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

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

Francesco Cravero[a#], Vasileios Englezos[a#], Kalliopi Rantsiou[a], Fabrizio Torchio[b], Simone Giacosa[a], Susana Río Segade[a], Vincenzo Gerbi[a], Luca Rolle[a] and Luca Cocolin[a*]

[a]Università di Torino, Dipartimento di Scienze Agrarie, Forestali e Alimentari, Largo Paolo Braccini 2, 10095 Grugliasco, Torino, Italy.

[b]Università Cattolica del Sacro Cuore, Istituto di Enologia e Ingegneria Agro-Alimentare, Via Emilia Parmense 84, 29122 Piacenza, Italy.

[*]Corresponding author: Luca Cocolin, Phone +39-011-6708553, Fax: +39-011-6708549, email: lucasimone.cocolin@unito.it.

[#]Francesco Cravero and Vasileios Englezos contributed equally to this article.

Abstract

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

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

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

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

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

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

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

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

2. Materials and methods

2.1. Grapes and preparation of the Brettanomyces bruxellensis inoculum

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

2.2. EW and ozone treatments

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

For the EW and WO treatments, samples were steadily sprayed for a contact time of 6 and 12 min 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 ºC), 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 (CO_2) 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 x 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*

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

2.5. Specific amplification for B. bruxellensis

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

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

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

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

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

2.8. DGGE analysis: Denaturing gradient gel electrophoresis

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

2.9. Chemical analyses

2.9.1. Main chemical composition

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

2.9.2. Volatile compound determination

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

2.10. Statistical analysis

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

3. Results

3.1. B. bruxellensis counts on grape berries surface

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

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

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

In Fig. 2 the growth dynamics of *B. bruxellensis* and *S. cerevisiae* population during inoculated alcoholic fermentation are presented. The fermentations of the musts obtained from the control and the treated grape berries, were characterized by a very similar *S. cerevisiae* population trend. Indeed, after four days of fermentation, the beginning of the stationary phase was registered with viable cell populations around 7.5 Log CFU/mL. This number remained stable for 7 days and then started to decline until the end of the monitored period, being around 6.9 Log CFU/mL. One exception was the WA trial, where *S. cerevisiae* decreased quickly after the seventh day of fermentation and reached 6.0 Log CFU/mL at the twentieth day. Population decreased probably as a result of the nutrient depletion (Cramer, Vlassides, & Block, 2002) and/or the presence of significant levels of ethanol.
As it can be seen in Fig. 2, the initial viable population of *B. bruxellensis* DSM 7001 in each fermentation trial was in accordance with the efficacy of each treatment. However, during fermentations, the evolution of *B. bruxellensis* was not influenced by the different treatments applied, in fact the maximum population was similar in all cases (around 7.0 Log CFU/mL). Towards the end of fermentation, more *B. bruxellensis* cells were found as *S. cerevisiae* viable population started to decline.

3.3. PCR and RT-PCR-DGGE results

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

3.4. Chemical composition of the wines at the end fermentation

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

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

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

4. Discussion

One possible approach to reduce the wine contamination by *B. bruxellensi*s is the use of electrolyzed water and ozone in post-harvest wine grapes thanks to their broad disinfectant action against 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 O_3 for 30 min were sufficient for a complete inactivation of a population with
369 a concentration of 10^6 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 at 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

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

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

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

5. Conclusion

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

Acknowledgments

We would like to thank the Alexander Stewart Hurley for proofreading and providing valuable linguistic advice.

References

Barata, A., Malfeito-Ferreira, M., & Loureiro, V., 2011. The microbial ecology of wine grape berries. *International Journal of Food Microbiology,* 153, 243-259. https://doi.org/10.1016/j.ijfoodmicro.2011.11.025.

Bellincontro, A., Catelli, C., Cotarella, R. & Mencarelli, F. (2017). Postharvest ozone fumigation of Petit Verdot grapes to prevent the use of sulfites and to increase anthocyanin in wine. *Australian Journal of Grape and Wine Research* 23, 200–206. http://dx.doi.org/10.1111/ajgw.12257

Benito, S., Palomero, F., Morata, A., Uthurry, C. & Suárez-Lepe, J.A., 2009. Minimization of ethylphenol precursors in red wines via the formation of pyranoanthocyanins by selected yeasts. *International Journal of Food Microbiology,* 132:145-152. https://doi.org/10.1016/j.ijfoodmicro.2009.04.015.

Boonkorn, P., Gemma, H., Sugaya, S., Setha, S., Uthaibutra, J., & Whangchai, K., 2012. Impact of high-dose, short periods of ozone exposure on green mold and antioxidant enzyme activity of tangerine fruit. *Postharvest Biology and Technology*, 67, 25-28. https://doi.org/10.1016/j.postharvbio.2011.12.012.

