A simple procedure for detecting *Dekkera/Brettanomyces bruxellensis* in wine environment by RNA-FISH using a novel probe

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ABSTRACT

Dekkera / Brettanomyces bruxellensis, considered the major contaminant in wine production, produces 4-ethylphenol, a cause of unpleasant odors. Thus, identification of this yeast before wine spoilage is crucial. Although challenging, it could be achieved using a simple technique: RNA-FISH. To reach it is necessary to design probes that allow specific detection/identification of *D. bruxellensis* among the wine microorganisms and in the wine environment and, if possible, using low formamide concentrations. Therefore, this study was focused on: a) designing a DNA-FISH probe to identify *D. bruxellensis* that matches these requirements and b) determining the applicability of the RNA-FISH procedure after the end of the alcoholic fermentation and in wine.

A novel DNA-FISH *D. bruxellensis* probe with excellent performance and specificity was designed. The application of this probe using an in-suspension RNA-FISH protocol (applying only 5% of formamide) allowed the early detection/identification of *D. bruxellensis* at extremely low cell densities ($5x10^2$ cell/mL). This was possible by flow cytometry independently of the growth stage of the target cells, both at the end of the alcoholic fermentation and in wine even in the presence of high *S. cerevisiae* cell densities.

Thus, this study aims to contribute to facilitate the identification of *D. bruxellensis* before wine spoilage occurs, preventing economic losses to the wine industry.

Keywords: *Dekkera/Brettanomyces bruxellensis*; Fluorescence *In Situ* Hybridization; DNA-FISH probes; Wine spoilage microorganisms; Flow-FISH

1. Introduction

During the winemaking process, microbial contamination or spoilage can occur with the development of microorganisms (lactic acid and acetic acid bacteria and yeasts) whose metabolism can negatively affect the organoleptic properties of wine. This results in the wine quality reduction and consequently in large economic losses to the wine industry.

Yeasts, *Saccharomyces* and non-*Saccharomyces* species, are directly involved in wine fermentation and can also act as spoilage microorganisms (Bartowsky and Henschke, 2008; Deak and Beuchat, 1996; Fleet and Heard 1993; Loureiro and Malfeito, 2003; Ribéreau-Gayon et al., 2006). These microorganisms are found in fresh must (*e.g*. *Saccharomyces cerevisiae; Pichia* spp.; *Rhodotorula* spp.; *Candida stellata*; *Hanseniaspora guilliermondii*; *Lachancea thermotolerants*; *Kluyveromyces marxianus*; *Torulaspora delbrueckii*; *Dekkera / Brettanomyces bruxellensis* and *Zygosaccharomyces bailii)* (Combina et al., 2005; Fleet, 2008; Lonvaud-Funel, 1996; Querol et al., 1990; Torija et al., 2001) and, some of them contribute to the sensorial profile of wine, influencing its organoleptic quality and complexity (Bisson and Kunkee ,1991; Ciani and Ferraro,1998; Clemente-Jimenez et al., 2004; Fleet, 2003, 2008; Jolly et al., 2013). However, several non-*Saccharomyces* species, those as ethanol tolerant as *S. cerevisiae* (*Z. bailii* and *D. bruxellensis* among others), may be found even in bottled wine and can produce its spoilage. *D. bruxellensis* (or its anamorph *B. bruxellensis*) is considered the major contaminant in wine production (Fugelsang, 1997; Guzon et al., 2018; Loureiro and Malfeito-Ferreira, 2003, Malfeito-Ferreira, 2018), being responsible for the development of the most unpleasant aromas in wine described as ‘‘barnyard-like’’ or ‘‘horse-sweat’’, provoked by the production of 4-ethylphenol (Fugelsang, 1997; Loureiro and Malfeito-Ferreira, 2003, Malfeito-Ferreira, 2018, Steensels et al., 2015).

Despite the large economic losses caused by *D. bruxellensis* to the wine industry, there is yet no specific isolation routine for this yeast. The existing methods for assessing its presence are time-consuming and very limited. Traditional plating on selective media is still the most commonly used method. However, it can yield false positives even employing the only selective medium that is commercially available to detect *D. bruxellensis*: Brettanomyces Specific Medium (BSM, Millipore) (Benito et al., 2009; Loureiro and Malfeito-Ferreira, 2003). Molecular methods, less time-consuming and more precise than the traditional ones, have also been applied for detecting this wine spoilage yeast, such as gene sequencing (Guzzon et al., 2018), Polymerase Chain Reaction (PCR)-based methods (Cocolin et al., 2004; Contreras et al., 2008; Ibeas et al., 1996; Phister and Mills, 2003; Renouf et al., 2007; Shimotsu et al., 2015), PCR by directly sampling (Cells-qPCR) (Soares-Santos et al., 2018), and Fluorescence *In Situ* Hybridization (FISH) (Röder et al., 2007; Serpaggi et al., 2010; Stender et al., 2001).

