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1 **Control of *Brettanomyces bruxellensis* on wine grapes by post-harvest treatments with**
2 **electrolyzed water, ozonated water and gaseous ozone**

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33 **Abstract**

34

35 In this study, we investigated the possible effect of electrolyzed water (EW), aqueous ozone (WO)
36 and gaseous ozone (GO) on *Brettanomyces bruxellensis* DSM 7001 strain artificially inoculated on
37 the grape surface and on its evolution during the subsequent, inoculated must fermentation. Culture-
38 dependent and -independent techniques were used to evaluate the effectiveness of treatments against
39 *B. bruxellensis*, as well as its presence during fermentation. Particularly, GO treatment of 24 h
40 decreased its presence by about 2.1 Log, making it possible to reduce significantly the concentration
41 of ethylphenols in the wine in relation to the control wine. EW and WO treatments caused less
42 relevant reductions. The results showed that all the treatments reduced the presence of this yeast on
43 grapes. However, in these experimental conditions it was not possible to achieve a complete removal
44 of this undesirable yeast.

45

46 Industrial Relevance: *Brettanomyces spp.* is considered a wine spoilage yeast due to its ability to
47 produce off-flavors (described as Brett character) and high levels of acetic acid. Broad disinfectant
48 action against microorganisms, eco-friendliness and easiness of on-site application are among the
49 main advantages of the ozone and the electrolyzed water. This study demonstrated the antimicrobial
50 potential of the EW, WO and GO treatments against *B. bruxellensis* inoculated on post-harvest grapes.

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53 **Keywords:** *Electrolyzed water; Ozone; Innovative sanitizing; Brettanomyces bruxellensis; Wine*
54 *grapes; Red wines*

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1. Introduction

During the alcoholic fermentation, yeasts convert sugars present in must, mainly to ethanol, but other compounds, important for the sensory characteristics of the wine, are produced as well, therefore their impact on wine quality could not be ignored (Fleet, 2008). The grape berries surface represents an important vector for yeast populations in the must. Especially when damaged berries are taken into consideration, they can carry a high number of undesirable yeast cell populations (Barata, Malfeito-Ferreira, & Loureiro, 2011; Guerzoni, & Marchetti, 1987; Pretorius, 2000). Among these, *B. bruxellensis* was isolated from several vineyards and in different stages of grape berry development, using mainly enrichment media (Renouf et al., 2006; Renouf, & Lonvaud-Funel, 2007). The yeasts belonging to the genus *Dekkera/Brettanomyces* are mainly responsible for wine spoilage during its storage in cellars, particularly in red wines. These yeasts are generally known for their capacity in the wines to produce off-flavors due to the activity of two enzymes: cinnamate decarboxylase and vinyl phenol reductase (Suarez, Suarez-Lepe, Morata, & Calderon, 2007). Vinyl- and ethyl-phenols are the off-flavor compounds produced by these enzymes from hydroxycinnamic acids, which are naturally present in grape must (Benito, Palomero, Morata, Uthurry, & Suárez-Lepe, 2009). 4-Ethylphenol has a low threshold of sensory perception (350 to 1000 µg/L as a function of wine characteristics) and different flavors, like pharmaceutical, horse-like, barnyard-like, horse blanket, wet dog, tar, tobacco, creosote, leathery and perhaps mousey descriptors (Campolongo, Siegumfeldt, Aabo, Cocolin, & Arneborg, 2014; Suarez et al., 2007). In addition, *Brettanomyces spp.* is a producer under certain conditions of the “mousy” off-flavour and of high concentrations of acetic acid from the sugar metabolism (Freer, Dien, & Matsuda, 2003.; Romano, Perello, De Revel, & Lonvaud-Funel, 2008; Snowdon, Bowyer, Grbin, & Bowyer, 2006). This species is considered dangerous because of its ability to survive in relatively high concentrations of ethanol (Suarez et al., 2007). Furthermore, *Brettanomyces spp.* growth control in wineries is very difficult due to its ability to tolerate normal concentrations of sulfur dioxide used in cellars (Cocolin, Rantsiou, Iacumin, Zironi, & Comi, 2004). Therefore, it may contaminate wineries with a low level of cleaning and disinfection. In fact, these yeasts can survive, proliferate and contaminate the wine during various steps of winemaking process. Several studies have demonstrated the risks of the presence of *Brettanomyces spp.* in wines, however it is very difficult to understand when contamination begins. As *B. bruxellensis* is frequently associated with barrel-red wines, wood used in storage and aging may be a common vector for the introduction of this species in wine in red wine (Suarez et al., 2007). However, some strain have been isolated from the vineyard (Renouf et al., 2007). In particular, Renouf (2007) where able to isolate *Brettanomyces spp.* from grape berries by using an optimized enrichment broth, able to recover their

90 populations in a culture- dependent manner indicating that grapes may act as a possible vector for the
91 introduction of this yeast species into the wine.

92

93 In the last years, new disinfecting agents are being proposed for fruits and vegetables treatment, such
94 as ozone and electrolyzed water (EW) (Boonkorn, et al., 2012; Guentzel, Lam, Callan, Emmons, &
95 Dunham, 2010; Hricova, Stephan, & Zweifel, 2008; Smilanick, Margosan, & Mlikota Gabler, 2002.).
96 EW has a broad spectrum of action against various microorganisms thanks to three combined actions:
97 hydrogen ions, oxidation-reduction potential and free chlorine, while, ozone is a strong oxidant able
98 to attack several cellular constituents of the microorganisms, in addition to this, eco-friendliness and
99 easiness of on site application are other main advantages of these agents (Khadre, Yousef, & Kim,
100 2001; Jermann, Koutchma, Margas, Leadley, & Ros-Polski, 2015).

101

102 On the grape, ozone is a sanitizer that leaves no residues, while a possible eventual residual of free
103 chlorine could be a problem for the formation in vinification of chloroanisoles and chlorophenols,
104 compounds responsible of the “cork taint” in the wines (Guentzel et al., 2010). However, to our
105 knowledge, relationships between use of EW and presence of anisols are still not described in
106 scientific literature. The ability of ozone and EW to sanitize has already been studied on both fresh
107 and withered wine grapes, highlighting not only an antimicrobial effect but also an improvement of
108 grape characteristics and wine quality (Bellincontro et al., 2017; Paissoni et al., 2017; Río Segade et
109 al., 2017). Considering the impact on fermentative yeasts, in grapes treated with ozone and EW,
110 apiculate yeasts were reduced by 0.5 Log CFU/mL when compared to untreated grapes, resulting in
111 a decrease of the acetic acid content in the wines (Cravero, et al., 2016a.; Cravero et al., 2016b).

