high extinction coefficient, quantum yield and stability) for obtaining stable and intense FI (Hohng *et al*., 2004; Zheng *et al*., 2014).

This study showed that the selection of a fluorophore with high photostability and quantum yield, such as ATTO 647N, can improve RNA-FISH performance and contribute to avoid inaccurate identification of microorganisms by RNA-FISH technique independently of the method used for analysis.

Performance and specificity evaluation of the 26S D. brux.5.1 RNA-FISH probe to identify D. bruxellensis

Most RNA-FISH probes developed until now for yeast identification are complementary to the D1 and D2 domains of 26S rRNA since they show a high degree of interspecies sequence variation for yeasts (Fell *et al*., 2000; Inácio *et al*., 2003; Xufre *et al*., 2006; Röder *et al*., 2007). Röder *et al.* (2007) developed several species-specific probes targeting the 26S rRNA D1/D2 domains of several *Dekkera*/*Brettanomyces* species (*D. bruxellensis*, *D. anomala*, *B. custersianus*, *B. nanus* and *B. naardenensis*) and investigated their specificity by screening the corresponding target species. They applied an 8-step protocol performed on glass slides in 4–16 h and limited to EM analysis. After that, Serpaggi *et al.* (2010) tested the fluorescence signals and specificity of three of these RNA-FISH probes (against the target, *D. bruxellensis,* and two non-target species*, S. cerevisiae* and *Z. bailii*) and their performance in red wine artificially contaminated with *D. bruxellensis*. They used a 7-step Flow-FISH protocol that includes two particularly time-consuming steps: a 24 h fixation and a 16 h hybridization. Applying this protocol, the highest target cell

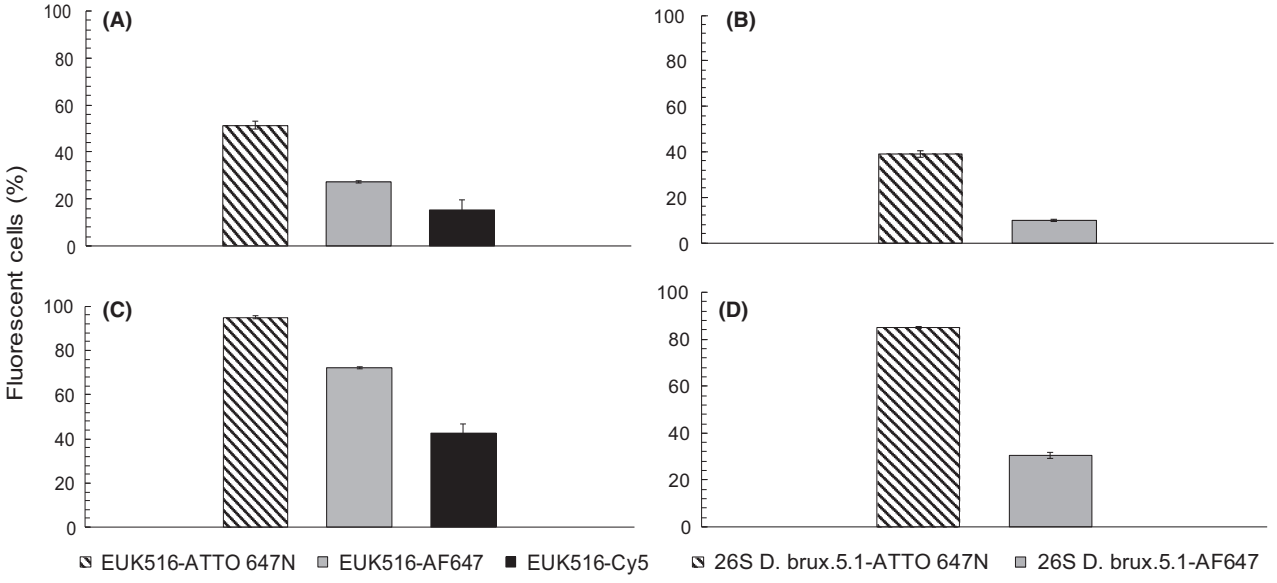

Fig. 3. Percentage of *D. bruxellensis* ISA 2101 (A, B) and CBS 2797 (C, D) cells hybridized with EUK516 labelled with ATTO 647N, AF647 and Cy5 (A, C) and hybridized with 26S D. brux.5.1 labelled with ATTO 647N and AF647 (B, D). In each assay, 1000 cells were analyzed in triplicate. Values represented correspond to the average of Flow Cytometry (FC) measurements and error bars to standard deviation (±SD).