FISH technique is based on the hybridization of synthetic fluorescently-labeled oligonucleotide probes targeted to specific regions of RNA (RNA-FISH) or DNA (DNA-FISH) of a given microorganism (Amann et al., 1995, 2008). Main advantages of this *in situ* technique, over other molecular methods are the possibility of: i) direct observation of targeted cells within their native environment by Epifluorescence Microscopy (EM); and ii) analyzing multiple parameters of individual cells within heterogeneous populations by flow cytometry (Flow-FISH) (Friedrich and Lenke, 2006).

Despite the potential of FISH technique for the detection of *Dekkera /Brettanomyces* species, it has been scarcely used with this aim (Longin et al., 2017). Various authors have already applied FISH with success. Stender et al. (2001) developed a Peptide Nucleic Acid (PNA) probe specific for *D. bruxellensis* and tested its specificity against various *Dekkera* species and other wine yeasts species using wine samples

naturally contaminated with *D. bruxellensis*. However, these artificially synthesized PNA probes are quite more expensive (about 10–20 fold) than DNA-FISH probes remaining impractical for wine industry routine analyses (Röder et al., 2007). Thus, Röder et al. (2007) developed several specific DNA-FISH probes targeting RNA sequences of *D. bruxellensis* and tested their specificity against five *Dekkera/Brettanomyces* isolated from wine using a slide-based protocol. Three of those probes were afterwards evaluated by Serpaggi et al. (2010) to detect and enumerate *Dekkera/Brettanomyces* cells in red wine using Flow-FISH. 26S-D.brux.5.1 probe showed the best results with *Dekkera/Brettanomyces* and failed to significantly hybridized *Saccharomyces cerevisiae* or *Zygosaccharomyces bailii*.

Using this probe, our research group (Branco et al., 2019) has recently contributed to facilitate the identification of *D. bruxellensis* in wine environment by using a rapid in-suspension RNA-FISH protocol and by overcoming some of the experimental difficulties that can be encountered when doing so (*e.g.* background autofluorescence and occurrence of false results associated to the fluorophores characteristics or to low specificity of the oligonucleotide probe). Applying the in-suspension RNA-FISH protocol 26S-D.brux.5.1 probe showed to be specific with 25% of formamide (FA). This compound is commonly applied in FISH assays to adjust the stringency conditions. However, considering its toxicity (reported by the National Toxicology Program as carcinogenic and classified by IARC as Group 1, carcinogenic to humans, IARC, 2006), the utilization of this compound in high concentrations must be avoided. Thus, the design and evaluation of novel DNA-FISH probes targeting *D. bruxellensis* RNA sequences that: i) allow identification of this spoilage microorganism requiring low or even none FA concentrations and ii) reach high specificity and hybridization efficiency, is of utmost importance. And so is to ensure its

applicability when spoilage by *D. bruxellensis* can occur, *i.e.* not only in wine (bottled or storage) but also after the end of alcoholic fermentation by *S. cerevisiae*.

Considering all the above mentioned, this study was focused on the design and performance evaluation of a DNA-FISH probe targeting the RNA of the wine spoilage yeast *D. bruxellensis* that owns high hybridization efficiency, high specificity and low FA requirements using an in-suspension RNA-FISH protocol. The potential of the novel DNA-FISH probe to detect *D. bruxellensis* in different stages of growth was also investigated. Finally, the applicability of the resulting RNA-FISH procedure for detecting this yeast in wine environment, after alcoholic fermentation by *S. cerevisiae* and in bottled wine, was determined.

2. Materials and Methods

2.1 Design and *in silico* evaluation of a *Dekkera bruxellensis* specific probe

To design a species-specific DNA-FISH probe to *D. bruxellensis* several 26S ribosomal RNA (rRNA) sequences of the target group were searched in the National Center for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov/, Altschul et al., 1990). They were aligned in BioEdit program (www.mbio.ncsu.edu/BioEdit/bioedit.html, Hall, 1999) and submitted to the Design Probes web tool of DECIPHER program (http://decipher.cee.wisc.edu/, Wright, 2016). Using this tool several species-specific DNA-FISH probes to *D. bruxellensis* were obtained. Then, the top-ten DNA-FISH probes were selected, according to the scores (measure of the specificity of the probes to the target group) and efficiencies given by the program. Their *in silico* evaluation was done in the following way: a) to reconfirm the specificity to the target microorganism the probes sequences were submitted to a BLASTN search on NCBI website (https://blast.ncbi.nlm.nih.gov, Altschul et al., 1997); b) the DNA single-stranded properties, including molecular weight, melting temperature, GC content, inter-molecular self-complementarity estimation and intra-molecular hairpin loop formation, were calculated applying Oligo Calc: Oligonucleotide Properties Calculator (Kibbe, 2007); and c) mathFISH program (http://mathfish.cee.wisc.edu ,Yilmaz et al., 2011) was used to carry out *in silico* simulations of the FISH performance of the probes including hybridization efficiency, probe affinity and FA melting point ($[FA]_m$) calculations as well as FA dissociation profiles predictions. Among all potential probes evaluated, the one with higher specificity and better FISH performance was selected for experimental evaluation.