112

113 However, studies assessing the effect of these innovative sanitizing techniques on *Brettanomyces spp.*
114 present on the grapes are missing. Therefore, the objective of this work was to evaluate the effect of
115 ozone (either in liquid or gaseous treatments) and EW on *B. bruxellensis* DSM 7001 on grape berries
116 used for red wine production. Its presence in wine grapes after the treatments and during the
117 fermentation process was studied by culture-dependent (traditional plate counts) and culture-
118 independent (PCR-denaturing gradient gel electrophoresis [DGGE] and reverse transcription PCR
119 [RT-PCR]-DGGE) techniques. The concentration of off-flavor compounds in the wines was
120 determined by Head Space Solid Phase Micro-Extraction (HS-SPME) coupled to Gas
121 Chromatography-Mass Spectrometry (GC-MS).

122

123 **2. Materials and methods**

124
125 *2.1. Grapes and preparation of the *Brettanomyces bruxellensis* inoculum*
126

127 Whole bunches of *Vitis vinifera* L. cultivar Barbera grapes were harvested from a vineyard located in
128 the Asti province (Piemonte, NW Italy). They were characterized by good phytosanitary conditions,
129 that is without signs of damage/infection by *Botrytis cinerea* or other grape pathogens, and all the
130 skin was intact. The grapes were subdivided in small clusters of 6-8 berries. Afterwards, they were
131 placed in a single layer into perforated boxes, forming batches of 2.0 ± 0.1 kg each. Each trial was
132 inoculated with *B. bruxellensis* DSM 7001 strain from DSMZ, German Collection of Microorganisms
133 and Cell Cultures (Braunschweig, Germany) (Campolongo et al., 2014). Even though the real load of
134 *B. bruxellensis* on grapes is normally lower, in this work, we inoculated about 6.0 Log cells/mL prior
135 to treatments, in order to accurately quantify the effects of the treatments on the yeast population.
136 Inoculum was prepared by introducing a pure *B. bruxellensis* DSM 7001 colony into 5 mL of DBDM
137 broth selective for *B. bruxellensis* (Campolongo, Rantsiou, Giordano, Gerbi, & Cocolin, 2010), after
138 about 10 days incubation at 25 °C, a small aliquot of this broth was spread into DBDM agar selective
139 medium for *B. bruxellensis*. The plates were incubated for 15 days at 25 °C, and then scraped using
140 sterile Ringer's solution (Oxoid, Milan, Italy), thus obtaining the solution used for the inoculum.
141 Afterwards, the yeast cells were stained with methylene blue dye and immediately the viable cell
142 population was counted by using a Thoma hemocytometer chamber (BRAND GMBH + CO KG,
143 Wertheim, Germany). Before inoculation, appropriate amounts of inoculum were calculated and
144 subsequently used to inoculate the grape berry surfaces at an initial cell population of 10^8 cells/mL.
145 Each grape aliquot was sprayed with 100 mL of inoculum. Inoculated grapes were left for 24 hours
146 at a constant temperature of about 25 °C to allow the inoculum to dry and stick to the grape skin.
147 Grape inoculation density was verified by randomly picking thirty berries from each perforated box.
148 Prior to inoculation, the absence of *B. bruxellensis* on grapes was checked by plate counts.

149
150 *2.2. EW and ozone treatments*
151

152 EW solution was generated using an EVA SYSTEM[®] 100 equipment (Industrie De Nora S.p.A,
153 Milano, Italy) as previously described by Cravero et al. (2016a), while an ozone generator (Model
154 C32-AG, Industrie De Nora SpA, MI, Italy) was used for aqueous (WO) and gaseous (GO) ozone
155 production (Cravero et al., 2016b).

156 For the EW and WO treatments, samples were steadily sprayed for a contact time of 6 and 12 min
157 with a nozzle connected to a peristaltic pump (SP311, Velp Scientifica, Usmate, MB, Italy). The EW

158 solution had a concentration of 400 mg/L of free chlorine, while the WO solution had an ozone
159 concentration of 5.00 ± 0.25 mg/L. During treatments, the flow and the temperature were maintained
160 constant at 200 mL/min and 25 °C, respectively. Control treatments were performed using tap water.
161 Two different times were used for the GO treatments (12 and 24 h) in a chamber saturated with
162 gaseous ozone at a concentration of 32 ± 1 µL/L. The treatment was performed in controlled
163 conditions of temperature (20 ± 1 °C), relative humidity (57 ± 3 %) and at constant concentration of
164 ozone, which was constantly monitored through a UV-photometric ozone analyzer BMT 964 (BMT
165 Messtechnik GmbH, Germany) that controls the generator output. Control treatments were performed
166 in another chamber for 12 and 24 h in contact with air, using the abovementioned temperature and
167 relative humidity conditions.

168 For each treatment, we have used three replicates and the experimental plan is summarized as follows:
169 **WA**: treated with tap water for 6 min (control); **WB**: treated with tap water for 12 min (control);
170 **EWA**: treated with electrolyzed water for 6 min; **EWB**: treated with electrolyzed water for 12 min;
171 **WOA**: treated with ozonated water for 6 min; **WOB**: treated with ozonated water for 12 min; **GA**:
172 untreated for 12 h (control); **GB**: untreated for 24 h (control); **GOA**: treated with ozone gas for 12 h;
173 **GOB**: treated with ozone gas for 24 h.