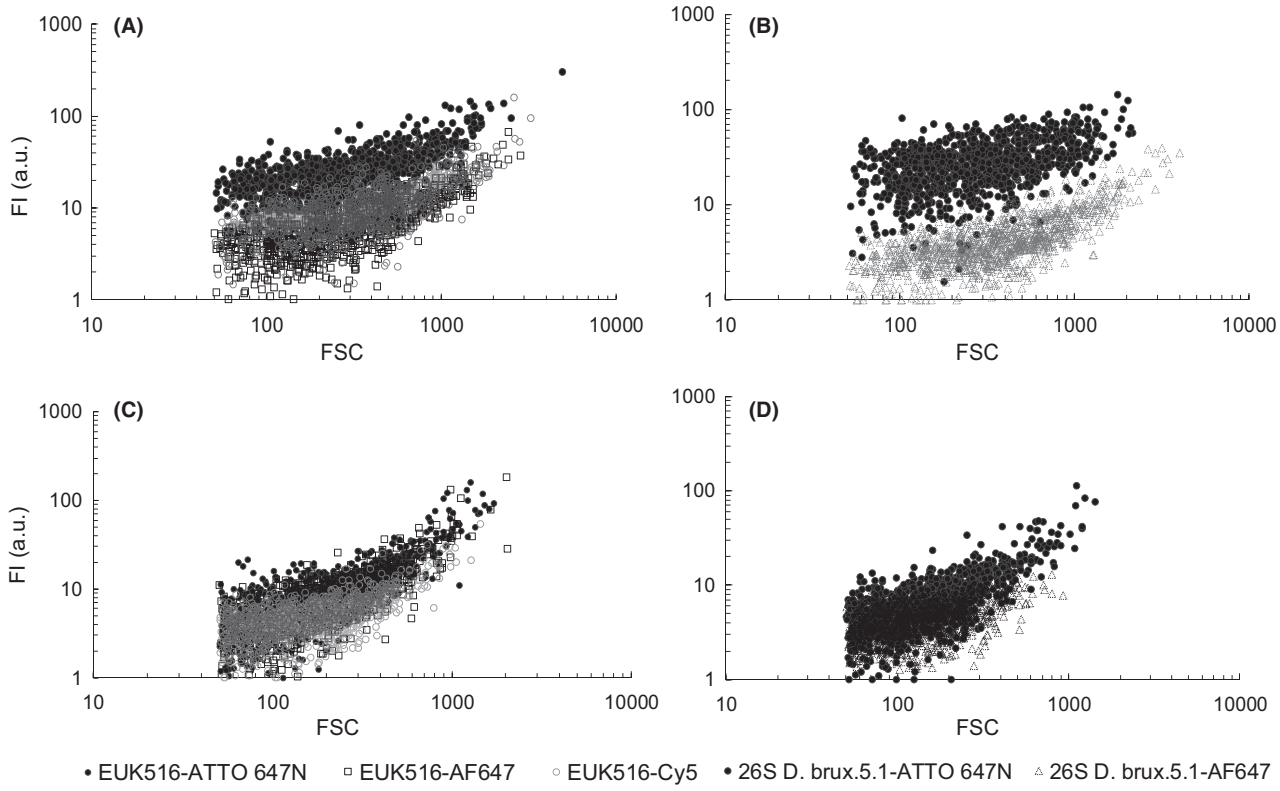

Fig. 4. Flow Cytometry (FC) results [Fluorescence Intensity (FI)/Forward SCattering (FSC)] of *D. bruxellensis* CBS 2797 (A, B) and ISA 2101 (C, D) cells hybridized with EUK516 (A, C) and 26S D. brux.5.1 (B, D) probes labelled with ATTO 647N, AF647 and Cy5 fluorophores.

hybridization percentage (91 ± 3%) and proper specificity of the probe were obtained with 26S D. brux.5.1 probe. Therefore, we selected this probe to identify *D. bruxellensis* in the wine environment using a simpler and faster in-suspension RNA-FISH protocol.