2.2 Analyses of the performance and specificity of the probe designed in wine environment

2.2.1 Strains and growth conditions

In this work, 15 wine yeast (*Candida krusei* CCLBH-YW101, *Hanseniaspora guilliermondii* CCLBH-YW701, *Torulaspora delbrueckii* CCLBH-YW301 and *Rhodotorula mucilaginosa* CCLBH-YW501 from the Culture Collection Laboratory of Biodegradation HERCULES, Évora, Portugal; *D. bruxellensis* ISA 1649, 1791, 2101, 2104 belonging to Instituto Superior de Agronomia, Lisboa, Portugal); *D. bruxellensis* CBS 2797, *Lachancea thermotolerans* CBS 2908 from the Centraalbureau voor Schimmelcultures; *H. guilliermondii* NCYC 2380 belonging to the National Collection of Yeast Cultures, Norwich, United Kingdom; *Kluyveromyces marxianus* PYCC 2671 and *T. delbrueckii* PYCC 4478 from the Portuguese Yeast Culture Collection, FCT/UNL, Caparica, Portugal; *Saccharomyces cerevisiae* CCMI 396 from the Culture Collection of Industrial Microorganisms of INETI, Lisbon, Portugal; and *Zygosacharomyces bailii* ATCC 58445 from the American Type Culture Collection); 2 lactic acid bacteria (*Lactobacillus plantarum* ISA 4395 and *Oenococcus oeni* ATCC BAA-1163) and 2 acetic acid bacteria (*Acetobacter aceti* ISA 4201 and *Gluconobacter oxydans* ISA 4270) were used.

Yeasts were maintained in YEPD-agar slants (20.0 g/L glucose, 20.0 g/L peptone, 10.0 g/L yeast extract and 20.0 g/L agar, pH 6.0) incubated at 30.0°C for 48–72 h and stored at 4.0ºC. Yeasts growth were carried out by harvesting the cells from one fresh YEPD-agar slant with YEPD medium (10.0 g/L yeast extract, 20.0 g/L peptone and 20.0 g/L glucose) and transferring them to an Erlenmeyer flask performing 50.0 mL of YEPD medium. Yeast cultures were incubated at 28.0°C and 120 rpm in an orbital shaker. Yeasts growth was monitored during 72 h by measuring the optical density at 600 nm (OD600) using a Multiskan Go Microplate Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, U.S.). Non-*Dekkera* log growth phase cells, harvested at 16 h,

and *D. bruxellensis* cells from five strains at lag (ISA 1649, 1791 and 2104 harvested after 18 h growth and ISA 2101 and CBS 2797 after 24 h growth), log (ISA 1649, 1791 and 2104 collected after 24 h, ISA 2101 after 29 h and CBS 2797 after 32h) and stationary phases (harvested after 48 h-growth) were used in this study. The lactic acid bacteria were maintained in MRS broth (MAN, ROGOSA, SHARPE broth, Thermo Fisher Scientific Inc., UK) and the acetic acid bacteria in GYC medium (50.0 g/L glucose, 10.0 g/L yeast extract, 5.0 g/L calcium carbonate, pH 4.5). Stock cultures of both lactic and acetic acid bacteria were prepared by incubation at 30.0°C for 72-96 h and stored at 4.0ºC. Lactic acid bacteria liquid cultures were prepared by transferring 1.0 mL of stock culture into 9.0 mL of MRS broth and by incubating at 25.0°C without agitation. Acetic acid bacteria growth was carried out by transferring 1.0 mL of stock culture into 9.0 mL of GYC medium followed by incubation at 30.0ºC with agitation at 120 rpm during 72 h.

2.2.2 DNA-FISH probes

The DNA-FISH probes used in this work were: EUK516 (a eukaryotic universal rRNA probe used as positive control for yeasts), EUB338 (a universal eubacterial rRNA probe used as negative control for yeasts and positive control for bacteria), NONEUB338 (control probe complementary to EUB338 used as negative control for bacteria) and a species-specific probe for *D. bruxellensis* (L-S-Dkb271-a-A-17, Dkb271). All the probes employed were probes labeled with ATTO 647N in the 5′-end.

2.2.3 RNA-FISH procedure

After incubation, the cultured cells were recovered by centrifugation and washed with 50.0 mL of PBS (Phosphate Buffered Saline solution: 130.0 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 ethanol and incubated for 1 h at room temperature, to maintain cellular integrity while permeabilizing the membranes. The fixed cells were preserved in 50/50 EtOH/PBS

(v/v) at -20.0°C until used. The fixed cells were washed with PBS and the volume of the resultant 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 [FA]% ranging from 0 to 45 (v/v)], 80.0 µL, was added to the pellet. The volume (1.0 µL) of the correspondent DNA-FISH probe stock solution (120 ng/µL) was then added to each FISH assay. The FISH assays carried out were: i) blanks (controls for natural and FISH induced autofluorescence) that were subjected to the RNA-FISH protocol without addition of the DNA-FISH probe, ii) controls with addition of EUK516-ATTO 647N (universal probe for eukaryotes, being the positive control for yeasts) EUB338-ATTO 647N (universal probe for eubacteria, being the positive control for bacteria and negative control for yeasts) or NONEUB338-ATTO 647N (negative control for bacteria) probes, and iii) tests with Dkb271-ATTO 647N probe.