174

175 *2.3. Laboratory-scale fermentations*

176

177 For each trial, before and after treatments, about 30 berries were randomly picked up, placed in sterile
178 bags, crushed and the must obtained was used for culture-dependent and -independent
179 microbiological analyses. Afterwards, all remaining grape berries were crushed in sterile bags and
180 the grape mash obtained (liquid, skins and seeds) was placed in a 2.5-L sterile glass bottle for the
181 laboratory-scale fermentations. The bottles were equipped with sterile airlocks containing sterile
182 vaseline oil, in order to let flow the carbon dioxide (CO₂) during the alcoholic fermentation while
183 avoiding external contaminations. All musts were inoculated with the commercial *Saccharomyces*
184 *cerevisiae* strain EC-1118 (Lallemand Inc., Montreal, Canada) strain was rehydrated according to the
185 manufacturer's instructions and inoculated for obtain a density of around 2.0×10^6 cells/mL in order
186 to standardize the fermentation process. Fermentations were performed under static conditions at
187 25 °C, and during the fermentation all bottles were shaken twice a day to soak the grape cap.
188 Fermentations were monitored by microbiological analysis at 0, 4, 7, 17 and 20 days after the
189 inoculum. Chemical analyses were performed after 7 days and at end (14 days) of fermentation.

190

191 *2.4. Microbiological analyses*

192

193 For culture-dependent analysis, 1 mL of sample from each trial was serially diluted in sterile Ringer's
194 solution (Oxoid, Milan, Italy) and plated into DBDM selective medium for *B. bruxellensis* and in the
195 non-selective Wallerstein laboratory nutrient medium agar (WLN) (Biogenetics, Milan, Italy). The
196 DBDM plates were incubated at 28 °C for 14 days, while WLN plates were incubated at 28 °C for 5
197 days and subsequently counted. The colonies grown on WLN plates were counted and grouped on
198 the basis of their color and morphology as described previously by Urso et al., (2008). After counting,
199 5 colonies from each group were streaked for isolation on YPD agar containing 1% (w/v) yeast
200 extract, 2% (w/v) bacteriological peptone and 2% (w/v) dextrose (Biogenetics, Milan, Italy). Isolates
201 were stored at -20°C in YPD Broth supplemented with 30% sterile glycerol (Sigma, Milan, Italy).

202

203 *2.5. Specific amplification for B. bruxellensis*

204

205 One millilitre of an overnight culture was centrifuged at 14.000 rpm for 10 min and the centrifuged
206 cells were subjected to DNA extraction using the methods proposed by Urso et al. (2008). The DNA
207 of pure colonies obtained from the DBDM medium were subjected to a specific amplification in order
208 to confirm the presence of *B. bruxellensis* in the samples. Particularly, D1-D2 loop of the 26S rRNA
209 gene of each isolate was amplified using the DB90F and DB394R primers as previously explained
210 by Cocolin et al. (2004).

211

212 *2.6. Interdelta-PCR to confirm dominance of the starter S. cerevisiae Lalvin EC1118® during the* 213 *fermentations*

214

215 At days 0, 4, 7, 17 and 20, from each trial, five colonies, with a *S. cerevisiae* morphotype on WLN
216 medium, were isolated and subjected to interdelta-PCR molecular fingerprinting analysis as
217 previously reported (Charpentier, Colin, Alais, & Legras, 2009.). After electrophoresis, the DNA
218 fingerprints were subjected to a cluster analysis by the software package Bionumerics, version 4.0
219 (Applied Maths, Kortrijk, Belgium), using the Unweighted Pair Group Method using Arithmetic
220 Averages (UPGMA) and the Pearson's coefficient.

221

222 *2.7. Direct extraction and PCR and reverse transcriptase (RT) amplification of DNA and RNA from* 223 *grapes and during fermentation*

224

225 For each treatment and sampling point, samples, for the extraction of both DNA and RNA, were
226 centrifuged for 10 min at 14000 rpm. Nucleic acid extraction was carried out by using the
227 MasterPure™ Complete DNA and RNA Purification kit (Epicentre, Madison, WI, USA) as described
228 by Rantsiou et al., (2013). Afterwards, a Nanodrop ND-1000 spectrophotometer (Celbio, Milan, Italy)
229 was used to check the quantity and quality of DNA. Subsequently, the DNA was quantified and
230 standardized at 100 ng/μL, while RNA was treated with the Turbo DNase (Ambion, Milan, Italy) to
231 digest the co-extracted DNA, using the manufacture's instructions. Lack of genomic DNA in the
232 RNA samples was checked by PCR amplification. The DNA and RNA extracts were subjected at
233 PCR and RT-PCR protocols as previously described by Rantsiou et al. (2013).

234

235 *2.8. DGGE analysis: Denaturing gradient gel electrophoresis*

236

237 The D-Code universal mutation detection system (Bio-Rad, Milan, Italy) was used for DGGE
238 analysis. The amplified products were loaded on a 0.8 mm thick polyacrylamide gel (8% (w/v)
239 acrylamide-bisacrylamide (37:5: 1)) with a denaturing gradient of 30 to 50%, in a 1X TAE buffer (0.8
240 mM Tris base and 0.02 mM EDTA, pH 8, adjusted with glacial acetic acid) at 130 V for 4 hours at
241 60 °C (Cocolin, Bisson, & Mills, 2000). The visualization of bands was carried out by immersing the
242 gels in 1X TAE buffer containing 1X SYBR Green (Sigma, Milan, Italy) for 20 min, and put under
243 UV using UVI pro platinum 1.1 Gel Software (Eppendorf, Hamburg, Germany).

244

245 *2.9. Chemical analyses*

246

247 *2.9.1. Main chemical composition*

248

249 Wine chemical composition was evaluated by high-performance liquid chromatography (HPLC)
250 using an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with
251 a refractive index detector and a diode array detector (DAD) set to 210 nm using the protocol reported
252 by Rolle et al., (2012). The chemical compounds quantified were: residual sugars (glucose and
253 fructose), organic acids (tartaric acid, malic acid, citric acid, succinic acid, lactic acid and acetic acid),
254 ethanol and glycerol.

