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Development of a molecular diagnostic test for the specific detection of *Brettanomyces bruxellensis* in red wine

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27

28 **Abstract:**

29 *Brettanomyces bruxellensis* is considered the main source of spoilage in red wine. This yeast,
30 by producing volatile phenols, is responsible for the development of unpleasant aromas
31 affecting the quality of final products and resulting in substantial economic losses for wine
32 producers. This work therefore describes the development of an easy to-use colorimetric
33 molecular diagnostic test for the rapid and specific detection of *B. bruxellensis* in wine.
34 Detection was achieved using a sandwich hybridization format in which the target RNA was
35 recognized by an immobilized DNA capture probe and a labeled DNA signal probe. The
36 proposed device was highly specific to *B. bruxellensis* and showed a linear relationship
37 between measured signal and target RNA concentration in the range 0.1-5 ng μL^{-1} , with a
38 limit of detection value of 0.1 ng μL^{-1} of total RNA. The colorimetric assay was validated on
39 red wine samples, with a detection limit of 10^2 CFU mL^{-1} . This study suggests that the
40 reported method could be used for early detection of spoilage yeasts in wine and other
41 alcoholic beverages.

42

43 **Key words:** *Brettanomyces bruxellensis*; molecular diagnostic; colorimetric detection ; RNA
44 target ; wine samples.

45

46 **Highlights**

- 47 - The proposed molecular-based assay was highly specific for *B. bruxellensis* diagnostic.
48 - Detection of 10^2 CFU mL^{-1} of the screening yeast was achieved in wine matrices.
49 - The pre-functionalized plates can be stored 2 months at 4°C before use.
50 - The complete wine analysis was performed in less than 24 h.

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57 1. Introduction

58 *Brettanomyces bruxellensis* is currently considered as the main cause of microbial
59 spoilage in red wine (Loureiro and Malfeito-Ferreira, 2003; Wedral et al., 2010). This yeast
60 affects the organoleptic properties of wines by producing volatile phenols, in particular 4-
61 ethylphenol and 4-ethylguaiacol, from hydroxycinnamic acids naturally present in grapes
62 (Suárez et al., 2007). At low levels, these volatile phenols can impart pleasant aroma notes of
63 smoke, spices or leather contributing to the production of a characterful wine. However,
64 phenol concentrations higher than 0.23 mg L⁻¹ for 4-ethylphenol and 0.047 mg L⁻¹ for 4-
65 ethylguaiacol (Chatonnet et al., 1990), can lead to wine spoilage with the development of
66 negative phenolic aromas and farmyard odors, described as “Brett. character” (Wedral et al.,
67 2010). In addition, *Brettanomyces* can be responsible for off-flavors and acetic acid
68 production leading to olfactory deviation of wines (Freer et al., 2003, Snowdon et al., 2006;).

69 A study carried out in Burgundy showed that *Brettanomyces* yeasts were present in 50%
70 of wines during the fermentation process and in 25% of bottled wines (Gerbaux et al., 2000).
71 Several factors, such as low SO₂ concentrations, poor fermentation management, and oak
72 barrel production, could favor the development of *Brettanomyces* in wine. Moreover, thanks
73 to its ability to form biofilms, this yeast can survive for long periods in wineries and resist the
74 sanitation procedures applied (Lebleux et al., 2020). As a high risk of spoilage is associated
75 with a *Brettanomyces* concentration of 10³ CFU mL⁻¹ (Renouf and Lonvaud-Funel, 2005),
76 winemakers generally take preventive measures such as SO₂ addition, flash pasteurization or
77 chitosan addition to avoid the proliferation of these yeasts in wine (Du Toit et al., 2005 ;
78 Ferreira et al., 2013). However, these methods do not eliminate bad phenolic tastes. Thus,
79 early detection of *B. bruxellensis* is necessary to prevent the production of volatile phenols
80 and thus, irreversible alteration of the wine.

81 As the size and morphology of *B. bruxellensis* are known to be influenced by
82 physiological state of the cells and environmental conditions, simple microscopic
83 identification is very difficult (Aguilar Uscanga et al., 2000, Tubia et al., 2018). Conventional
84 microbiological culture approaches are commonly used to isolate *Brettanomyces* from wine
85 samples (Millet and Lonvaud-Funel, 2000; Renouf and Lonvaud-Funel, 2007). However,
86 these methods have two limitations. Firstly, they are time consuming, requiring 7 to 10 days
87 to obtain initial results. Secondly, they are unable to detect cells in a viable but non culturable
88 (VBNC) state, induced by environmental stresses.

89 In this context, rapid, sensitive, specific and easy-to-use tools are needed for the detection
90 of *B. bruxellensis* to meet the technical and economic expectations of wine production. In
91 recent years, several methods, mainly molecular one have been reported, based on DNA
92 detection, such as qPCR, dot blot, or flow cytometry (Cecchini et al., 2013, Cocolin et al.,
93 2004; De Bellis et al., 2022; Soares-Santos et al., 2018, Tessonière et al., 2008; Willenburg
94 and Divol, 2012). But these techniques, although widely used for the detection of
95 microorganisms, require expensive laboratory equipment as well as trained personnel (Paniel
96 et al., 2013).