All the FISH assays were incubated in the dark in a water-bath for 2 h at 46.0°C under continuous shaking. After centrifugation, the cells were washed with 100.0 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 [FA]% 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 S1**. For the cells treated with HB without FA the WB used was the HB. Finally, the cells were pelleted by centrifugation, resuspended in 500.0 µL of PBS and analyzed by EM and FC. The process was performed under aseptic conditions and the centrifugations were carried out for 5 min at 13000 rpm and 4°C.

2.3 Epifluorescence Microscopy (EM) and Flow Cytometry (FC) analysis

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) D605/55m], FITC (ex D480/30x, dm 505DCLP, em D535/40m) and Cy5 (ex HQ620/60x, dm Q660LP, em HQ700/75m). Images were recorded and analyzed with the Motic Images Plus 2.0^{LM} software.

Muse® Cell Analyzer and MuseSoft 1.4.0.0 software were used for FC analysis. For each RNA-FISH assay the percentage of cells that become fluorescent after FISH treatment and their Fluorescence Intensities (FI), using the red (680/30) photodiode detector, were analyzed. And, for doing it, 1000 or 5000 events were acquired. FI values were recorded on a gate that was first defined in a FI-versus-Forward Scatter (FSC) density plot considering the results of the blanks, controls and test assays. The probe-conferred Fluorescence Intensity (FIpro), *i.e*. summation of the Fluorescence Intensities of cells detected after RNA-FISH treatment using any of the probes tested (EUB338-NONEUB338-, EUK516- or Dkb271-ATTO 647N), was directly calculated from the results given by the flow cytometer. The Fluorescence Intensity specifically conferred to the cells by Dkb271-ATTO 647N probe after FISH treatment (FIspe) was calculated by the following formula: (Σ FI of the fluorescent cells detected after RNA-FISH treatment using Dkb271-ATTO 647N probe) - (Σ FI of the fluorescent cells detected after RNA-FISH treatment in the corresponding negative control). The percentage of fluorescent cells obtained after RNA-FISH treatment were calculated according to the following formula: [(Σ of fluorescent cells detected in the positive control or in the assay with

257 Dkb271-ATTO 647N) - (Σ of fluorescent cells detected in the blank assay)] *100/1000
258 or 100/5000. Each sample was run in triplicate.

259 **2.4 Applicability of the in-suspension RNA-FISH protocol for *Dekkera***
260 ***bruxellensis* cells by FC and EM at the end of the fermentation and in wine**

261 The applicability of the in-suspension RNA-FISH protocol for *D. bruxellensis*
262 cells by FC and EM was determined. To this end, various samples artificially
263 contaminated with several cell densities of lag phase *D. bruxellensis* strains were
264 analyzed: a) at the end of a Synthetic Grape Juice (SGJ) fermentation performed with *S.*
265 *cerevisiae*, and b) in commercial red and white wine.

266 The alcoholic fermentation was performed in 250.0 mL of SGJ prepared as
267 described in Pérez-Nevado et al. (2006), at 25.0°C for 8 days without agitation. *S.*
268 *cerevisiae* was inoculated in SGJ with an initial cell density of $1x10^5$ cells/mL. The
269 alcoholic fermentation was performed in triplicate and daily samples were taken to
270 determine cell density, sugars consumption and ethanol production. To determine the cell
271 density 1.0 mL of cells were collected and centrifuged at 13000 rpm for 5 min. The pellet
272 was washed with PBS 1x, centrifuged again at 13000 rpm for 5 min and then resuspended
273 in 1.0 mL of PBS 1x. Afterwards, the cell density was determined by counting in
274 Neubauer chamber. Sugars consumption and ethanol production was determined using
275 the D-fructose/D-glucose and ethanol, UV-method following the manufacture
276 instructions (NZYTech, Lda, Portugal).

277 When the sugars were totally consumed by *S. cerevisiae* (8^{th} day), mixed cultures
278 of *D. bruxellensis/S. cerevisiae* were performed at different cell concentrations ratios:
279 3000, 600, 200, 100, 50, 20, 10 and 2, respectively. This assay was performed mixing
280 $1x10^5$ or $3x10^7$ cells/mL of *S. cerevisiae* cells, from the alcoholic fermentation in SGJ,

281 with different cell densities of *D. bruxellensis* ($1x10^2$, $5.0x10^2$, $1.0x10^3$, $2.0x10^3$, $5.0x10^3$,
282 $1.0x10^4$ and $5.0x10^4$ cells/mL) in SGJ.