The in-suspension RNA-FISH procedure selected was one previously applied by us for detecting yeast cells (González-Pérez *et al.*, 2017): a 4-step protocol that allows both EM and FC analysis and takes < 4 h. Also, the fluorophores used in this work were different than those previously applied for labelling 26S D. brux.5.1 probe (Röder *et al.*, 2007 and Serpaggi *et al.*, 2010). We used ATTO 647N rather than Cy3 or Alexa Fluor® 488 since this fluorophore showed to improve FISH performance and contribute to avoid inaccurate identification of yeasts in the wine environment by RNA-FISH technique.

Several yeast species are present during winemaking at high cell densities which might interfere with *D. bruxellensis* identification by RNA-FISH. Those yeast species can be in different growth phases when *D. bruxellensis* can start to grow (end of the alcoholic fermentation). Considering this, but also that: (i) early detection of *D. bruxellensis* is preferred for enabling measures to be taken before wine spoilage; and (ii) the RNA-FISH signals are dependent on the rRNA content of the target cells and, consequently, on their growth stage [the most intense signals are obtained in the mid-log phase and the less intense in the lag phase (Waldron and Lacroute, 1975; Warner, 1999)]; in this work, we evaluated the specificity of 26S D. brux.5.1 probe against *D. bruxellensis* strains at lag phase and other various wine yeasts at mid-log phase. For doing this, 10 non-target wine yeasts (*C. krusei*; two strains of *H. guilliermondii*; *K. marxianus*; *L. thermotolerants*; two strains of *T. delbrueckii*; *S. cerevisiae*; *R. mucilaginosa* and *Z. bailii*) and two strains of *D. bruxellensis* were used (Table 1). Also, the FISH performance of the probe in the conditions needed to ensure its specificity was determined.

In a first step, no formamide was used as it was done both by Röder *et al.* (2007) and by Serpaggi *et al.* (2010) when they tested this probe with other RNA-FISH protocols. Whereas the controls gave the expected results (Fig. S1 A, B and Table 2), the species-specific probe 26S D. brux.5.1-ATTO 647N was not specific for *D. bruxellensis* using the in-suspension RNA-FISH protocol (a high percentage of fluorescent non-target cells were detected in the tests performed with 26S D. brux.5.1-ATTO 647N, Fig. 5A and B and Table 2). Thus, in a second step formamide curve was

Table 2. Results obtained by epifluorescence microscopy for RNA-FISH-treated cells with 0 and 25% of formamide (FA).

	Probes					
	EUB338-ATTO 647N		EUK516-ATTO 647N		26S D. brux.5.1-ATTO 647N	
	Fluorescent signal [a] [FA]%					
Yeast species	0	25	0	25	0	25
D. bruxellensis CBS 2797	–	–	+/+	+/+	+/+	+/+
D. bruxellensis ISA 2101	–	–	+/+	+/+	+/+	+/+
C. krusei CCLBH-YW101	–	–	+/+	+/+	+/+	–
H. guillermondii NCYC 2380	–	–	+/+	+/+	+/–	–
H. guilliermondii CCLBH-YW701	–	–	+/+	+/+	+	–
K. marxianus PYCC 2671	–	–	+/+	+/+	+/+	–
L. thermotolerants CBS 2803	–	–	+/+	+/+	+	–
R. mucilaginosa CCLBH-YW501	–	–	+/+	+/+	–	–
S. cerevisiae CCMI 396	–	–	+/+	+/+	+/–	–
T. delbruekii CCLBH-YW301	–	–	+/+	+/+	+	–
T. delbruekii PYCC 2478	–	–	+/+	+/+	+	–
Z. bailli ATCC 58445	–	–	+/+	+/+	+/–	–

a. Signal intensities were classified into four categories: –, no signal; +/–, low; +, medium; +/+, high.

constructed for 26S D. brux.5.1-ATTO 647N probe to evaluate the mismatch discrimination of the probe (Stahl and Amann, 1991). The formamide curves were obtained for a target, *D. bruxellensis* CBS 2797, and for the non-target yeast that showed the highest percentage of fluorescent cells with zero per cent of formamide, *C. krusei*. The formamide concentration on the hybridization buffer was varied in the range 0–45% and, correspondingly, was also the composition of the washing buffer (Table 3). With formamide at 25 per cent, the fluorescence signal response remained maximal for the target yeast whereas it became extremely low for the non-target yeast (Fig. 5C). Considering these results, in a third step we re-evaluated the specificity of the 26S D. brux.5.1-ATTO 647N probe with formamide at 25 per cent against all the target and non-target yeast strains (Table 2, Fig. 5). Once again, the controls gave the expected results for all the yeast strains tested (Fig. S1 C and D). The FC results with all non-target yeast cells showed a relevant decrease in the percentage of fluorescent cells hybridized with 26S D. brux.5.1-ATTO 647N probe and of their FI when compared with those corresponding to the assays carried out without formamide (Fig. 5A and B). In addition, the non-target yeast cells were not detectable by EM (Table 2). Even if the percentage of fluorescent target cells (both *D. bruxellensis* strains) slightly decreases with formamide at 25 per cent (Fig. 5A), a high fluorescent signal was still detectable either by FC or by EM (Fig. 5B, Table 2). This means that 26S D. brux.5.1-ATTO 647N probe allows specific detection of *D. bruxellensis* by applying a simple and fast in-suspension RNA-FISH protocol using formamide at 25 per cent.