Boutou, S., & P. Chatonnet., 2007. Rapid headspace solid-phase microextraction/gas chromatographic/mass spectrometric assay for the quantitative determination of some of the

main odorants causing off-flavours in wine. *Journal of Chromatography*, 1141,1-9. https://doi.org/10.1016/j.chroma.2006.11.106.

Campolongo, S., Rantsiou, K., Giordano, M., Gerbi, V., & Cocolin, L., 2010. Prevalence and biodiversity of Brettanomyces bruxellensis in wine from Northwestern Italy. *American Journal Enology and Viticulture*, 61:4. https://doi.org/10.5344/ajev.2010.10034.

Campolongo S., Siegumfeldt H., Aabo T., Cocolin L. & Arneborg N., 2014. The effects of extracellular pH and hydroxycinnamic acids influence the intracellular pH of *Brettanomyces bruxellensis* DSM 7001. *LWT-Food Science and Technology*, 59, 1088-1092. https://doi.org/10.1016/j.lwt.2014.06.006.

Chatonnet, P., Dubourdieu, D., Boidron, J.N., & Lavigne V., 1993. Synthesis of volatile phenols by *Saccharomyces cerevisiae* in wines. *Journal of the Science of Food and Agriculture*,1993, 62, 191-202. https://doi.org/10.1002/jsfa.2740620213.

Charpentier, C., Colin, A., Alais, A. & Legras, J.L., 2009. French Jura flor yeasts: genotype and technological diversity. *Antonie Van Leeuwenhoek*, 95, 263-273. https://doi.org/10.1007/s10482-009-9309-8.

Ciani, M. & Ferraro, L., 1997. Role of oxygen on acetic acid production by in winemaking *Brettanomyces/Dekkera*. *Journal of the Science of Food and Agriculture*,1997, 75, 489-495. https://doi.org/10.1002/(SICI)1097-0010(199712)75:4<489::AID-JSFA902>3.0.CO;2-9.

Cocolin, L., Bisson, L.F., & Mills, D.A., 2000. Direct profiling of the yeast dynamics in wine fermentations. *FEMS Microbiology Letters*, 189, 81-87. https://doi.org/10.1111/j.1574-6968.2000.tb09210.x.

Cocolin, L., Rantsiou, K., Iacumin, L., Zironi, R., & Comi, G., 2004. Molecular detection and identification of *Brettanomyces/Dekkera bruxellensis* and *Brettanomyces/Dekkera anomalus* in spoiled wines. *Applied and environmental microbiology*, 70 (3), 1347-1355. https://doi.org/10.1128/AEM.70.3.1347-1355.2004.

Chatonnet, P., Dubourdieu, D., & Boidron, J. N. (1995). The influence of *Brettanomyces/Dekkera* sp. yeasts and Lactic Acid Bacteria on the ethylphenol content of red wines. *American Journal of Enology and Viticulture*, 46: 463-468. Cramer, A.C., Vlassides, S. & Block D.E., 2002. Kinetic model for nitrogen-limited wine fermentations. *Biotechnology and bioengineering*, 77 (1), 49-60. https://doi.org/10.1002/bit.10133.

Cravero, F., Englezos, V., Torchio, F., Giacosa, S., Río Segade, S., Gerbi, V., Rantsiou, K., Rolle, L., & Cocolin, L., 2016a. Post-harvest control of wine-grape mycobiota using electrolyzed water. *Innovative Food Science and Emerging Technologies*, 35, 21-28. https://doi.org/10.1016/j.ifset.2016.03.010

Cravero, F., Englezos, V., Rantsiou, K., Torchio, F., Giacosa, S., Río Segade, S., Gerbi, V., Rolle, L., & Cocolin, L., 2016b. Ozone treatments of post harvested wine grapes: Impact on fermentative yeasts and wine chemical properties. *Food Research International*, 87, 134-141. https://doi.org/10.1016/j.foodres.2016.06.031.

Fleet, G.H., 2008. Wine yeast for the future. *FEMS Yeast Research*, 8, 979-995. https://doi.org/10.1111/j.1567-1364.2008.00427.x.

Freer, S.N., Dien, B., & Matsuda, S., 2003. Production of acetic acid by Dekkera/Brettanomyces yeasts under conditions of constant pH. *World Journal of Microbiology & Biotechnology*, 19, 101-105. https://doi.org/10.1023/A:1022592810405.