283 To simulate wine contaminated by *D. bruxellensis*, commercial white and red
284 wine were artificially contaminated with lag phase *D. bruxellensis* cells. For this, *D.*
285 *bruxellensis* strains isolates were grown in YEPD medium during 24 h (ISA 2101 and
286 CBS 2797) or 18 h (ISA 1649, 1791, 2104) at 28.0°C and 120 rpm. Then, the white and
287 red wines were artificially contaminated with *D. bruxellensis* cells at different cell
288 densities: i) $1x10^2$, $5x10^2$, $2.0x10^3$, $5.0x10^3$, $1.0x10^4$ and $5.0x10^4$ cells/mL for ISA 2101
289 and CBS 2797 strains; and ii) $5x10^2$ cells/mL for ISA 1649, 1791, 2104 strains. All the
290 cells were subjected to the RNA-FISH procedure described in section 2.2.3 and analyzed
291 by FC and EM.
292

3. Results and Discussion

3.1 Design of a *Dekkera*/*Brettanomyces bruxellensis* specific probe and its *in silico* evaluation

The main challenge in RNA-FISH applications is the design of probes that fitting the conditions for being a DNA-FISH probe (high affinity and low prospect of hairpin and self-dimer formation *inter alia*) owns the desired level of specificity and hybridization efficiency (Amann et al., 1995; Keller and Manak, 1989). The affinity of the DNA-FISH probe is its predilection to bind to its target under given hybridization conditions (being determined by the probe melting temperature, and thus by the GC content and probe length, Owczarzy et al., 1998) and its specificity is its aptness to bind only to its target (that can be assessed by determining the number of existing matches of the probe sequence to the target organisms using sequence databases). Then, to detect/identify *D. bruxellensis* in a fast and reliable way for avoiding wine spoilage by this yeast, the development of a DNA-FISH probe with a good performance is required and it must fulfill the requirements described above.

Decipher program was applied for designing potential *D. bruxellensis* DNA-FISH probes and the aligned sequences with GenBank accession numbers D32098.1, D32102.1, KY107614.1, HF547277.1, KY107603.1, KY107610.1, KY107611.1, KY107612.1, KY107613.1, KY107614.1, JQ014666.1, JQ014673.1, JQ014665.1, JQ014667.1, GU291284.1, EU011655.1, EF550257.1, AM491366.1, DQ406715.1 and JX094787.1 were used as input. From the DNA-FISH probes given by the DECIPHER's design probes web tool, the top ten in terms of score and specificity were selected for their *in silico* evaluation, **Table 1** (including theoretical calculations of the GC content, probe length, possibility of hairpin and self-dimer formation, specificity to the target organism and hybridization efficiency).

All the probes selected own high affinity (**Table 1**): GC content between 40-60% (that is required to avoid nonspecific hybridization) and short length (which facilitates its entrance in the cells). The analyses made with Oligo Calc: Oligonucleotide Properties Calculator (Kibbe, 2007) revealed the possibility of hairpin formation for Dkb220 probe and self-dimer formation for Dkb220 and Dkb222 probes (**Table 1**). In this way, these probes were automatically discarded as candidates. The specificity to the target organism, evaluated according with the matches obtained in BLAST nucleotide (Altschul et al., 1990), revealed that Dkb248, Dkb252 and Dkb282 showed the highest number of matches (151) to *D. bruxellensis* (**Table 1**). In accordance with the evaluation of the specificity, Dkb248 and Dkb252 as well as the other remaining probes that showed matches to organisms from the same ecosystem of *D. bruxellensis*, Dkb246 and Dkb197 (**Table 1**), were ruled out. The last parameter used to select the best candidate among the top-ten probes was the theoretical hybridization efficiency calculated using math FISH program (Yilmaz et al., 2011). Since Dkb271, Dkb268 and Dkb261 possess the same number of matches to the target organism and none to other organisms of *D. bruxellensis* ecosystem, those with lower hybridization efficiencies were discarded: Dkb268 and Dkb261 (with 94.49% and 94.99%, respectively). Thus, having considered all the criteria, only two probes remained: i) the probe with the highest number of matches to the target organism (151) and none to organisms of *D. bruxellensis* ecosystem, Dkb282, that did not show the highest hybridization efficiency (93.03 % with 0% of FA); and ii) Dkb271, the probe that exhibited the highest hybridization efficiency (99.89 % with 0% of FA) and a high specificity (148 sequences matched the target organism, **Tables 1** and **S2**). The differences that exist between the specificities of these two probes are not as important as those observed between their hybridization efficiencies. Thus, the best *D. bruxellensis* specific probe resulting from the design process and the *in silico* evaluation was Dkb271.