97 Alternatively, biosensors defined as analytical devices combining a recognition element
98 associated to a transducer, could also help to meet this challenge. While many biosensors have
99 been used for pathogen detection in food, clinical, and environmental applications, only a few
100 works have been performed for *Brettanomyces* analyses, probably due to a lack of as noted by
101 Tubia et al. (2018) in a recent review. Some of these biosensors, known as genosensors, are
102 based on DNA detection using specific DNA probes associated with an optical fiber (Cecchini
103 et al., 2012), or surface plasmon resonance (Manzano et al., 2016). It may be noted that these
104 biosensors have not been applied to the analysis of wine samples, questioning on the
105 announced detection limit in this complex food matrix. Moreover, DNA detection could lead
106 to the counting of dead cells, which would overestimate the results obtained. Furthermore,
107 antibodies specific to *B. bruxellensis* have been used as recognition element in several
108 immunosensors, mainly associated with electrochemical detection (Borisova et al., 2017;
109 Villalonga et al., 2019). To our knowledge, no antibodies specific to *B. bruxellensis* are
110 commercially available, implying that this element must be produced for each immunosensor
111 development, using a mode of production involving several immunizations of a host animal.
112 All these immunosensors were applied to wine analysis, resulting to detection limit range
113 between 10 to 100 CFU mL⁻¹. Unfortunately, the authors did not address the potential
114 interferences of wine chemical compounds, such as phenols, in the electrochemical analysis.
115 In this context, the aim of this work was to develop a rapid, easy-to-use assay for early
116 detection of *B. bruxellensis* in wine, by finding an alternative to DNA detection and the use of
117 antibody. We proposed the development of a system based on the detection of RNA, and
118 more specifically ribosomal RNA, known as the major structural component of the ribosome,
119 and whose genes are used for microbial taxonomic classification (Antil et al., 2022).
120 Detection was achieved using a sandwich hybridization format in which the target RNA is
121 recognized by two DNA probes, one immobilized on a support and named capture probe, and
122 the second, named signal probe, conjugated to digoxigenin. The hybridization event is then

123 revealed by colorimetric detection, leading to a signal proportional to the concentration of the
124 trapped target. The DNA probes were designed on the basis of the *B. bruxellensis* 26S rRNA
125 sequence. Once the assay parameters have been optimized, sensitivity was first tested on pure
126 cultures, then evaluated with spiked and natural red wine samples. Specificity was also
127 verified using yeasts and bacteria commonly found in red wines.

128 **2. Material and methods**

129 **2.1 Bacterial strains and culture conditions**

130 Thirteen microbial strains were used in this study. *Brettanomyces bruxellensis*
131 ATCC10560, *B. anomala* ATCC58985, *B. naardenensis*, *Zygosaccharomyces bailii* and
132 *Pichia kluyveri* were provided by Centre International de Ressources Microbiennes, (INRAE,
133 Jouy-en-Josas, France), *Metschnikowia pulcherrima*, *Saccharomyces cerevisiae* and
134 *Torulaspora delbrueckii* by the Institut Coopératif du Vin, (Lattes, France), *Acetobacter aceti*
135 ATCC 15973TM by Laboratoires Humeau (La Chapelle sur Erdre, France), and *Lactobacillus*
136 *plantarum* ATCC 14917 (now *Lactiplanctibacillus plantarum*), *L. plantarum* WCF S1,
137 *Pediococcus pentosaceus*, and *Oenococcus oeni* by UMR PAM, (University Bourgogne
138 Franche-Comté, France). All yeast strains were grown at 28°C in YPD medium (10 g L⁻¹
139 yeast extract, 20 g L⁻¹ bacteriological peptone, 20 g L⁻¹ D-glucose) without agitation.
140 *Acetobacter aceti* was incubated at 30°C in GYC medium (10 g L⁻¹ yeast extract, 20 g L⁻¹
141 CaCO₃, 20 g L⁻¹ D-glucose) with agitation at 120 rpm and other lactic bacteria were cultivated
142 at 30°C in MRS medium (Difco™ Lactobacilli MRS Broth, Becton, Dickinson and Co.,
143 USA), without agitation. Culturable method was used as reference method for *B. bruxellensis*
144 counting, using YPD agar medium supplemented with ampicilline (100 µg.mL⁻¹) to prevent
145 bacterial growth and cycloheximide (50 µg.mL⁻¹) to prevent *Saccharomyces* growth (Couto et
146 al., 2005; Willenburg and Divol, 2012). 100 µL of sample (pure culture or wine) was spread
147 at 10-fold dilutions on YPG supplemented medium. Three replicates were performed for each
148 dilution and the number of CFU mL⁻¹ was determined after 6 days of incubation at 28°C.

149 **2.2 Total RNA extraction from pure yeast and bacteria cultures for the test** 150 **development and for the specificity assays**

151 Total RNAs were extracted from *B. bruxellensis* strain, from others *Brettanomyces* species
152 relatively close to *B. bruxellensis*, and from the non-*Brettanomyces* yeast strains as follow .:

153 1,5 mL of each overnight culture was centrifuged at 8 000 × g for 5 min, and the pellet was
154 subjected to RNA extraction using the E.Z.N.A.[®] Yeast RNA Kit (Omega Bio-Tek, Norcross,
155 USA) following the manufacturer's instructions. Total RNA was also extracted from the five
156 bacterial strains typically isolated from wines using the Tri-Reagent extraction method (Da
157 Silva et al., 2017). RNA concentrations were determined using the Quant-iT[™] RiboGreen[™]
158 RNA Assay Kit (Fisher Scientific, Illkirch, France).

159 All the RNA extracts were fragmented before analysis using a 10 X fragmentation buffer (100
160 mM ZnCl₂, 100 mM TrisHCl pH 7). Briefly, 1 μL of 10 X buffer was added to 10 μL of RNA
161 and samples were incubated at 65°C for 15 min. Fragmentation reaction was stopped with 1
162 μL of EDTA 0.5 M pH 8 and samples were stored at -80°C until use. Fragmented RNA
163 extracts from *B. bruxellensis* were used to develop the molecule diagnostic test and
164 fragmented RNA extracts from other yeasts and bacteria were applied to the specificity
165 assays.

166 2.3 DNA probe design and use

167 Capture and signal probes were both designed to hybridize with two adjacent
168 sequences located on the 26S rRNA of *B. bruxellensis* species. The probe specificity was
169 checked *in silico* using the nucleotide Basic Local Alignment Search Tool (BLAST,
170 <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) against GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) and Silva (<http://www.arb-silva.de/>) databases. The 35 bp capture probe
172 was biotinylated in 5', and the 36 bp signal probe was labelled with a digoxigenin (DIG) in 3'
173 (Table 1). A 71 bp synthetic oligonucleotide corresponding to the complementary strand of
174 both capture and signal probes without any chemical modification was used as positive
175 control (PC) to check hybridization conditions. Probes and PC were synthesized by
176 Microsynth (Switzerland) and were diluted in DNase-RNase free water to obtain 100 μM
177 stock solutions. Capture probe solutions were prepared at the appropriate concentration in 0.1
178 M TrisHCl, 0.3 M NaCl, pH 7.6 and were denatured by 3 min at 90°C, 2 min at 4°C and then
179 5 min at room temperature, before use.