Applicability of the in-suspension RNA-FISH method in red and white wine

Once determined the conditions to be used for specific analysis of *D. bruxellensis* in the wine environment using the in-suspension RNA-FISH protocol described here, a preliminary evaluation of its applicability in wine was performed. An approximate value of the Limit Of Detection (LOD, spike amount of target organism in dilution that could be detected in 95% of replicates) was estimated using a dilution to extinction approach. Samples with different concentrations of *D. bruxellensis* (0, 1.0×10^2, 5.0×10^2, 1.0×10^3 and 1.0×10^4 cells ml^{-1}) were analyzed by the RNA-FISH procedure combined with FC or EM in triplicate. The concentrations were selected considering that at 2×10^3 CFU ml^{-1}, *D. bruxellensis* can produce 4-ethylphenol (Barata *et al.*, 2008), responsible for the off-flavours associated with wine spoilage by this yeast.

For each sample, four different assays were carried out: a blank without probe (data not shown), a positive control using a universal probe for eukaryotes (EUK516-ATTO 647N), a negative control using a universal probe for eubacteria (EUB338-ATTO 647N) and the test with 26S D. brux.5.1-ATTO 647N probe. The expected results were obtained for all the blanks and the controls both by FC (Fig. S2) and by EM (data not shown).

The lowest *D. bruxellensis* cell density detected among those tested by the RNA-FISH protocol combined with EM analysis, both in red and white wines, was 1.0×10^4 cells ml^{-1} (data not shown). However, by FC fluorescent cells were detected in samples contaminated with lower cell densities (5.0×10^2 cells ml^{-1}). The statistical analysis of the FC results, in terms of FI and % of fluorescent cells, showed that for both wines, (i) no significant differences were found between the signals obtained for the samples containing 1.0×10^2 and 0 cells ml^{-1} ($P > 0.05$); and (ii) these signals were significantly different to those corresponding to samples contaminated with cell densities higher than 1.0×10^2 cells ml^{-1} ($P < 0.05$). Thus, the approximate LOD value determined for Flow-FISH was 1.0×10^2 cells $ml^{-1} < \text{LOD} < 5.0 \times 10^2$ cells ml^{-1} (Fig. 6).

The FC results also revealed that the percentage of fluorescent cells in white wine were higher than in red wine (Fig. 6). This may be caused by the higher