Before evaluating this probe experimentally, the *in silico* analyses obtained for Dkb271 were compared with those obtained for the probe 26S D. brux.5.1 (**Table 1**), designed by Röder et al. (2007) and experimentally evaluated by us previously (Branco et al., 2019) giving good results for the identification of *D. bruxellensis* in wine using an in-suspension RNA-FISH protocol. Dkb271 probe showed higher hybridization efficiency (99.89 % against 98.24 %) and, particularly a higher specificity (148 sequences against 21 that match with *D. bruxellensis*) than the 26S D. brux.5.1 probe. Thus, theoretically, Dkb271 is a better probe than 26S D. brux.5.1. Therefore, it was selected for further experimental evaluation.

In summary, the *in silico* analyses indicated that Dkb271 probe ought to be a promisor candidate for specifically detect *D. bruxellensis* with high affinity.

3.2 Evaluation of Dkb271 probe specificity and performance in wine environment

Various experimental conditions are usually applied for the evaluation and optimization of DNA-FISH probes specificity and performance. To test the specificity, it is useful to construct a formamide curve (for evaluating the mismatch discrimination potential, Stahl and Amann, 1991) as well as to test the probe against non-target organisms of the same ecosystem of the target organism (for examining the possibility of non-specific hybridization of the probe). Likewise, to improve the DNA-FISH probe performance is crucial to evaluate the RNA-FISH results obtained for different assays (blanks, controls and tests with the probe) using adequate experimental conditions, including the use of a fluorophore with high photostability and quantum yield as label for the probe (Fuchs et al., 2000). The success of RNA-FISH technique to detect the target cells is also dependent on the cellular rRNA content and, consequently, on their growth stage (low rRNA content can lead to low hybridization efficiency of the probe and,

368 consequently, to low fluorescence signals that could induce false negatives (Amann et al.,
369 1995; Waldron and Lacroute, 1975; Warner, 1999).

370 Thus, in the present study to experimentally test the specificity of the *D.*
371 *bruxellensis* probe designed by us, Dkb271, firstly the formamide curves (Fluorescence
372 Intensity specifically conferred to the cells by Dkb271-ATTO 647N probe after FISH
373 treatment/formamide percentage, FIspe/[FA]%), for the target (*D. bruxellensis*) and a
374 non-target wine yeast (*Candida krusei*) were constructed. *D. bruxellensis* cells with low
375 RNA content (at lag phase of growth) and *Candida krusei* cells with high RNA content
376 (at log phase) were used in this assay for determining the [FA]% that ensure specific
377 detection of *D. bruxellensis* even in these adverse conditions.

378 Once determined this [FA]%, it was used to test the specificity of the probe to *D.*
379 *bruxellensis* against log phase cells of 15 yeasts (10 non-target and 5 target yeasts) and 4
380 bacteria isolates usually present in wine environment.

381 Finally, the performance of the Dkb271-ATTO 647N probe to detect *D.*
382 *bruxellensis* at different growth phases by FC and EM with the in-suspension RNA-FISH
383 method previously used was investigated.

384 The specificity of the probe was evaluated by EM, in terms of absence/presence
385 of fluorescent signals and of their relative intensity, and by FC considering the
386 hybridization efficiency (both the percentage of cells that become fluorescent and their
387 fluorescence intensities). The in-suspension RNA-FISH protocol previously developed
388 by us (González-Pérez et al., 2017) was used for all the experiments (since it allows
389 simultaneous detection/identification of yeast and bacteria, which are the microorganisms
390 normally present in wine environment). For each of the microorganism and conditions
391 tested, various assays were carried out in parallel: a blank (without probe addition), a
392 negative and a positive control (using EUB338, EUK516 or NONEUB 338 probes

393 depending on the type of organism) and the test of the species-specific probe Dkb271.
394 For labeling the probes, a fluorophore with suitable photophysical and photochemical
395 properties, ATTO 647N, was used (Branco et al., 2019; Hohng et al., 2004; Roy et al.,
396 2008; Vogelsang et al., 2009).

397 The results of the FIspe/[FA]% curves obtained by FC showed the maximal FIspe
398 for *D. bruxellensis* (target) and none for *C. krusei* cells (non-target) with [FA]=5% (**Fig.**
399 **1**). The probe-conferred Fluorescence Intensity / Forward Scattering (FIpro/FSC) dot-
400 plots corresponding to the tests and the controls are shown in the Supplementary Data
401 (**Fig. S1**). To confirm the specificity of the probe to the target microorganism the probe
402 was tested using [FA]=5% against 5 target and 14 non-target wine microorganisms,
403 including those previously tested, at log phase.

404 For all the yeast cells tested, the expected results were obtained by FC (**Fig. S2**
405 **and Table 2**) and EM (data not shown) for the blanks and the controls (no signals were
406 detected for the blanks, a high percentage of fluorescent yeast cells that emit intense
407 fluorescence were observed for the positive controls and low or none for the negative
408 controls). On the other hand, all the assays performed with bacteria isolates (*A. aceti*, *G.*
409 *oxydans*, *L. plantarum and O. oeni*) gave extremely reduce number of fluorescent cells
410 with scarce fluorescence or none both by EM and FC (**Figs. 2 and S2 and Table 2**).
411 These results confirmed that the conditions tested as well as the fluorophore selected,
412 ATTO 647N, are proper to specifically detect yeast in wine environment since completely
413 avoid bacteria false positives.