Guerzoni, E. and Marchetti R., 1987. Analysis of yeast flora associated with grape sour rot and of the chemical disease markers. *Applied and environmental microbiology*, 53 (3), 571-573.

Guentzel, J. L., Lam, K. L., Callan, M. A., Emmons, S. A., & Dunham, V. L., 2010. Postharvest management of gray mold and brown rot on surfaces of peaches and grapes using electrolyzed oxidizing water. *International Journal of Food Microbiology*, 143, 54-60. https://doi.org/10.1016/j.ijfoodmicro.2010.07.028.

Guzzon, R., Nardin, T., Micheletti, O., Nicolini, G. & Larcher R., 2013. Antimicrobial activity of ozone. Effectiveness against the main wine spoilage microorganisms and evaluation of impact

on simple phenols in wine. *Australian Journal of Grape and wine research* 19,180–188,2013. https://doi.org/10.1111/ajgw.12018.

Hricova, D., Stephan, R., & Zweifel, C., 2008. Electrolyzed water and its application in the food industry. *Journal of Food Protection*, 71, 1934-1947. https://doi.org/10.4315/0362-028X-71.9.1934.

Jermann, C., Koutchma, T., Margas, E., Leadley, C., & Ros-Polski, V., 2015. Mapping trends in novel and emerging food processing technologies around the world. *Innovative Food Science and Emerging Technologies*, 31, 14-27. https://doi.org/10.1016/j.ifset.2015.06.007.

Khadre, M.A., Yousef, A.E., & Kim, J.G., 2001. Microbiological aspects of ozone applications in food: A review. *Journal of Food Science*, 66, 1242-1252. https://doi.org/10.1111/j.1365-2621.2001.tb15196.x.

Laureano, J., Giacosa, S., Río Segade, S, Torchio, F., Cravero, F., Gerbi, V., Englezos, V., Carboni, C., Cocolin, L., Rantsiou, K., Faroni, L. D. R., & Rolle, L. (2016). Effect of continuous exposure to ozone gas and electrolyzed water on the skin hardness of table and wine grape varieties. *Journal of Texture Studies*, 47,1, 40-48. doi:10.1111/jtxs.12158.

Paissoni, M.A., Río Segade, S., Giacosa, S., Torchio, F., Cravero, F., Englezos V., Rantsiou, K., Carboni, C., Gerbi, V., Teissedre, P.L., Rolle, L. (2017). Impact of post-harvest ozone treatments on the skin phenolic extractability of red winegrapes cv Barbera and Nebbiolo (Vitis vinifera L.). *Food Research International*, 98, 68-78 https://doi.org/10.1016/j.foodres.2016.11.013

Pretorius, I. S., 2000. Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast*, 16, 675–729. https://doi.org/10.1002/1097-0061(20000615)16:8<675::AID-YEA585>3.0.CO;2-B.

Rantsiou, K., Campolongo, S., Alessandria, V., Rolle, L., Torchio, F., & Cocolin, L., 2013. Yeast populations associated with grapes during withering and their fate during alcoholic fermentation of high-sugar must. *Australian Journal of Grape and Wine Research*, 19, 40-46. https://doi.org/10.1111/ajgw.12000.

Renouf, V., Falcou, M., Miot-Sertier, C., Perello, M.C., De Revel G. & Lonvaud-Funel A., 2006. Interactions between *Brettanomyces bruxellensis* and other yeast species during the initial stages of winemaking. *Journal of applied microbiology*, 100, 1208-1219. https://doi.org/10.1111/j.1365-2672.2006.02959.x.

Renouf, V., & Lonvaud-Funel A., 2007. Development of an enrichment medium to detect *Dekkera/Brettanomyces bruxellensis*, a spoilage wine yeast, on the surface of grape berries. *Microbiological Research*, 162, 154-167. https://doi.org/10.1016/j.micres.2006.02.006.

Río Segade, S., Vilanova, M., Giacosa, S., Perrone, I., Chitarra, W., Pollon, M., Torchio F., Boccacci, P., Gambino, G., Gerbi V., Rolle L. (2017). Ozone improves the aromatic fingerprint of white grapes. *Scientific Reports*, 7, 16301. doi:10.1038/s41598-017-16529-5

Rolle L., Giordano M., Giacosa S., Vincenzi S., Río Segade S., Torchio F., Perrone B., & Gerbi V., 2012. CIEL*a*b* parameters of white dehydrated grapes as quality markers according to chemical composition, volatile profile and mechanical properties. Analitica Chimica Acta, 732, 105-113. https://doi.org/10.1016/j.aca.2011.11.043.