255

256 *2.9.2. Volatile compound determination*

257

258 Ethyl phenols of each wine were quantified by Head Space Solid Phase Micro-Extraction (HS-SPME)
259 coupled to Gas Chromatography-Mass Spectrometry (GC-MS), using the protocols previously
260 described by Campolongo et al. (2010). In a vial of 20 mL, we added 5 mL of the wine sample (pH
261 7), 5 mL of MilliQ water, 200 μ L of a solution of internal standard (3,4-dimethyl-phenol) and 3 g of
262 NaCl (Boutou, & Chatonnet, 2007). For the HS-SPME a DVB/CARBOXEN/PDMS fiber of 1 cm of
263 length was used for 20 minutes at 45 °C, with automatic stirring. Analyses were performed on an
264 Agilent 7890C gas chromatograph (Little Falls, DE, USA) coupled to an Agilent 5975 mass selective
265 detector and a DB-WAX capillary column (30 m x 0.25 mm inner diameter, 0.25 mm film thickness,
266 J&W Scientific Inc., Folsom, CA, USA). The software used was Agilent G1702-90057 MSD
267 ChemStation. The chromatographic program was: 35 °C for 2 minutes, gradient of 20 °C/min until
268 170 °C for 1 minute, gradient of 3 °C/min until 210 °C for 15 minutes. Detection and standards curves
269 were achieved in electron impact mode (EI) with selection ion monitoring (SIM) mode and
270 metabolites were measured by comparing peaks area of specific ions with those of the internal
271 standard (3,4-dimethylphenol). The volatile compounds evaluated were the off-flavors produced by
272 *B. bruxellensis*, namely 4-vinylguaiacol (4-VG), 4-vinylphenol (4-VP), 4-ethylguaiacol (4-EG) and
273 4-ethylphenol (4-EP).

274

275 2.10. Statistical analysis

276

277 The microbiological and chemical results were submitted to one-way Analysis of Variance
278 (ANOVA). To highlight statistical differences among treatments, we used the Tukey-HSD post-hoc
279 test with a confidence level of 95%. The statistical analyses were performed with the software
280 package IBM SPSS Statistics (version 21.0, IBM Corp., Armonk, NY, USA).

281

282 3. Results

283

284 3.1. *B. bruxellensis* counts on grape berries surface

285

286 The load of *B. bruxellensis* DSM 7001 population on grape berries surface was about 5.3 Log
287 CFU/mL in all the trials, data obtained by sampling done 24 hours after inoculation. Fig. 1 shows the
288 decrease of *B. bruxellensis* population after the treatments with EW, WO and GO. All treatments
289 reduced greatly the presence of this yeast, but not completely. Particularly, GOB treatment decreased
290 its population by 2.1 Log. EW and WO treatments obtained comparable reductions, more precisely
291 1.2 Log for EWA, 1.4 Log for EWB, and 1.3 Log for WOB, respectively.

292 As it can be seen from Fig. 1, also the control treatments reduced the *B. bruxellensis* load on grape
293 berries surface. Indeed, GA and GB treatments reduced the population of *B. bruxellensis* DSM 7001
294 by 0.9 and 1.7 Log, respectively, whereas control treatments with water reduced the population by
295 0.6 Log for WA and 0.7 Log for WB.

296

297 3.2. *B. bruxellensis* and *S. cerevisiae* growth dynamics during the fermentation

298

299 In Fig. 2 the growth dynamics of *B. bruxellensis* and *S. cerevisiae* population during inoculated
300 alcoholic fermentation are presented. The fermentations of the musts obtained from the control and
301 the treated grape berries, were characterized by a very similar *S. cerevisiae* population trend. Indeed,
302 after four days of fermentation, the beginning of the stationary phase was registered with viable cell
303 populations around 7.5 Log CFU/mL. This number remained stable for 7 days and then started to
304 decline until the end of the monitored period, being around 6.9 Log CFU/mL. One exception was the
305 WA trial, where *S. cerevisiae* decreased quickly after the seventh day of fermentation and reached
306 6.0 Log CFU/mL at the twentieth day. Population decreased probably as a result of the nutrient
307 depletion (Cramer, Vlassides, & Block, 2002) and/or the presence of significant levels of ethanol.

308 As it can be seen in Fig. 2, the initial viable population of *B. bruxellensis* DSM 7001 in each
309 fermentation trial was in accordance with the efficacy of each treatment. However, during
310 fermentations, the evolution of *B. bruxellensis* was not influenced by the different treatments applied,
311 in fact the maximum population was similar in all cases (around 7.0 Log CFU/mL). Towards the end
312 of fermentation, more *B. bruxellensis* cells were found as *S. cerevisiae* viable population started to
313 decline.

314

315 3.3. PCR and RT-PCR-DGGE results

316

317 The PCR and RT-PCR-DGGE analyses were included in this study in order to increase the
318 information about the vitality and presence of *B. bruxellensis* DSM 7001 before and after the
319 treatments, as well as its presence during the alcoholic fermentations. The RNA and DNA profiles
320 for all stages of sampling were equal between them and agreed with the results obtained by plate
321 counts using DBDM medium. In fact, the band of *B. bruxellensis* DSM 7001 was present in all
322 samples and in all steps of the fermentation period. In Fig. 3 the profiles of the RT-PCR-DGGE at
323 the end of fermentation is reported, where the bands of the *B. bruxellensis* DSM 7001 can be seen in
324 all samples.

325

326 3.4. Chemical composition of the wines at the end fermentation

327

328 The main chemical compounds for each wine produced in this study are presented in Table 1. All
329 fermentations consumed all the sugars from the medium after 14 days (< 2.0 g/L of residual sugars,
330 fructose and glucose), without stuck fermentations. As it can be seen in Table 1, most data did not
331 show significant differences between the samples of EW, WO and GO treatments. The only
332 significant difference was found in the amount of acetic acid present in the wines produced from GO
333 treated grapes. Indeed, the concentration of this compound was high for EW and WO treatments,
334 reaching levels up to 0.8–0.9 g/L, whereas the wines produced from treated grapes with GO showed
335 acetic acid concentrations between 0.5 and 0.7 g/L. This high concentration of acetic acid in these
336 wines could be explained by the presence of *B. bruxellensis* during the fermentation.

337

338 3.5. Vinyl- and ethyl-phenols presence at the end of the fermentations

339

340 At the end of the fermentation, to better understand the impact of the different treatments on wine
341 quality, we have assessed the presence in the wine of the off-flavors: 4-vinylphenol, 4-vinylguaiacol,
342 4-ethylguaiacol and 4-ethylphenol. In Fig. 4 the concentrations of the volatile phenols found in the
343 wines at the end of the fermentations are reported. In all samples the concentrations of the
344 vinylphenols (4-vinylphenol, 4-vinylguaiacol) was quite low. In addition, these values have not
345 highlighted differences between wines produced from treated and untreated grapes. In fact, all wines
346 produced had a concentration of vinylphenols between 90 and 450 µg/L. As shown by the data
347 presented in Fig. 4, all wines contained high levels of ethylphenols that exceed their threshold. The
348 concentrations of ethylphenols in the wines produced from EW and WO treated grapes were not
349 significantly different from those of their respective controls (W). In fact, the values recorded in these
350 wines rang all around 800 µg/L. On the other hand, wines produced from GO treated grapes showed
351 significant differences in the concentrations of ethylphenols when compared with the respective
352 controls (G). Indeed, the GA wine had a high concentration of the total ethylphenols with 1817 µg/L,
353 while the GOA wine accounted for 820 µg/L. In the GB and GOB wines, the total ethylphenols
354 concentration slightly decreased to 1031 and 576 µg/L, respectively.