414 The RNA-FISH tests performed with Dkb271-ATTO 647N probe using [FA]=5%
415 revealed the specificity of the probe in these conditions by FC since: i) when tested against
416 the target cells (5 *D. bruxellensis* strains), 20 to 60 % of *D. bruxellensis* cells showed
417 intense fluorescence (**Fig. 2 and Table 2**), and ii) with the non-target yeast and bacteria

418 cells, the fluorescent signals as well as the percentage of fluorescent cells were as low as
419 or lower than the values obtained for the respective negative controls (**Fig S2 and Table**
420 **2**). These findings were in accordance with the EM observations (data not shown).

421 Altogether these results showed that, to achieve the desired specificity and
422 performance for the Dkb271 probe with the in-suspension RNA-FISH procedure applied,
423 the use of ATTO 647N as label is recommended and only a reduce concentration of
424 formamide is necessary ([FA]=5%). In a previous work performed by our research group
425 (Branco et al., 2019) in which the same in-suspension RNA-FISH protocol was used by
426 us with 26S D. brux.5.1-ATTO 647N probe, specific detection of *D. bruxellensis* was
427 only achieved using higher concentrations of formamide ([FA]%= 25). This highlights
428 again that the probe designed in this work, Dkb271, is a promisor candidate for safe and
429 specific detection/identification of *D. bruxellensis*.

430 The capacity of the probe to detect *D. bruxellensis* at different growth phases was
431 investigated with cells of five *D. bruxellensis* strains (ISA 1649, 1791, 2101, 2104 and
432 CBS 2797). The controls performed gave the expected results (**Fig. S3**) and the tests done
433 using the Dkb271-ATTO 647N probe (**Fig. 3**) revealed that *D. bruxellensis* cells were
434 detectable independently of their growth stage by EM (data not shown) and FC (**Fig. 3**).
435 Despite differences were observed between strains, the fluorescence intensities (FIspe,
436 **Fig. 3 A**, and FIpro, **Fig. 3 C-H** and **Fig. S3 A-C**) and percentage of fluorescent cells
437 (**Fig. 3B** and **Fig. S2 B-F**) values confirmed that the novel probe Dkb271-ATTO 647N is
438 suitable to specifically detect *D. bruxellensis* in the conditions tested even at lag and
439 stationary growth stages (at which the cellular rRNA content is low). As expected, the
440 highest percentage of fluorescent cells and the most intense signals were detected by FC
441 (**Fig. 3 A**, **B**, **D**, **G**) for all the *D. bruxellensis* strains tested at log phase of growth.

In summary, the experimental evaluation of the Dkb271-ATTO 647N probe confirms the *in silico* results, which point out that this novel probe is specific for *D. bruxellensis* and has good RNA-FISH performance. It exhibits a good hybridization efficiency with low FA requirements ([FA]% = 5) contrasting to what was observed for the 26S D. brux.5.1-ATTO 647N probe (it was found to be species-specific only with a [FA]% = 25) (Branco et al., 2019). Besides that, using Dkb271-ATTO 647N probe it is possible to detect *D. bruxellensis* at all growth phases. The detection at lag phase of growth is advantageous for the early detection of this yeast in winemaking and, thus, for implementation of remediation strategies before wine spoilage occurs.

3.3 Applicability of the RNA-FISH procedure for detecting *D. bruxellensis* cells at the end of the fermentation and in wine

During spontaneous wine fermentation non-*Saccharomyces* yeasts, such as species from the genera *Hanseniaspora* and *Candida*, are predominant during the first 1-3 days of the alcoholic fermentation (Pretorius, 2000). Subsequently, the strongly fermentative and highly ethanol-tolerant strains of *S. cerevisiae* take over the fermentation process until completion reaching high cell densities, 10^7-10^8 cells/ mL (Fleet and Heard, 1993; Pretorius, 2000).

In this way, *S. cerevisiae* is always present in high cell densities levels at the end and after the alcoholic fermentation, when wine spoilage by *D. bruxellensis* can occur, (Fleet and Heard, 1993; Pretorius, 2000). Hence, the determination of the applicability of the in-suspension RNA-FISH procedure to detect *D. bruxellensis* in wine environment in the presence of high cell densities of *S. cerevisiae* (at log or stationary phase of growth) and with lag phase *D. bruxellensis* at low cell densities is of utmost importance. Thus, the applicability of the RNA-FISH procedure described in this work was evaluated using artificially contaminated Synthetic Grape Juice (SGJ) and wine.