Romano A., Perello M.C., De Revel G., & Lonvaud-Funel A., 2008. Growth and volatile compound production by *Brettanomyces/Dekkera bruxellensis* in red wine. *Journal of applied microbiology*, 104, 1577-1585. https://doi.org/10.1111/j.1365-2672.2007.03693.x.

Schwarz, M., Wabnitz, T.,C., & Winterhalter P., (2003). Pathway leading to the formation of anthocyanin-vinylphenol adducts and related pigments in red wines. *Journal Agricultural Food Chemistry*, *51*, 3682-3687. doi: 10.1021/jf0340963

Smilanick, J.L., Margosan, D.M., & Mlikota Gabler, F., 2002. Impact of ozonated water on the quality and shelf-life of fresh citrus fruit, stone fruit, and table grapes. *Ozone: Science & Engineering*, 24 (5), 343-356. http://dx.doi.org/10.1080/01919510208901625.

Snowdon E.M., Bowyer M.C., Grbin P.R. & Bowyer P.K., 2006. Mousy off-flavor: A Review. *Journal agricultural and food chemistry*,54, 6465-6474. http://dx.doi.org/10.1021/jf0528613.

Suarez, R., Suarez-Lepe, J. A., Morata, A., & Calderon, F., 2007. The production of ethylphenols in wine by yeasts of the genera *Brettanomyces* and *Dekkera*: a review. *Food Chemistry*, 102, 10-21. https://doi.org/10.1016/j.foodchem.2006.03.030.

Urso, R., Rantsiou, K., Dolci, P., Rolle, L., Comi, G., & Cocolin, L., 2008. Yeast biodiversity and 400 dynamics during sweet wine production as determined by molecular methods. *FEMS Yeast Research*, 8,1053-1062. https://doi.org/10.1111/j.1567-1364.2008.00364.x.

Zuehlke, J.M., Petrova, B., & Edwards, C.G., 2013. Advances in the control of wine spoilage by *Zygosaccharomyces* and *Dekkera/Brettanomyce*s. *Annual reviews of Food Science and Technolgy*. 4 (1) 57-78. https://doi.org/10.1146/annurev-food-030212-182533.

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

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

Figure captions

Fig. 1. Decrease of *B. bruxellensis* DSM 7001 population after the treatments with EW, WO and GO registered by the counts before and after the treatments on the DBDM medium. Data are expressed as average value ± standard deviation (n = 3). Different Latin letters indicate significant differences among the treatments, according to the Tukey-HSD post-hoc test ($p < 0.01$). **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.

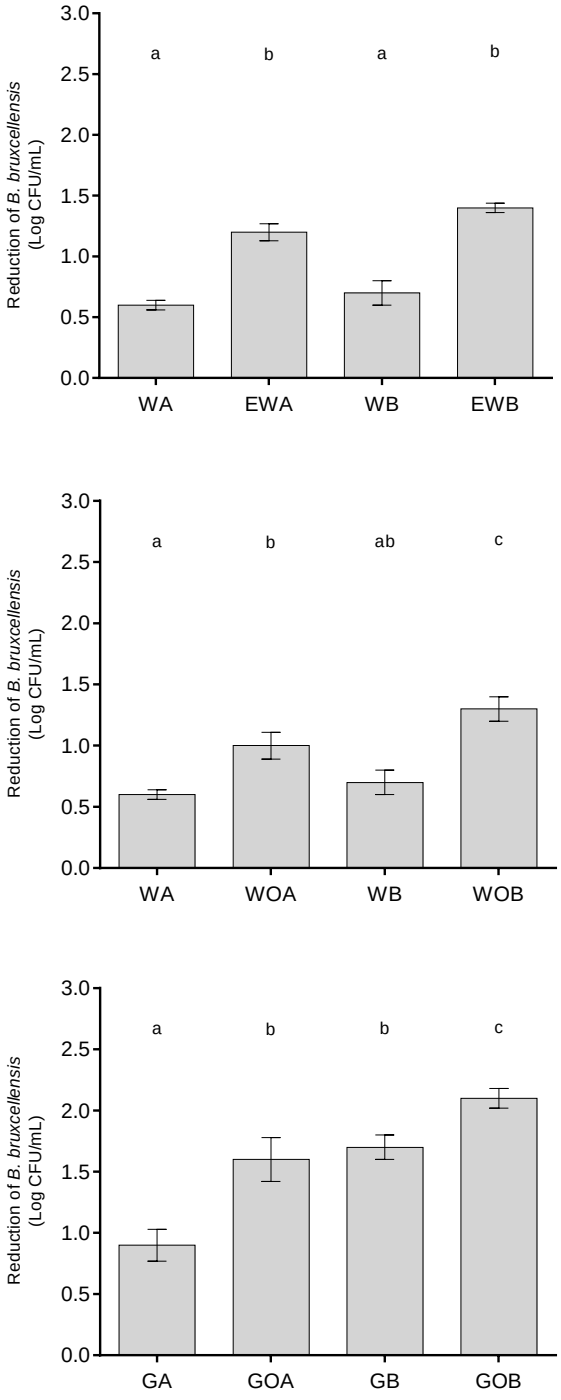
Fig. 2. Counts (CFU/mL) of *S. cerevisiae* Lalvin EC1118® [●] and *B. bruxellensis* DSM 7001 [○] in control (broken line) and treatments (solid line) of the **EW**, **WO** and **GO** trials. **A**: treatments of 6 min (EW and WO) and 12 h (GO); **B**: treatments of 12 min (EW and WO) and 24 h (GO). *B.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