355

356 **4. Discussion**

357 One possible approach to reduce the wine contamination by *B. bruxellensis* is the use of electrolyzed
358 water and ozone in post-harvest wine grapes thanks to their broad disinfectant action against
359 microorganisms, eco-friendliness and easiness of on-site application. In this context, almost all

360 treatments with EW and ozone had a significantly higher effect on yeast vitality respect to the
361 controls, even though controls have slightly reduced the charges of *Brettanomyces*. However, none
362 of the treatments applied in this study were able to completely reduce *B. bruxellensis* cells.
363 Particularly, the results showed greater efficacy of the treatments with gaseous ozone, where the
364 longer treatment time influenced the yeast counts. In fact, the GOB treatment decreased the
365 population of *B. bruxellensis* by 2.1 Log, while EW and WO treatments reduced its population in a
366 range of 1.0 to 1.4 Log. The effectiveness of aqueous ozone on *B. bruxellensis* was already
367 highlighted in another study (Guzzon, Nardin, Micheletti, Nicolini, & Larcher, 2013), where it was
368 shown that 5 mg/L of O₃ for 30 min were sufficient for a complete inactivation of a population with
369 a concentration of 10⁶ CFU/mL. The results obtained here confirmed the low ozone tolerance of this
370 yeast, although the ozone treatments used did not guarantee its complete elimination. This fact can
371 be explained by the different treatment times and substrate used in the two different studies. Indeed,
372 many studies have shown how the effectiveness of ozone is influenced by many factors including
373 concentration, contact time, and substance on which it works (Khadre et al., 2001.; Jermannet et al.,
374 2015). In fact, other studies done on the use of EW and WO and GO on post-harvest grapes showed
375 lower reductions in yeast charges, around of 0.5 Log (Cravero et al., 2016a, b). These two studies
376 showed the antimicrobial property of EW, WO and GO on the population present on grapes surface,
377 where the treatments have reduced of about 0.5 Log the counts of apiculate yeasts, resulting in a
378 decrease of the acetic acid content in the wines produced by spontaneous fermentation from the
379 treated grapes. Comparing the results obtained in this study with those obtained in the two works of
380 Cravero et al., 2016 a, b, it is shown how the treatments are much more efficient on *B. bruxellensis*
381 respect the other yeast species. Particularly, the reduction of *B. bruxellensis* is twice that of the
382 apiculate yeast in treatments with EW and WO and even four times higher in GO treatments. In
383 addition to this, Renouf et al., (2006, 2007), demonstrated that the concentration of *B. bruxellensis*
384 found in fresh grape must after crushing is 2.0 Log CFU / mL. As a consequence, the reduction of 2.1
385 Log observed in grapes treated with GO for 24 h, could help to limit the spreading of this undesirable
386 yeast and prevent the contamination of the winery from the vineyard.

387 During the fermentation time, *S. cerevisiae* population was dominated thanks the inoculated Lalvin
388 EC-1118[®], as demonstrated by the results of interdelta-PCR and cluster analysis using the similarity
389 coefficient of 90% (data not shown). However, towards the end of fermentation, more *B. bruxellensis*
390 cells were found as *S. cerevisiae* viable population started to decline. This is correlated with the higher
391 ethanol tolerance of *B. bruxellensis* than *S. cerevisiae* in conditions of low sugar concentrations
392 (Renouf et al., 2006). It is important to take into account that, at the end of fermentation, the
393 population of *B. bruxellensis* was lower after GO treatments when compared with that after EW and

394 WO treatments, this fact is reflected in the data of the acetic acid present in the wines. In fact, the
395 level of the acetic acid in the GO wines was low respect the concentration present on the EW and
396 WO wines, although the high charges of *Brettanomyces* have produced very high acetic acid levels
397 in all wines, making them all impaired. Other studies confirm the capacity of *B. bruxellensis* to
398 produce acetic acid during the alcoholic fermentation (Freer et al., 2003), or even have demonstrated
399 that the production of acetic acid by *B. bruxellensis* depends on its cell concentration at the end of
400 fermentation, and on the presence or not of the oxygen at that stage (Ciani, & Ferraro, 1997).
401 Therefore, the results obtained here are in agreement with other studies since the populations of this
402 yeast in GA, GB, GOA and GOB trials, at the end of the fermentation, were lower with respect to the
403 other trials.

404 Additionally, the concentrations of the phenols confirm the high charge of *B. bruxellensis* observed
405 by microbiological analysis (plant counts and DGGE analysis) during fermentation. The sensory
406 threshold of vinylphenols, that can be responsible for a depreciating ‘phenolic’ or ‘pharmaceutic’
407 characteristic, has been described to be 725 µg/L (Chatonnet, Dubourdieu, Boidron, & Lavigne,
408 1993). Therefore, the concentrations found in the wines produced in this study cannot influence
409 negatively the wine aroma. Rather, as the concentrations were below 500 µg/L, these compounds
410 help to improve the aromatic quality of wines with pleasant flowery and spicy notes, more, several
411 studies have highlighted that vinylphenols are able to bind to wine anthocyanins stabilizing the color
412 over time (Schwarz, Wabnitz & Winterhalter 2003, Chatonnet et al., 1993). Vinylphenols are also
413 produced by different yeasts, including *S. cerevisiae*, and same lactic acid bacteria, while only the 4-
414 ethylphenol and 4-ethylguaiacol are typically produced by *B. bruxellensis* in significant quantities to
415 damage the wine (Chatonnet, Dubourdieu, & Boidron. 1995; Zuehlke, Petrova, & Edwards, 2013).