180

181 **Table 1** : DNA probes and positive control used in the developed DNA-based assay.

Oligonucleotide	Sequence (5'-3')	Modification
-----------------	------------------	--------------

name		
Capture probe	CCGGCTAGCCACAAGATGCCCGCGCTATAACACCC	5' Biotin
Signal probe	TCCGACAGAATCGAAGGGCCACATTCACGAACCCCG	3' Digoxigenin
Positive control (PC)	CGGGGTTCGTGAATGTGGCCCTTCGATTCTGTTCGGAGGGTGTATA GCGCGGGCATCTTGTGGCTAGCCGG	None

182

183 2.4 Molecular-based assay development for *B. bruxellensis* detection

184 Streptavidin was purchased from Thermo Fischer Scientific and all the buffer components
185 from Sigma (France). Hammerstein-grade casein was purchased from MP Biomedicals
186 (France) and anti-digoxigenin-horseradish peroxidase conjugate (Anti-DIG-HRP) was
187 purchased from Roche (France). The experimental protocol was adapted from Da-Silva et al.
188 (2017), MaxiSorp™ microplates (Nunc, Denmark) were coated with 100 µL of 1 µg mL⁻¹
189 Streptavidin in HEPES 0.1 M, pH 7.2, for 1h30 at room temperature. Then, the Streptavidin
190 excess was eliminated by 3 washing steps using 1X PBS (137 mM NaCl, 2.7 mM KCl, 10
191 mM Na₂HPO₄, 1.76 mM KH₂PO₄; pH 7.4). One hundred microliters of 0.1 µM denatured
192 capture probe were added to each well and the microplate was incubated for 30 min at room
193 temperature. Wells were washed 3 times with 1X HYB buffer. Non-specific sites were
194 blocked using 1% Hammerstein casein in binding buffer (1X PBS, 1 mM MgCl₂, 0.01%
195 Tween 20 ; pH 7.4) for 45 min at room temperature, and casein excess was removed by three
196 washing steps using 1X HYB buffer. Then, pre-hybridization mix containing 25 µL of 4X
197 HYB buffer, 10 µL of 1 µM labelled signal probe, 10 µL of target RNA (or PC) and 55 µL of
198 RNase-free water was incubated for 10 min at 65°C, under stirring. The PC were prepared
199 from the stock solution in the range from 0.05 to 500 nM and the RNAs were diluted from the
200 extract solution in the range from 0.01 to 50 ng µL⁻¹. Three times (5, 10 and 20 min) and three
201 temperatures of incubation (55, 60 and 65°C) were assessed for the pre-hybridization step.
202 One hundred microliters of the pre-hybridization mix were added to the wells and the
203 microplate was incubated for 30 min at 65°C. Wells were then washed 3 times with 1X POP
204 buffer. One hundred microliters of anti-DIG-HRP conjugate at 50 mU mL⁻¹ in PBS-T-BSA
205 (1X PBS, BSA 0.1 %, Tween 20 0.05%; pH 7.4) were added to the wells and the microplate
206 was incubated for 20 min at room temperature. Wells were washed 3 times using 1X POP
207 buffer. Hybridization revelation was performed using colorimetric detection by adding 100
208 µL of ready-to-use 3,3',5,5'-Tetramethylbenzidine substrate (TMB) (Sigma, France) to each

209 well. After 15 min of incubation at room temperature, the absorbance values were directly
210 measured at 620 nm using the Multiskan Ascent™ (Thermo Scientific, France) and assays
211 were carried out in triplicate. For each triplicate, normalized optical density (OD_{620N}) was
212 calculated according to $OD_{620N} = OD_{620A} - OD_{620C}$, where OD_{620A} is the mean OD₆₂₀
213 measured at various concentrations of RNA (or PC), OD_{620C} is the mean OD₆₂₀ measured
214 without RNA, or without PC. The precision was determined by the relative standard deviation
215 (RSD), calculated for each series of triplicates. Due to the low RSD (between 0.5 and 3 %),
216 associated to the colorimetric detection, the lowest value of OD being 2 times higher than the
217 value of the back noise was considered as the limit of detection (LOD) to be safe in the
218 screening.

219 2.5 . Specificity of the assay

220 Firstly, the RNA extracts from the seven yeasts and from the five bacteria often encountered
221 in wines were assessed in the molecular assay, using the protocol presented in 2.4. RNA
222 solutions were diluted in the range from 0.1 to 5 ng μL^{-1} , and obtained results were compared
223 to control performed with the same concentrations of RNA extracted from *B. bruxellensis*.

224 A second assay was specifically done with *B. bruxellensis* and *S. cerevisiae*. A first sample
225 was obtained by mixing 15 ng of RNA from *B. bruxellensis* with 1500 ng of RNA from *S.*
226 *cerevisiae*, and the mixture was included in the assay. A second sample was done by mixing
227 $1 \cdot 10^6$ cells of *B. bruxellensis* with $1 \cdot 10^7$ cells of *S. cerevisiae*. RNAs were extracted from this
228 cell suspension and assessed in the test as previously described. For each assay, controls were
229 done either with the same concentration of RNA or cells of either *B. bruxellensis* or *S.*
230 *cerevisiae*.

231 2.6 Long-term stability and storage of the pre-functionalized microplate

232 Microplates pre-functionalizing was assessed to reduce experiment time. Streptavidin,
233 capture probe, and casein were successively added to the wells of several strip plates
234 following the protocol described above. Strip plates were then stored at 4°C without any
235 buffer on wells. Hybridization and enzymatic revelation were carried out just after coating
236 (day 0) and at 6, 16, 30, 49 and 54 days of storage. Stability was assessed using PC
237 concentrations of 0.5 and 5 nM.