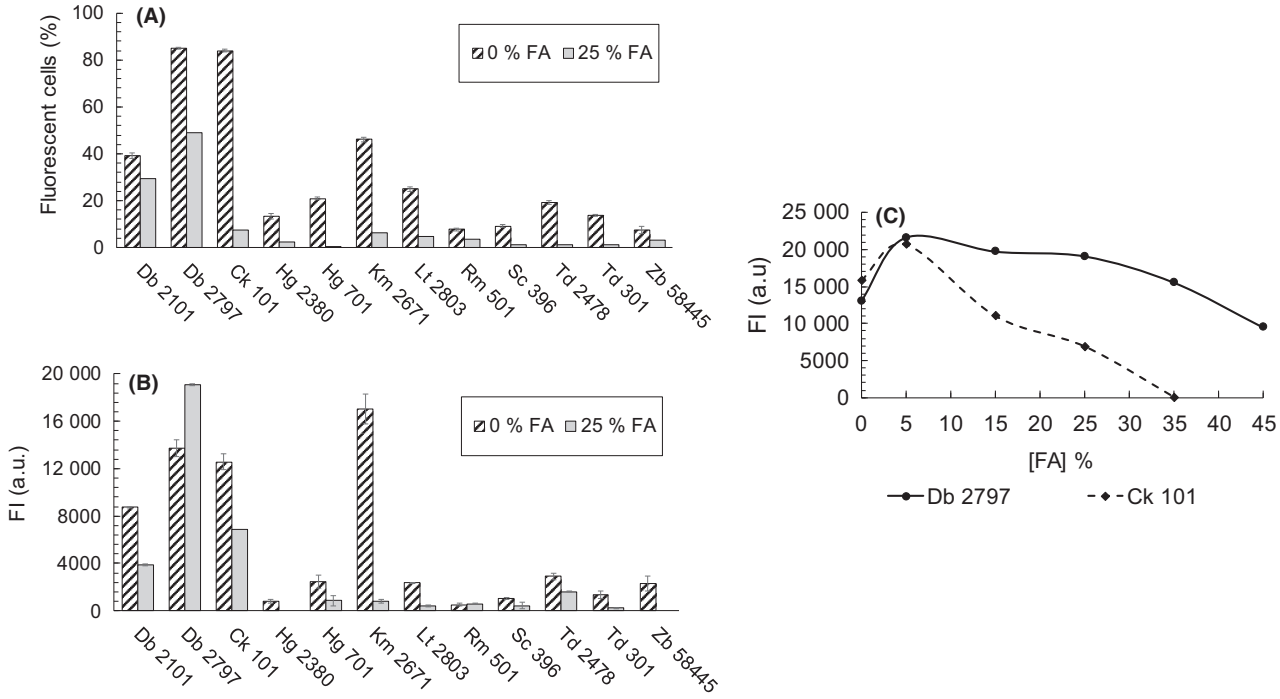

Fig. 5. Percentage of fluorescent cells (A) and fluorescence intensity (FI) (B) of *D. bruxellensis* ISA 2101 (Db 2101), *D. bruxellensis* CBS 2797 (Db 2797), *C. krusei* CCLBH-YW101 (Ck 101); *H. guilliermondii* NCYC 2380 (Hg 2380); *H. guilliermondii* CCLBH-YW701 (Hg 701); *K. marxianus* PYCC 2671 (Km 2671); *L. thermotolerans* CBS 2803 (Lt 2803); *R. mucilaginosa* CCLBH-YW501 (Rm 501); *S. cerevisiae* CCMI 396 (Sc 396); *T. delbrueckii* PYCC 4478 (Td 4478); *T. delbrueckii* CCLBH-YW301 (Td 301) and *Z. bailii* ATCC 58445 (Zb 58445) cells hybridized with 26S D. brux.5.1-ATTO 647N performing the FISH procedure with 0% and 25% (A, B) formamide concentration ([FA]). Formamide denaturation curve, FI/[FA]%, obtained by flow cytometry (FC) for the target (Db 2797) and non-target cells (Ck 101) hybridized with 26S D. brux.5.1-ATTO 647N (C). In each assay, 1000 cells were analyzed in triplicate. Values represented correspond to the average of FC measurements and error bars to standard deviation (±SD).

Table 3. Composition of Washing Buffer (WB) used. The stringency of the WB was dependent on the concentration of formamide used in the hybridization step [FA]%. The final volume was made up to 50.0 ml of dd H_2O.

	$V_{stock\ solutions}$ (ml) for preparing WB [FA]%				
Stock solutions	5	15	25	35	45
5 M NaCl	6.30	3.18	1.49	0.70	0.30
1 M Tris/HCl	1	1	1	1	1
0.5 M EDTA	0	0	0.5	0.5	0.5

concentration of polyphenols found in red wine since these compounds prevented the probes to enter the cell (Serpaggi *et al.*, 2010). The results obtained by Flow-FISH also revealed that the percentage of the *D. bruxellensis* cells increased with the cell density inoculated (Fig. 6), in agreement with the previous studies (Serpaggi *et al.*, 2010).

Whereas more work is required to validate the method, the fast and simple in-suspension procedure described in this study allowed to detect *D. bruxellensis* in lag phase by Flow-FISH in wine samples artificially inoculated. The preliminary results point out the possibility to detect this yeast at cell densities of 5×10^2 cells ml^{-1}, lower than the ones associated with wine spoilage, 2×10^3 cells ml^{-1} (Barata *et al.*, 2008). This will represent a step forward for facilitating the early detection of *D. bruxellensis*, avoiding large economic losses to the wine industry associated with the development of undesirable organoleptic characteristics by this yeast. The results of our work invite to continue the exploration of the in-suspension RNA-FISH technique for developing a simple, rapid and accurate procedure to specifically detect *D. bruxellensis* by flow cytometry (Flow-FISH) and epifluorescence microscopy in wine.