416 On the other hand, the ethylphenols (4-ethylphenol and 4-ethylguaiacol) has a lower threshold of
417 sensory perception (350 to 1000 µg/L as a function of the characteristics of wine) and different off-
418 odors (Suarez et al., 2007). In the wines obtained in this study, like see in the Fig 4., concentrations
419 are all higher than the perception threshold, so all wines are irretrievably damaged. Interestingly,
420 gaseous ozone reduced the capacity of *B. bruxellensis* to produce ethylphenols. In fact, the
421 concentrations of 4-ethylphenol are halved in GOA and GOB wines with respect to GA and GB. This
422 result is very important because it highlights that the use of gaseous ozone prior to grape crushing
423 may reduce the risk of “off-flavors” in the wines even if the grapes were inoculated by *B. bruxellensis*.

424

425 **5. Conclusion**

426

427 This first study demonstrated the partial efficacy of the EW, WO and GO treatments in reducing *B.*
428 *bruxellensis* inoculated in post-harvested grapes. The results showed a relatively high reduction of *B.*
429 *bruxellensis* in the must produced by grapes treated with GO at 24 h decreasing by 2.1 Log. EW and
430 WO treatments have obtained lower reductions ranging between 1.0 and 1.4 Log. However, none of
431 the treatments applied in this study was able to completely reduce *B. bruxellensis* cells. In fact, at the
432 end of the fermentations, all wines had high amounts of ethylphenols, which are above the threshold
433 of perception. This could be explained by the high inoculum of *B. bruxellensis* for all tests, used to
434 better understand the impact of these treatments against it. These preliminary results showed that the
435 use of EW, WO and, in particular, GO could be considered good sanitizing agents in order to reduce
436 the population of *B. bruxellensis* on the grapes surface and in the musts. However, grapes are only
437 one of the potential sources of *B. bruxellensis* infections, and therefore treatments on grapes cannot
438 completely remove this issue from wine production. A better knowledge of the free chlorine present
439 in EW, ozone concentration, and contact time that modulate the efficiency of these treatments will
440 allow a greater reduction in *B. bruxellensis* cells.

441

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443

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446

447 **References**

448

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Table 1: Chemical data of the wines at the end of fermentation.

WA: treated with water for 6 min; **WB:** treated with water for 12 min; **EWA:** treated with EW (400 mg/L of free chlorine) for 6 min; **EWB:** treated with EW (400 mg/L of free chlorine) for 12 min; **WOA:** treated with ozonated water (5.00 ± 0.25 mg/L) for 6 min; **WOB:** treated with ozonated water (5.00 ± 0.25 mg/L) for 12 min; **GA:** treated with air for 12 h; **GB:** treated with air for 24 h; **GOA:** treated with ozone gas (32 ± 1 µL/L) for 12 h; **GOB:** treated with ozone gas (32 ± 1 µL/L) for 24 h. All data are expressed as average value ± standard deviation (n = 3). Different Latin letters within the same column indicate significant differences among the treatments, according to the Tukey-HSD post-hoc test (*p* < 0.01). Sign.: ** and ns indicate significance at *p* < 0.05 and not significant, respectively

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EW	Citric Acid (g/L)	Tartaric Acid (g/L)	Malic Acid (g/L)	Glycerol (g/L)	Acetic Acid (g/L)	Ethanol (%v/v)	Succinic Acid (g/L)	Lactic Acid (g/L)
WA	0.14 ± 0.01	5.1 ± 0.2	1.4 ± 0.1	10.7 ± 0.2	0.94 ± 0.07	13.6 ± 0.1 ab	1.0 ± 0.1	0.36 ± 0.01
EWA	0.14 ± 0.01	4.4 ± 0.1	1.2 ± 0.1	11.0 ± 0.1	0.82 ± 0.05	13.8 ± 0.1 b	1.1 ± 0.1	0.34 ± 0.02
WB	0.14 ± 0.01	4.8 ± 0.4	1.3 ± 0.1	10.5 ± 0.2	0.91 ± 0.11	13.5 ± 0.1 a	1.0 ± 0.1	0.35 ± 0.02
EWB	0.12 ± 0.03	4.7 ± 0.3	1.2 ± 0.3	10.8 ± 0.3	0.82 ± 0.03	13.8 ± 0.1 b	1.1 ± 0.1	0.35 ± 0.03
Sign.	ns	ns	ns	ns	ns	*	ns	ns
WA	0.14 ± 0.01	5.1 ± 0.2	1.4 ± 0.1	10.7 ± 0.2	0.94 ± 0.07	13.6 ± 0.1	0.9 ± 0.1	0.36 ± 0.01
WOA	0.11 ± 0.01	5.4 ± 0.1	1.1 ± 0.1	10.6 ± 0.1	0.85 ± 0.13	13.6 ± 0.1	1.0 ± 0.1	0.37 ± 0.01
WB	0.14 ± 0.01	4.8 ± 0.4	1.3 ± 0.1	10.5 ± 0.2	0.91 ± 0.11	13.5 ± 0.1	1.0 ± 0.1	0.35 ± 0.02
WOB	0.15 ± 0.01	4.9 ± 0.3	1.3 ± 0.1	10.5 ± 0.1	0.83 ± 0.03	13.7 ± 0.1	1.0 ± 0.1	0.34 ± 0.01
Sing.	ns	ns	ns	ns	ns	ns	ns	ns
GA	0.13 ± 0.01	4.9 ± 0.4	1.4 ± 0.3	10.2 ± 0.1	0.60 ± 0.06	13.8 ± 0.1	1.1 ± 0.1	0.33 ± 0.02
GOA	0.12 ± 0.03	4.7 ± 0.4	1.2 ± 0.1	10.2 ± 0.1	0.71 ± 0.04	13.7 ± 0.1	1.0 ± 0.1	0.35 ± 0.02
GB	0.13 ± 0.01	5.1 ± 0.4	1.2 ± 0.1	10.1 ± 0.1	0.59 ± 0.07	13.8 ± 0.1	1.1 ± 0.1	0.33 ± 0.01
GOB	0.17 ± 0.03	4.7 ± 0.1	1.8 ± 0.2	9.9 ± 0.1	0.48 ± 0.05	13.8 ± 0.1	1.1 ± 0.1	0.30 ± 0.03
Sign.	ns	ns	ns	ns	ns	ns	ns	ns