Firstly, the RNA-FISH procedures were applied to SGJ samples containing stationary phase *S. cerevisiae* cells ($1.0x10^5$ and $3.0x10^7$ cells/mL) and lag phase *D. bruxellensis* cells at different cell densities ($1.0x10^2$, $5.0x10^2$, $1x10^3$, $2.0x10^3$, $5.0x10^3$, $1.0x10^4$ and $5.0x10^4$ cells/mL of two strains ISA 2101 and CBS 2797)

The results showed that *S. cerevisiae* completely metabolize the sugars (glucose and fructose) in 8 days producing 95.2 g/L of ethanol and reach a population of $3.0x10^7$ cell/mL (**Fig. S4**). As expected, the values of FIpro and percentage of fluorescent cells obtained for the positive controls (EUK516-ATTO 647N) were always higher than those for the corresponding negative controls (EUB338-ATTO647N) and for the sample of SGJ without inoculation (**Fig. S5**). Using Dkb271-ATTO647N probe in SGJ samples containing *S. cerevisiae* at high cell densities ($1.0x10^5$ and $3.0x10^7$ cells/mL), the lowest *D. bruxellensis* cell density detected by EM, among those tested, was $1.0x10^4$ cells/mL for both strains (**Fig. 4 C**). Nevertheless, by FC, it was possible to find *D. bruxellensis* cells exhibiting detectable fluorescent signals (**Fig. 4A**) at extremely low cell densities, $5x10^2$ cells/mL (**Fig. 4B**). These results showed that Flow-FISH (FISH technique combined with FC analysis) is a suitable approach to detect *D. bruxellensis* at low cell densities, after alcoholic fermentation, when *S. cerevisiae* strains are present at high cell densities.

Wine spoilage by *D. bruxellensis* can occur not only just after alcoholic fermentation, but also during wine storage or in bottled wines even at extremely low cell density levels, $2x10^3$ CFU/mL (Barata et al., 2008; Renouf et al., 2007; Rodrigues et al., 2001). In this manner, a reliable method to early detect low cell densities of *D. bruxellensis* in wine is crucial to avoid its adulteration. Thus, the applicability of the RNA-FISH procedure combined with FC or EM analysis to detect lag phase *D. bruxellensis* cells in artificially contaminated commercial red and white wines using

492 Dbk271-ATTO 647N probe was also determined. First, the absence of *D. bruxellensis*
493 contamination in the white and red wines used was confirmed by Flow-FISH. The
494 positive and negative controls performed (using EUK516-ATTO647N and EUB338-
495 ATTO647N probes, respectively) in samples without artificial inoculation of *D.*
496 *bruxellensis* cells gave the same results by FC (**Fig. S6**). After that, the in-suspension
497 RNA-FISH protocol was applied in red and white wines artificially inoculated with *D.*
498 *bruxellensis* strains (ISA 2101 and CBS 2797) at different cell densities ($5x10^2$, $2.0x10^3$,
499 $5.0x10^3$, $1.0x10^4$ or $5.0x10^4$ cells/mL). The positive and negative controls performed gave
500 the expected FC results (**Fig. S6**). Furthermore, by EM it was possible to detect *D.*
501 *bruxellensis* cells using Dkb271-ATTO 647N probe at cell densities of $1.0x10^4$ cells/mL
502 and $5.0x10^4$ cells/mL (**Fig. 5**). Lower densities were not detectable by EM being in
503 agreement with the results obtained in SGJ. On the other hand, by FC it was possible to
504 specifically detect fluorescent cells at extremely low cell densities (**Fig. 6 A-D**). *D.*
505 *bruxellensis* strains ISA 2101 and CBS 2797 inoculated in red and white wine were
506 detected in the cell densities range $5x10^2$-$5.0x10^4$ cells/mL (no fluorescent cells were
507 detected at $1x10^2$ cells/mL, data not shown). Considering this, red and wine samples
508 artificially inoculated with other three *D. bruxellensis* strains (ISA 1649, 1791 and 2104)
509 at the lowest cell density detected for the ISA 2101 and CBS 2797 strains ($5x10^2$
510 cells/mL). Once again, the controls gave the expected results (**Fig. S7**). The results
511 presented in **Fig. 6 E** and **F** confirmed that using Flow-FISH and Dbk271-ATTO 647N
512 probe *D. bruxellensis* cells were detected in red and white wine containing $5x10^2$ cells/mL
513 of any of the *D. bruxellensis* strains tested.

514 These results showed that it is possible to detect *D. bruxellensis* by Flow-FISH
515 with the novel Dkb271-ATTO 647N probe: i) in the presence of high cell densities of *S.*
516 *cerevisiae* just after alcoholic fermentation (when it is present in cell densities above 10^4

cell/mL); and also ii) in red and white wines below the cell density that was previously correlated by Barata et al. (2008) with the production of 4-ethylphenol, $2x10^3$ cells/mL.

In conclusion, this study contributes to the future implementation of an easy and fast methodology to detect *D. bruxellensis* by Flow-FISH technique before wine deterioration. This is advantageous for the employment of effective preservative strategies that allow to avoid economic losses to the wine industry. It will be possible with the use of proper species-specific DNA-FISH probes to detect *D. bruxellensis* such as that developed and evaluated in this work, Dkb271-ATTO 647N.

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