Experimental procedures

Strains and growth conditions

In this work, 12 wine yeasts were used (Table 1). They were maintained in YEPD-agar slants (20 g l^{-1} glucose, 20 g l^{-1} peptone, 10 g l^{-1} yeast extract and 20 g l^{-1} agar, pH 6.0) incubated at 30°C for 48–72 h and stored at 4°C. Yeast cultures were prepared by harvesting the cells of one YEPD-agar fresh slant with YEPD medium (10 g l^{-1} yeast extract, 20 g l^{-1} peptone and 20 g l^{-1} glucose) and transferring them to an Erlenmeyer performing 50 ml of YEPD medium. They were incubated at

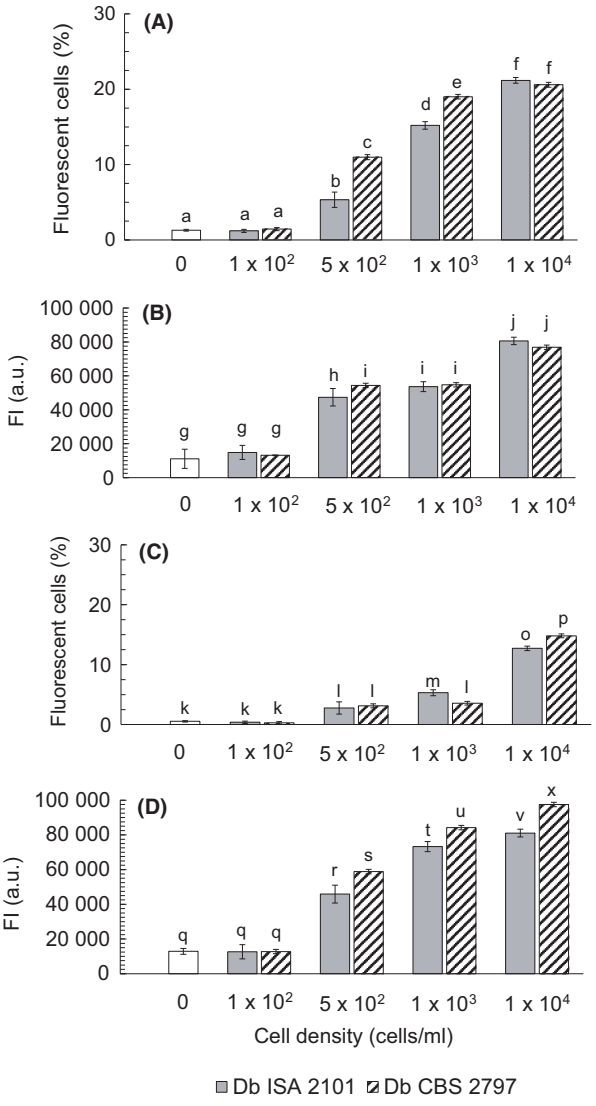

Fig. 6. Percentage of fluorescent cells (A, C) and Fluorescence Intensity (FI) (B, D) of *D. bruxellensis* strains CBS 2797 (Db 2797) and ISA 2101 (Db 2101) after RNA-FISH treatment using 26S D. brux.5.1-ATTO 647N probe in white (A, B) and red wine (C, D) artificially inoculated at different cell densities: 0 (without inoculation), 1.0×10^2, 5.0×10^2, 1.0×10^3 and 1.0×10^4 cells ml^{-1}. In each assay, 5000 cells were analysed in triplicate. Values represented correspond to the average of three FC measurements and error bars to standard deviation (±SD). Different letters located over the error bars indicate significant differences ($P < 0.05$).

28°C with 120 rpm of agitation. Yeast growth was assessed by measurement at 600 nm in a Multiskan Go Microplate Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Determination of total polyphenols in grape must and wine

The Total Polyphenol (TP) content of the white grape must and wine (both from a blend of Antão Vaz and Syrian grape variety) and red grape must and wine (both from Touriga Nacional grape variety) were determined using the Folin–Ciocâlteu micromethod adapted for wine analysis using Gallic Acid (GA) as standard (Waterhouse, 2001). A series of solutions containing different concentrations of the standard were prepared, and their absorbance at 765 nm (A_{765}) was measured in a Multiskan Go Microplate Spectrophotometer (Thermo Fisher Scientific Inc.). A calibration curve, A_{765}/[GA](mg l^{-1}), was used to quantify the TP content of wine and must samples in terms of mg gallic acid equivalents (GAE) per litre of wine (mg GAE l^{-1}), after measuring the A_{765} of the samples (or of their dilutions when necessary, $A_{765} > 0.5$). They were expressed as means ± standard deviation of triplicate analysis.