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599 **Figure captions**

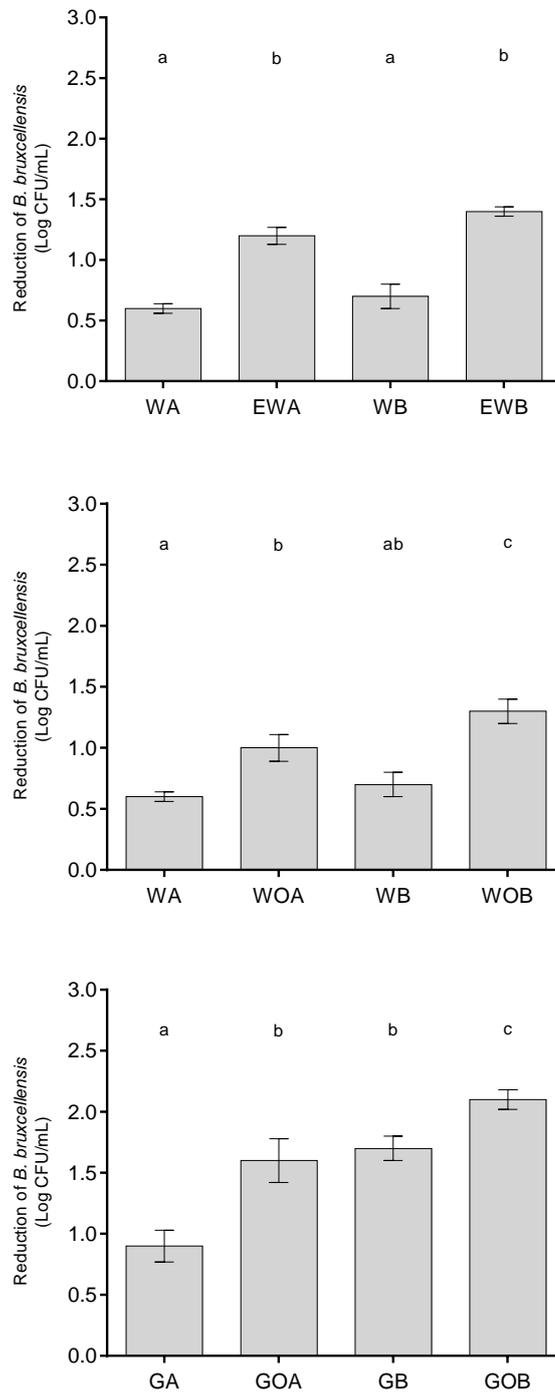
600 **Fig. 1.** Decrease of *B. bruxellensis* DSM 7001 population after the treatments with EW, WO and GO
601 registered by the counts before and after the treatments on the DBDM medium. Data are expressed
602 as average value \pm standard deviation ($n = 3$). Different Latin letters indicate significant differences
603 among the treatments, according to the Tukey-HSD post-hoc test ($p < 0.01$). **WA:** treated with water
604 for 6 min; **WB:** treated with water for 12 min; **EWA:** treated with EW (400 mg/L of free chlorine)
605 for 6 min; **EWB:** treated with EW (400 mg/L of free chlorine) for 12 min; **WOA:** treated with
606 ozonated water (5.00 ± 0.25 mg/L) for 6 min; **WOB:** treated with ozonated water (5.00 ± 0.25 mg/L)
607 for 12 min; **GA:** treated with air for 12 h; **GB:** treated with air for 24 h; **GOA:** treated with ozone gas
608 (32 ± 1 μ L/L) for 12 h; **GOB:** treated with ozone gas (32 ± 1 μ L/L) for 24 h.

609
610 **Fig. 2.** Counts (CFU/mL) of *S. cerevisiae* Lalvin EC1118[®] [●] and *B. bruxellensis* DSM 7001 [○] in
611 control (broken line) and treatments (solid line) of the **EW**, **WO** and **GO** trials. **A:** treatments of 6
612 min (EW and WO) and 12 h (GO); **B:** treatments of 12 min (EW and WO) and 24 h (GO). *B.*
613 *bruxellensis* counts were determined on the DBDM medium and species identification was performed
614 by specific amplification using the DB90F and DB394R primers. *S. cerevisiae* counts were
615 determined on WLN medium and the identification was reached through RFLP analysis of the ITS1-
616 5.8S ribosomal RNA (rRNA)-ITS2. The counts were reported as average value \pm standard deviation
617 ($n = 3$).

618
619 **Fig. 3.** RT-PCR-DGGE profile of the samples at the end of fermentation. **WA:** treated with water for
620 6 min; **WB:** treated with water for 12 min; **EWA:** treated with EW (400 mg/L of free chlorine) for 6
621 min; **EWB:** treated with EW (400 mg/L of free chlorine) for 12 min; **WOA:** treated with ozonated
622 water (5.00 ± 0.25 mg/L) for 6 min; **WOB:** treated with ozonated water (5.00 ± 0.25 mg/L) for 12
623 min; **GA:** treated with air for 12 h; **GB:** treated with air for 24 h; **GOA:** treated with ozone gas ($32 \pm$
624 1 μ L/L) for 12 h; **GOB:** treated with ozone gas (32 ± 1 μ L/L) for 24 h. **Bb:** *Brettanomyces bruxellensis*
625 DSM 7001 strain.