238 2.7 Analysis from red wine samples

239 Analytical performances of the assay were assessed on several red wines, collected in
240 French wineries by the Institut Coopératif du Vin (ICV, Lattes, France). All samples were
241 spread in triplicates on YPD agar medium to check the presence of culturable *Brettanomyces*.
242 For the first experiment, a red wine without culturable *B. bruxellensis* were spiked with 5
243 different concentrations of *B. bruxellensis* cells, ranging between $1.3 \cdot 10^1$ to $1.3 \cdot 10^5$ CFU mL⁻¹.
244 Total RNA of those wine samples, together with the wine control were extracted as follows:
245 Firstly, 25 mL of each sample were pre-filtered through 12- μ m polycarbonate membranes and
246 then, filtered on 0.45- μ m polycarbonate filters. Then, total RNA extractions were directly
247 performed on the 0.45 μ m filters using the E.Z.N.A.[®] Yeast RNA Kit, with a slight
248 modification of the protocole using 800 μ L of lysis buffer instead of the 480 μ L
249 recommended in the kit instructions. RNA extraction was performed in triplicate for each
250 wine sample and analyzed as previously described. For the second experiment, two strategies
251 were assessed to analyze ten red wines (DV12, DV13, DV14, DV16, DV18, DV19, DV20,
252 DV22, SE13, SE9) using the developed molecular-based assay. The first strategy was based
253 on a direct analysis of 25 mL of each wine samples, as described above. The second strategy
254 involved a pre-enrichment step, performed as follows. Five mL of each wine sample was
255 added into 20 mL of YPD liquid medium and incubated at 28°C during 15 or 24 hours. The
256 25 mL was then filtered, and RNA extracted as described above. Each wine sample was
257 prepared and analyzed in triplicate.

258

259 **3. Results and discussion**

260 **3.1 Molecular-based assay design and optimization**

261 The molecular diagnostic assay investigated is based on a sandwich hybridization strategy
262 in which targeted RNA was bound between a capture DNA probe and a labelled signal probe.
263 Some authors have shown that this double hybridization format improves certain analytical
264 parameters, such as sensitivity and specificity, compared with a single hybridization method
265 (Pividori et al., 2001). Indeed, the targeted sequence is larger, as it is identified with two
266 different complementary probes. To adapt the protocol to routine analysis in terms of
267 equipment and experiment time, hybridization time and temperature were optimized.
268 Hybridization time of 10, 20, 30, 40 and 50 min were tested using concentrations ranging
269 from 0.5 to 5 ng μ L⁻¹ of total RNA extracted from pure culture of *B. bruxellensis* (data not
270 shown). Between 10 and 30 min of hybridization, increasing signals were observed, but
271 hybridization times longer than 30 min did not increase the sensitivity. Furthermore,

272 hybridization time of 50 min led to lower signals. Since the best results were obtained with a
273 hybridization time of 30 min, this condition was kept for all the assays. To enable the
274 diagnostic tool to be used in laboratories with limited resources, we have chosen to keep the
275 equipment to a minimum. Thus, we increased the hybridization temperature from 46°C to
276 65°C to perform pre-hybridization and hybridization steps in the same oven. Calibration
277 curves were run in parallel at 46° and 65°C, and similar results were obtained for both
278 temperatures tested. For subsequent experiments, a hybridization temperature of 65°C was
279 applied.

280 3.2 Performance evaluation of the developed assay

281 To assess the sensitivity of our system, a calibration curve was performed using different
282 PC concentrations ranging from 0.1 to 100 nM, according to the parameters defined
283 previously. The measured colorimetric responses are shown in Figure 1A. As expected, the
284 signal obtained is proportional to the concentration of PC. Linear responses were observed
285 from 0.1 to 5.0 nM of PC with a correlation coefficient (R^2) value of 0.9918 (Fig. 1B).
286 Furthermore, the relative standard deviation average of 6% indicated an excellent
287 reproducibility of the assay. Different concentrations of total RNA extracted from *B.*
288 *bruxellensis* were then evaluated to validate the assay (Fig. 2). As previously described, the
289 colorimetric signal was proportional to the RNA concentration tested. Linear responses were
290 measured between 0.1 ng μL^{-1} and 5 ng μL^{-1} , with a detection limit (LOD) of 0.1 ng μL^{-1} of
291 total RNA. The R^2 value of 0,9940 as well as the low RSD mean once again reflect the
292 excellent reproducibility of the developed assay. These two experiments were repeated
293 several times, on different days, either with freshly prepared PC solutions or with RNA newly
294 extracted from *B. bruxellensis* culture, giving similar results with R^2 between 0.9878 and
295 0.9921 (data not shown).

296

297 3.3 Evaluation of the specificity of the assay.

298 Specificity was assessed using 25 ng μL^{-1} of total RNA extracted from different microbial
299 strains. Seven yeast strains, close to *B. bruxellensis* or found in red wines, as well as 5
300 bacterial strains typically isolated from red wines were selected for cross-reactivity testing
301 (Fig. 3A). A clear distinction was observed between the results obtained with *B. bruxellensis*
302 and those obtained with the other strains. Indeed, for *B. bruxellensis* a high signal, with an
303 optical density value of 1.01 was observed whereas no signal or low signals were measured
304 for the other strains, attesting the high specificity of the reported assay.

305 As *Saccharomyces cerevisiae* is known to be the main yeast strain present during wine
306 fermentation, it was important to determine whether the presence of RNA from this specie
307 could interfere with the detection of the targeted RNA. In a first experiment, pure *B.*
308 *bruxellensis* RNA was mixed with a 100-fold amount of pure *S. cerevisiae* RNA, and the
309 obtained RNA solution was evaluated in the nucleic acid assay. At the same time, the same
310 quantity of each RNA was tested separately. The results obtained showed that no interference
311 was due to the presence of excess of non-specific RNA (Fig. 3B). In a second experiment,
312 overnight cultures of *B. bruxellensis* and *S. cerevisiae* was mixed in a 1:10 ratio,
313 corresponding to 1. 10^6 : 1.10^7 cells, and RNAs were immediately extracted as previously
314 described. RNAs were also extracted from the same number of *B. bruxellensis* or *S. cerevisiae*
315 cells. The results obtained (Fig. 3B) are similar to those obtained for the previous assay,
316 underlining the specificity of the molecular-based assay developed.