RNA-FISH analysis

RNA-FISH probes. The probes used were as follows: (i) universal eukaryote- and eubacteria-specific probes, EUK516 and EUB338 (used as positive and negative controls, respectively); and (ii) a species-specific probe for *D. bruxellensis* (26S D. brux.5.1) previously described in the literature (Röder *et al.*, 2007). All probes were labelled with red-emitting fluorophores in the 5′-end (EUB338 and EUK516 labelled with Cy5, AF647 or ATTO 647N and 26S D. brux.5.1 labelled only with AF647 or ATTO 647N fluorophores).

RNA-FISH procedure. After incubation, the cultured cells were recovered by centrifugation and washed with 50 ml of PBS (Phosphate Buffered Saline solution: 130 mM NaCl, 8.0 mM NaH_2PO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4, pH 7.2). Then, the cells were fixed with absolute EtOH and incubated for 1 h at room temperature, to maintain cellular integrity while making the membranes permeable. The fixed cells were preserved in 50/50 EtOH/PBS (v/v) at −20°C until used. The fixed cells were washed with PBS, and the cellular suspension containing 10^6 cells was transferred to 1.5 ml microtubes and centrifuged. The Hybridization Buffer [HB: 0.9 M NaCl, 20 mM Tris–HCl, 0.1% SDS aqueous solution, pH 7.2, with formamide concentrations ranging from 0 to 45% (v/v)], 80 μl, was added to the pellet. The volume (1 μl) of the correspondent RNA-FISH probe stock solution (120 ng μl^{-1}) was then added to each FISH assay. The FISH assays carried out were as follows: (i) blanks (controls for FISH-induced autofluorescence) that were subjected to standard FISH conditions without the addition of the RNA-FISH probe, (ii) controls: positive with EUK516 and negative with EUB338 both labelled with ATTO 647N, AF647 or Cy5 and iii) tests with 26S D. brux.5.1 labelled with ATTO 647N and AF647. All the FISH assays were incubated in the dark in a water bath for 2 h at 46°C under continuous shaking. After centrifugation, the cells were washed with 100 μl of

Washing Buffer (WB) for 30 min in a water bath maintaining the same conditions used for hybridization. The stringency of the WB was adjusted according to the formamide percentage used in the hybridization step following the protocol of Snaidr *et al.* (1997) with some modifications. The composition of the WB used for cells treated with HB with FA (5–45%) is summarized in Table 3. For the cells treated with HB without FA, the WB used was the HB. Finally, the cells were pelleted by centrifugation, resuspended in 500 μl of PBS and analyzed by epifluorescence microscopy and by FC. The process was performed under aseptic conditions, and the centrifugations were carried out for 5 min at 13 000 rpm and 4°C.

Flow cytometry (FC)

Muse® Cell Analyzer and MuseSoft 1.4.0.0 software were used for FC analysis. For each FISH assay, 1000 events were acquired, and the Fluorescence Intensity (FI) was analyzed using the red (680/30) photodiode detector. Each sample was run in triplicate. It was recorded on a gate that was first defined in a FI-versus-forward scatter (FSC) density plot (considering controls, FISH samples and blanks). In each assay, the percentage and FI of the fluorescent cells were analyzed. The percentage of fluorescent cells of the positive controls and tests with 26S D. brux.5.1 probe were calculated according to the following formulas:

$$\frac{(\sum(\text{fluorescent cells in the positive control}) - \sum(\text{fluorescent cells in the negative control}) \times 100}{1000}$$

$$\frac{(\sum(\text{fluorescent cells in the test}) - \sum(\text{fluorescent cells in the negative control})) \times 100}{1000}$$

Epifluorescence microscopy

Fluorescence images were taken with a Moticam PRO 282B camera mounted on a BA410E Motic microscope coupled to a 100-W Quartz Halogen Koehler illumination with intensity control and to an epi-attachment (EF-UPRIII) and a power supply unit (MOTIC MXH-100). The microscope was equipped with the Motic filter sets Cy3, [excitation (ex) D540/40x, dichroic mirror (dm) 565DCLP and emission (em) D605/55 m], FITC (ex D480/30x, dm 505DCLP and em D535/40 m) and Cy5 (ex HQ620/60x, dm Q660LP and em HQ700/75 m). Images were recorded and analyzed with the Motic Images Plus 2.0^{LM} software (Motic, Hong Kong, China).