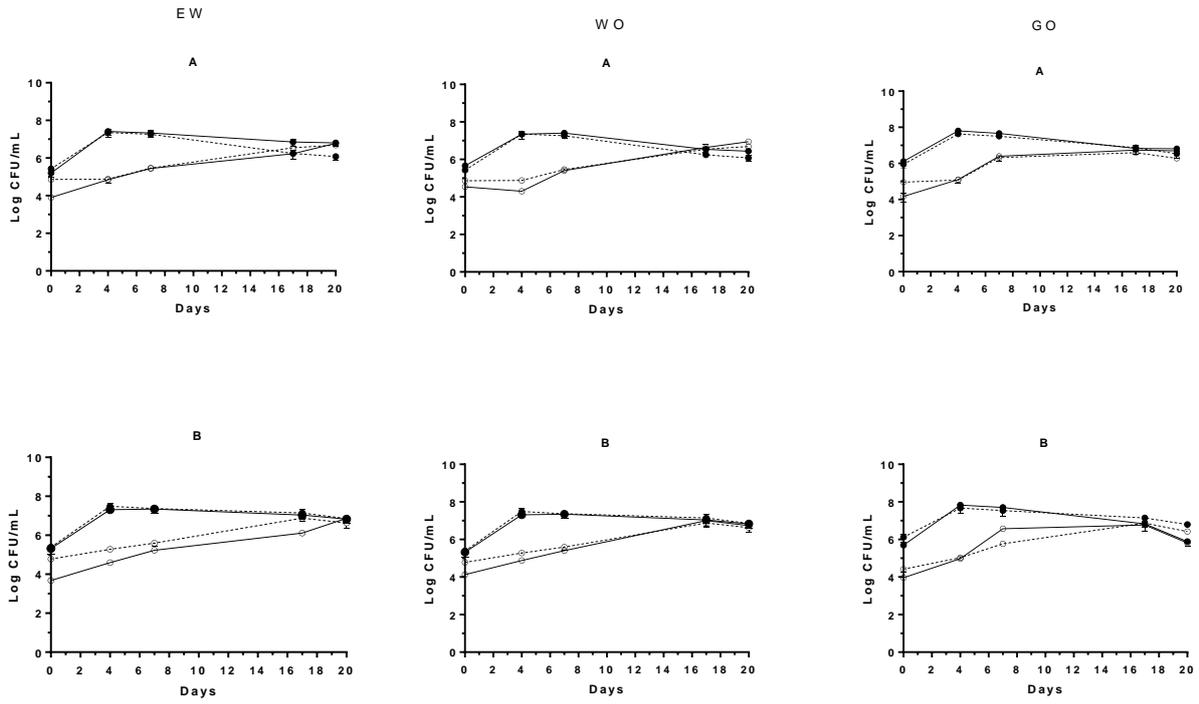
626
627 **Fig. 4.** Vinyl- and ethyl-phenols present at the end of fermentation in the wines treated with EW, WO
628 and GO. All data are expressed as average value \pm standard deviation ($n = 3$). Different Latin letters
629 indicate significant differences among the treatments, according to the Tukey-HSD post-hoc test (p
630 < 0.05). Sign.: **, *** and ns indicate significance at $p < 0.01$, $p < 0.001$ and not significant,
631 respectively. **WA:** treated with water for 6 min; **WB:** treated with water for 12 min; **EWA:** treated
632 with EW (400 mg/L of free chlorine) for 6 min; **EWB:** treated with EW (400 mg/L of free chlorine)

633 for 12 min; **WOA**: treated with ozonated water (5.00 ± 0.25 mg/L) for 6 min; **WOB**: treated with
634 ozonated water (5.00 ± 0.25 mg/L) for 12 min; **GA**: treated with air for 12 h; **GB**: treated with air for
635 24 h; **GOA**: treated with ozone gas (32 ± 1 μ L/L) for 12 h; **GOB**: treated with ozone gas (32 ± 1
636 μ L/L) for 24 h. **4-VG**: 4-vinylguaiacol; **4-VP**: 4-vinylphenol; **4-EG**: 4-ethylguaiacol; **4-EP**: 4-
637 ethylphenol.
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650 Fig 2
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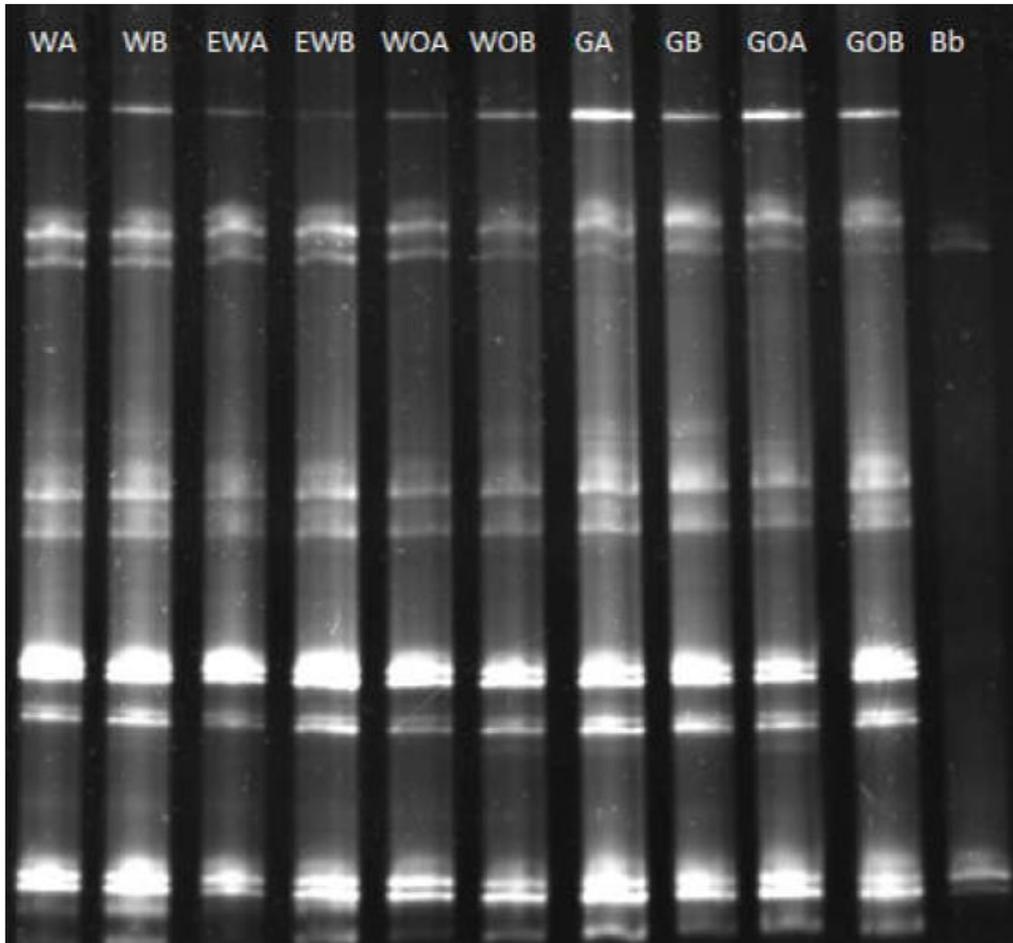


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672 **Fig. 3**

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