317 3.4 Long-term stability and storage of the molecular-based assay

318 Given the importance of a rapid detection of *Brettanomyces* contamination in wineries, we
319 evaluated the possibility to reduce experiment time by pre-functionalizing microplate with
320 streptavidin and capture probe. The pre-functionalized microplates were then stored at 4°C
321 and stability assessed during 54 days with two concentrations of PC. Responses over time
322 were compared to the value of the initial response on day 0, and to values obtained with new
323 microplate, functionalized prior to testing, which was evaluated under the same experimental
324 conditions (Fig. 4). Whatever the storage period, for both PC concentrations, the responses
325 obtained with the pre-functionalized plates were of the same order of magnitude than the day
326 0 measurements, and those obtained with the new, freshly prepared microplates. Storage
327 conditions appear to be a good way of preserving the immobilized components in each well,
328 enabling stabilization of the capture probe. These results demonstrate that the microplates can
329 be pre-coated and stored at 4°C until use for at least 54 days, resulting in a 3-hour reduction in
330 experiment time and enabling the assay to be completed in just 2 hours. This result shows
331 better stability than those obtained for similar assays where the stability of coated plates was
332 validated for only 30 days (Da-Silva et al, 2017). This could be due to the variation in the
333 nucleic acid composition of our capture probe which is composed of 63 % of GC whereas the
334 one used in the Da Silva study is composed of only 48.5% GC.

335

336 3.5. Application of the molecular-based assay to wine analysis

337 Validation of the filtration protocol developed for wine analyse was carried out using a red
338 wine sample containing no cultivable *B. bruxellensis*. This sample was spiked with 5 known
339 concentrations of *B. bruxellensis* cells, ranging from 13 to $1.3 \cdot 10^5$ CFU mL⁻¹. As shown in
340 Figure 5, no response was obtained for the non-spiked wine (WC), and absorbance values
341 increase proportionally with the concentration of *B. bruxellensis* cells, validating the filtration
342 protocol applied for the analysis of wine sample. The results obtained for the two lowest cell
343 concentrations were not significantly different from the control, indicating that the LOD of
344 the assay, under the conditions applied is around $1.3 \cdot 10^3$ CFU mL⁻¹.

345 To confirm this result, ten red wines, containing between 10 to 10^3 cultivable *B. bruxellensis*
346 mL⁻¹ were evaluated in the molecular assay, using two samples preparation strategies in an
347 attempt to be more sensitive. Either 25 mL of each sample was directly filtered as before, or 5
348 mL of wine sample was subjected to a pre-enrichment step, in YEP medium during 15 or 24
349 hours prior to filtration. For both strategy, RNA extraction was performed as described above.
350 As shown in figure 6, only wine samples containing at least $3 \cdot 10^3$ CFU mL⁻¹ produced a
351 significant response without an enrichment step (DV 14,18, 19, 20, 22), confirming previous
352 results. We can observe that the enrichment step considerably increases the values obtained,
353 even with the shorter enrichment time. The differences between the two enrichment times are
354 negligible for most samples, except for DV12, with a 6-fold lower response for 24h
355 enrichment, and for DV20 samples with a 10-fold higher response for 24h enrichment,
356 although the response obtained after 15 h is significantly interpretable. Thus, our results
357 showed that a 15 h pre-incubation of the tested wines reduces the LOD to a value around 10^2
358 CFU mL⁻¹. The LOD obtained is of the same order of magnitude as that of recent
359 immunosensors reported in literature (Borisova et al., 2017), or of RNA-FISH assay ($5 \cdot 10^2$
360 cell mL⁻¹) recently developed (Branco et al., 2020). Estimated costs of the reported method
361 have been calculated for the analysis of 10 wine samples. Each RNA extraction costs 6 euros,
362 and the microplate testing of 10 samples in triplicates costs 5 euros for reagents and small
363 equipment. This corresponds to an estimated cost of 65 euros (excluding personal time) for
364 the analysis of 10 samples. In addition, it is important to note that the analyses developed use
365 only inexpensive, and easy-to-use equipment that can be set up in an oenology laboratory,
366 unlike flow cytometry and epifluorescence microscopy, which are expensive research
367 equipment requiring experienced personnel.

368

369 **4. Conclusion**

370 This study reported the development of a molecular diagnostic test for the detection of *B.*
371 *bruxellensis* in wine. The sandwich hybridization format applied enabled direct analysis of the
372 extracted RNA, without any amplification step such as the PCR step commonly performed in
373 molecular analytic techniques. Our device was highly specific to *B. bruxellensis* and showed a
374 linear relationship between measured signal and target RNA concentration in the range 0.1-5
375 ng μL^{-1} , with an LOD value of 0.1 ng μL^{-1} total RNA. The reported assay was successfully
376 applied to the analysis of red wine samples, leading to a LOD of 10^2 CFU mL^{-1} . The method
377 developed, targeting rRNA has the advantage to detect only the cells in a viable physiological
378 state, including the VBNC cells, not detected by a traditional cultural approach. In addition,
379 optimized microplates functionalization reduced analysis time, with a complete analysis,
380 including the 15 h pre-incubation step, RNA sample extraction and microplate assay in less
381 than 24h. The results demonstrated that the method developed could be used for an early
382 detection of spoilage yeasts in wine and other alcoholic beverages such as beer and cider. This
383 method can be considered a simple, effective, and environmentally friendly analytical tool, as
384 it minimizes the use of hazardous chemicals, uses little energy and limits waste production.

385 Further work could focus on transferring the molecular method to a biosensor format
386 associated with optical or electrochemical detection to increase sensitivity, and also to
387 implement the proposed method in the beverage industries. The reported method could also
388 be adapted to the detection of other microorganisms of interest in wine and cider production,
389 such as the lactic acid bacteria and *S. cerevisiae* involved in fermentations, or acetic acid
390 bacteria as spoilage microorganisms, enabling the design of multi-target detection from RNA
391 extracted from the sample to be analyzed.

392

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398

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401 **Authors contribution** : EDS, AS and CG carried out the research and analysed the data. LB
402 conceived and directed research. EDS and LB wrote the manuscript. All authors read,
403 amended, and approved the manuscript.

404

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504

Figure captions :

505 **Figure 1.** Calibration curve (A) and linear part of the calibration plot (B) obtained in the
506 presence of different concentrations of PC with the nucleic acid-based assay using
507 colorimetric detection. Error bars are standard deviations of the mean with n =3

508 **Figure 2.** Calibration curve obtained for different concentration of *B. bruxellensis* total RNA.
509 Error bars are standard deviations of the mean with n=3.