Autofluorescence tests

The autofluorescence of various matrixes (white grape must and wine from a blend of Antão Vaz and Syrian grape variety and red grape must and wine from Touriga Nacional grape variety) and of the cells of 12 wine yeast strains (the same used for testing the specificity of the probe, Table 1) before and after fixation was evaluated by epifluorescence microscopy using the Cy3, FITC and Cy5 filter sets before the analysis of cells by RNA-FISH. Fluorescence images were taken as described in the section 'Epifluorescence microscopy'.

Applicability of the in-suspension RNA-FISH method in red and white wine

A preliminary evaluation of the applicability of the in-suspension RNA-FISH method for detecting *D. bruxellensis* cells by FC and EM in red and white wine was performed. Both red (Touriga Nacional) and white wines (Antão Vaz and Syrian) were contaminated artificially with *D. bruxellensis* cells at lag growth phase. For this, *D. bruxellensis* strain (ISA 2101 and CBS 2797) isolates were grown in YEPD medium during 24 h at 28°C and 120 rpm. Before artificial contamination, the lack of *D. bruxellensis* in the wines was confirmed by microscopic observation and also by RNA-FISH as described in the section 'RNA-FISH procedure'. Then, the cells were inoculated at different cell densities (1.0×10^2, 5.0×10^2, 1.0×10^3 and 1.0×10^4 cells ml^{-1}) in the white and red wines. The artificially contaminated samples were subjected to the RNA-FISH procedure and analyzed by FC and EM as described in the corresponding sections. For FC analyses, 5000 events were acquired and the percentage of fluorescent cells were calculated according to the following formula:

$$\frac{(\sum(\text{fluorescent cells in the test}) - \sum(\text{fluorescent cells in the negative control})) \times 100}{5000}$$

The significant difference of the results obtained by FC was calculated to permit comparison of means as described by Fry (1993). The statistical analysis was performed in Microsoft Excel. First, the Levene's test was used to check the assumption of equal variances, and then, one-way ANOVA (if the variances were equal)

or Welch tests (if the variances were unequal) were applied to determine the significance of the difference between means.

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Conflicts of interest

None declared.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Percentage of cells (A, C) and Fluorescent Intensities (FI) (B, D) of *D. bruxellensis* ISA 2101 (Db 2101), *D. bruxellensis* CBS 2797 (Db 2797), *C. krusei* CCLBH-YW101 (Ck 101); *H. guilliermondii* NCYC 2380 (Hg 2380); *H. guilliermondii* CCLBH-YW701 (Hg 701); *K. marxianus* PYCC 2671 (Km 2671); *L. thermotolerans* CBS 2803 (Lt 2803); *R. mucilaginosa* CCLBH-YW501 (Rm 501); *S. cerevisiae* CCMI 396 (Sc 396); *T. delbrueckii* PYCC 4478 (Td 4478); *T. delbrueckii* CCLBH-YW301 (Td 301) and *Z. bailii* ATCC 58445 (Zb 58445) cells hybridised with EUK516-ATTO 647N and EUB338-ATTO 647N performing the FISH procedure with 0% (A, B) and 25 % (C, D) of formamide (FA). In each assay 5000 cells were analysed in triplicate. Values represented correspond to the average of Flow Cytometry (FC) measurements and error bars to standard deviation (±SD).

Fig. S2. Percentage of cells (A,C,E,G) and Fluorescent Intensities (FI) (B,D,F,H) of *D. bruxellensis* ISA 2101 (A-D) and *D. bruxellensis* CBS 2797 (E-H) cells in white wine (A, B and E,F) and in red wine (C,D and G,H) hybridized with EUK516-ATTO 647N (Positive control) and EUB338-ATTO 647N (Negative control) performing the FISH procedure with 25 % of formamide (FA). In each assay 5000 cells were analyzed in triplicate. Values represented correspond to the average of Flow Cytometry (FC) measurements and error bars to standard deviation (±SD).