510 **Figure 3.** Specificity evaluation of the assay. A) Total RNA (25 ng) extracted from *B.*
511 *bruxellensis* and from 12 yeast and bacteria strains were assessed in the assay. B) The assay
512 was performed either with pure RNA (RNA) from *B. bruxellensis* (*B.b.*, 15 ng); or from *S.*
513 *cerevisiae* (*S.c.*, 1500 ng) and a mix both RNA (*B.b.*+ *S.c.*, 15 ng and 1500 ng respectively),
514 or with RNA extracted (Cells) from $1 \cdot 10^6$ cells of *B. bruxellensis* (*B.b.*), from $1 \cdot 10^7$ cells of *S.*
515 *cerevisiae* (*S.c.*) and from a mix of those two strains (*B.b.*+ *S.c.*, $1 \cdot 10^6$ cells + $1 \cdot 10^7$ cells
516 respectively). For A and B, absorbance values are expressed as normalized optical density.
517 Error bars are standard deviations of the mean with n=3.

518 **Figure 4.** Long-term stability evaluation. Microplates pre-functionalized with the capture
519 probe were stored at 4°C during 54 days, and hybridization, using 0.5 nM (+) and 5 nM (++)
520 of PC were performed at different days of storage (Stored, in yellow). Each tested day, a
521 complete analyse, with a new functionalized microplate, and using the same reagents was
522 performed (New, in violet). Absorbance values are expressed as normalized optical density.
523 Error bars are standard deviations of the mean with n =3.

524 **Figure 5.** Wine sample analysis using the nucleic molecular assay. Total RNAs were
525 extracted from wine sample without *B. bruxellensis* addition (WC), and from wine samples
526 spiked with different concentrations of *B. bruxellensis* cells. Absorbance values are expressed
527 as normalized optical density. Error bars are standard deviations of the mean with n =3.

528 **Figure 6.** Red wine samples analysis. RNAs were extracted from 25 mL of each wine
529 samples, or after an enrichment step of either 15h or 24h of 5 mL of each wine samples. Same
530 volumes of extracted RNA were analyzed in all the assays. Absorbance values are expressed
531 as normalized optical density. Error bars are standard deviations of the mean with n =3. The
532 10 wine samples were plated on YPG agar medium to determine the cultivable *B.*
533 *bruxenllensis*.

534

535

Figure 1

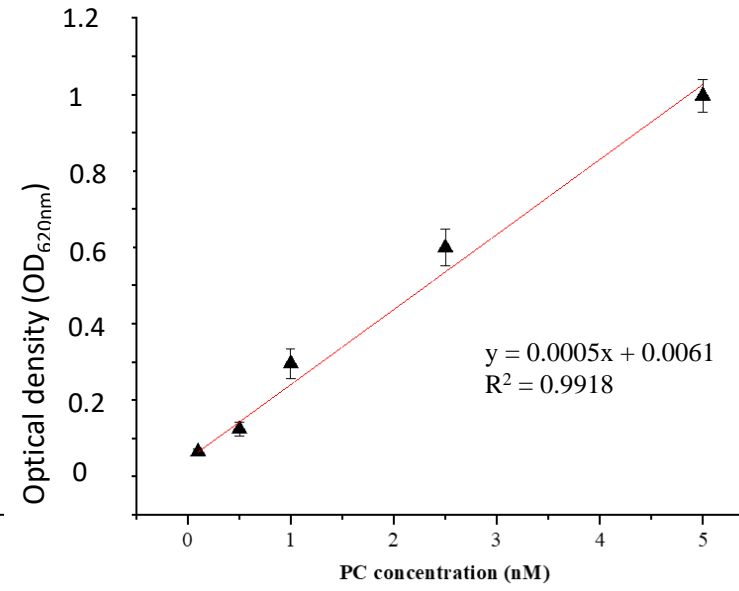
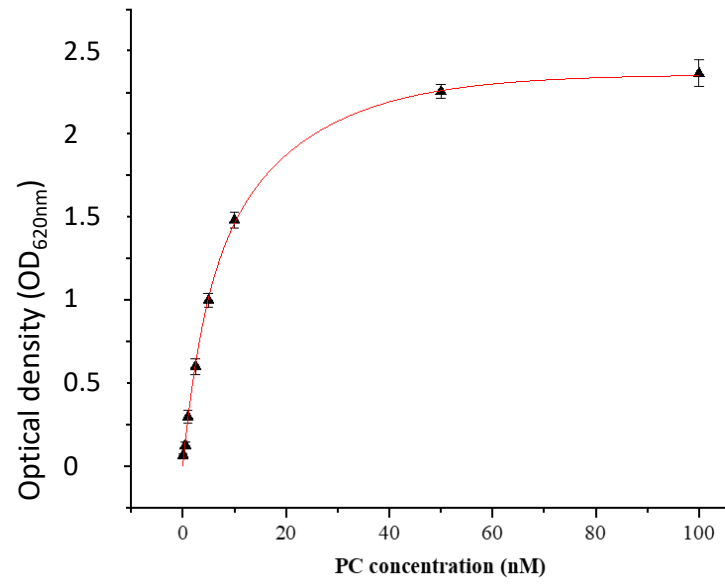


Figure 2

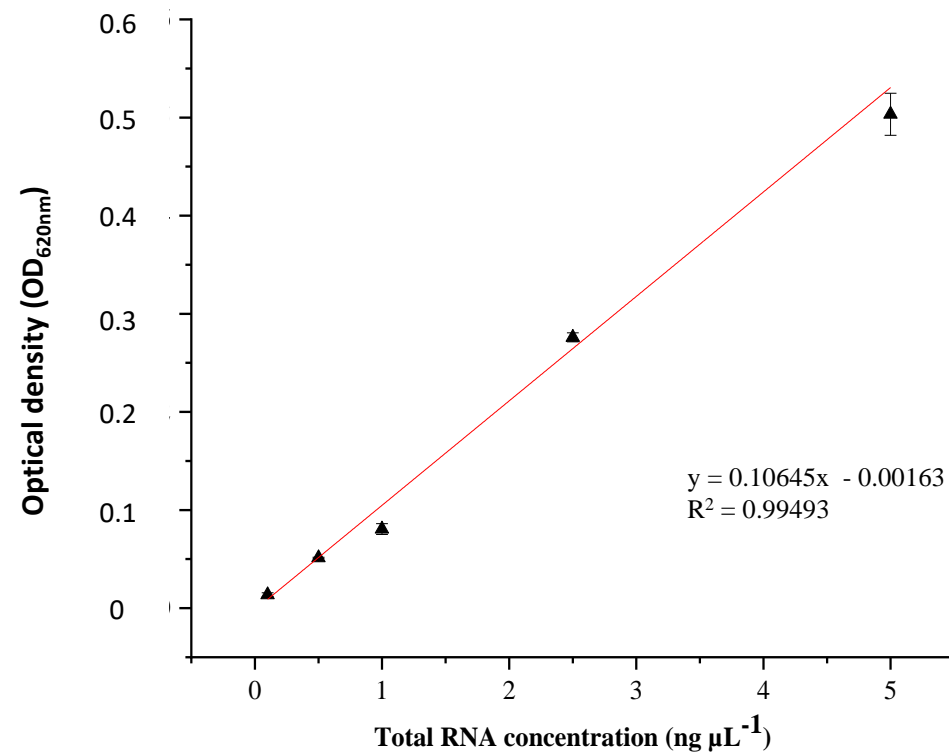


Figure 3

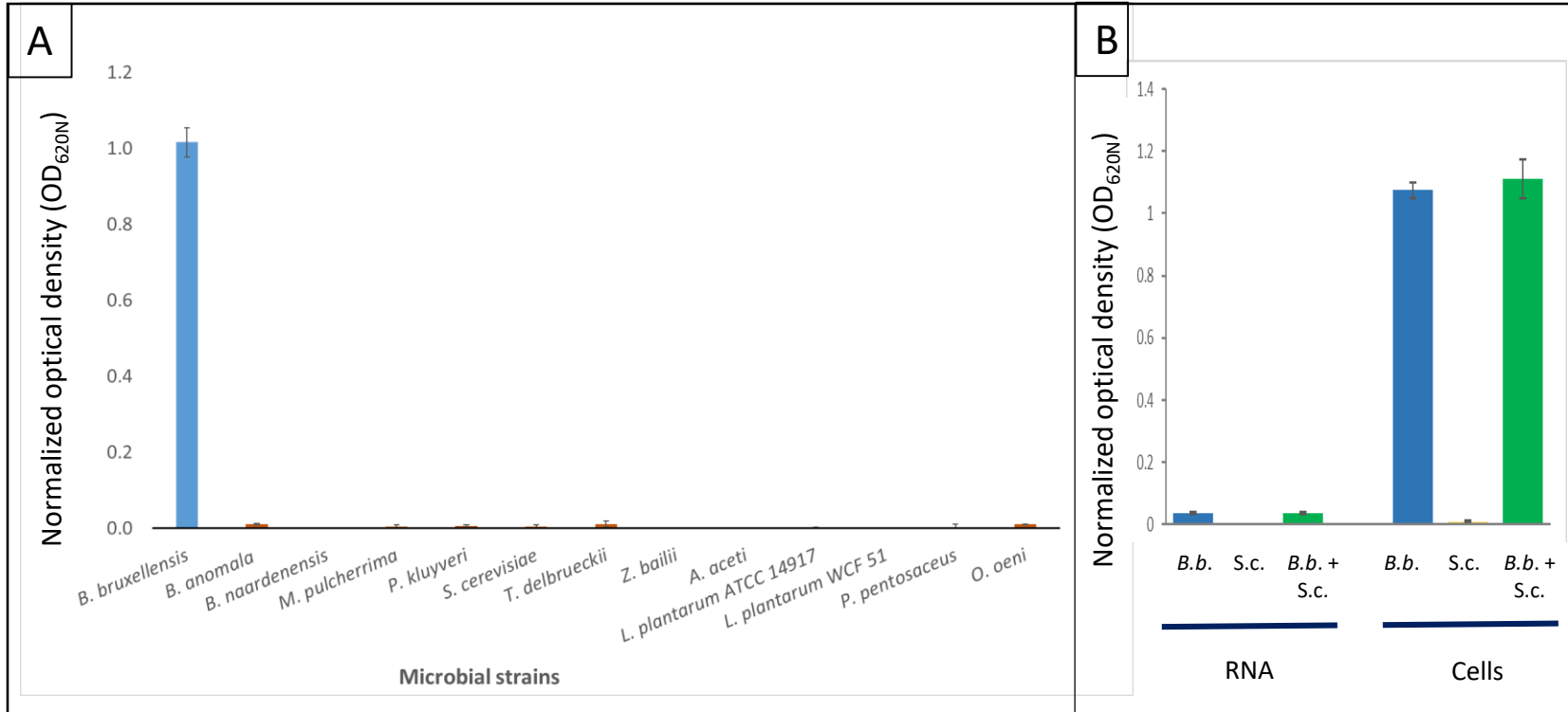


Figure 4

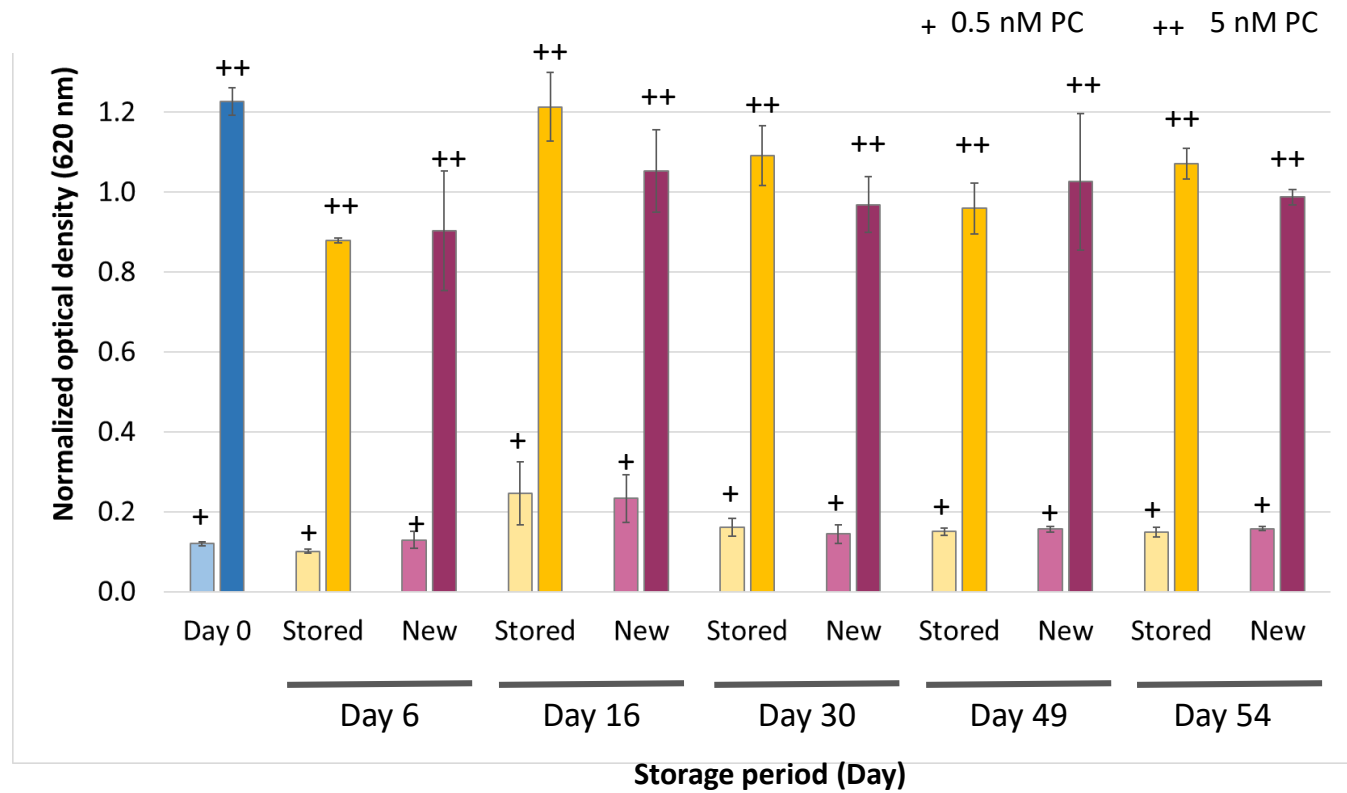


Figure 5

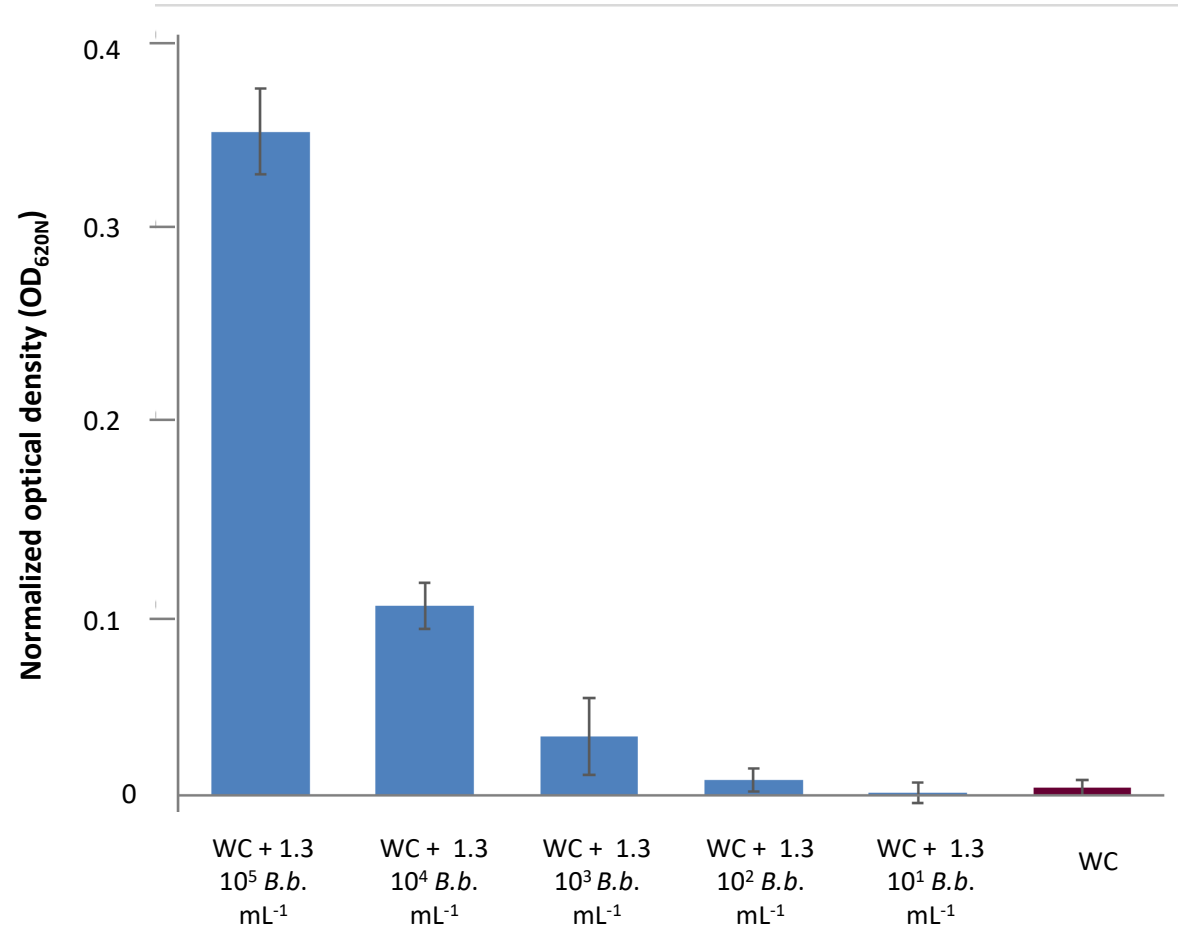


Figure 6