Fig. 3. RT-PCR-DGGE profile of the samples 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. **Bb**: *Brettanomyces bruxellensis* DSM 7001 strain.

Fig. 4. Vinyl- and ethyl-phenols present at the end of fermentation in the wines treated with EW, WO and GO. All data are expressed as average value ± standard deviation (n = 3). Different Latin letters indicate significant differences among the treatments, according to the Tukey-HSD post-hoc test ($p < 0.05$). Sign.: **, *** and ns indicate significance at $p < 0.01$, $p < 0.001$ and not significant, respectively. **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)

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 µL/L) for 12 h; **GOB**: treated with ozone gas (32 ± 1
636 µL/L) for 24 h. **4-VG**: 4-vinylguaiacol; **4-VP**: 4-vinylphenol; **4-EG**: 4-ethylguaiacol; **4-EP**: 4-
637 ethylphenol.
638

639 **Fig. 1**



640
641
642
643
644
645
646
647
648
649

Fig 2

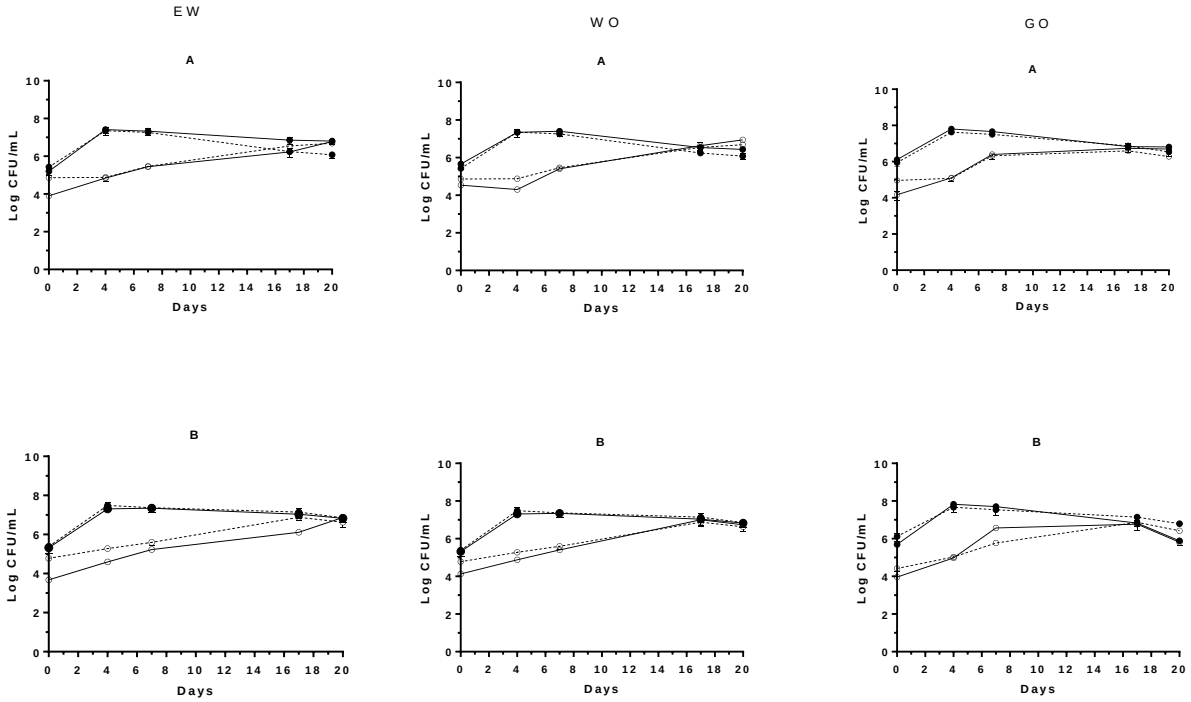
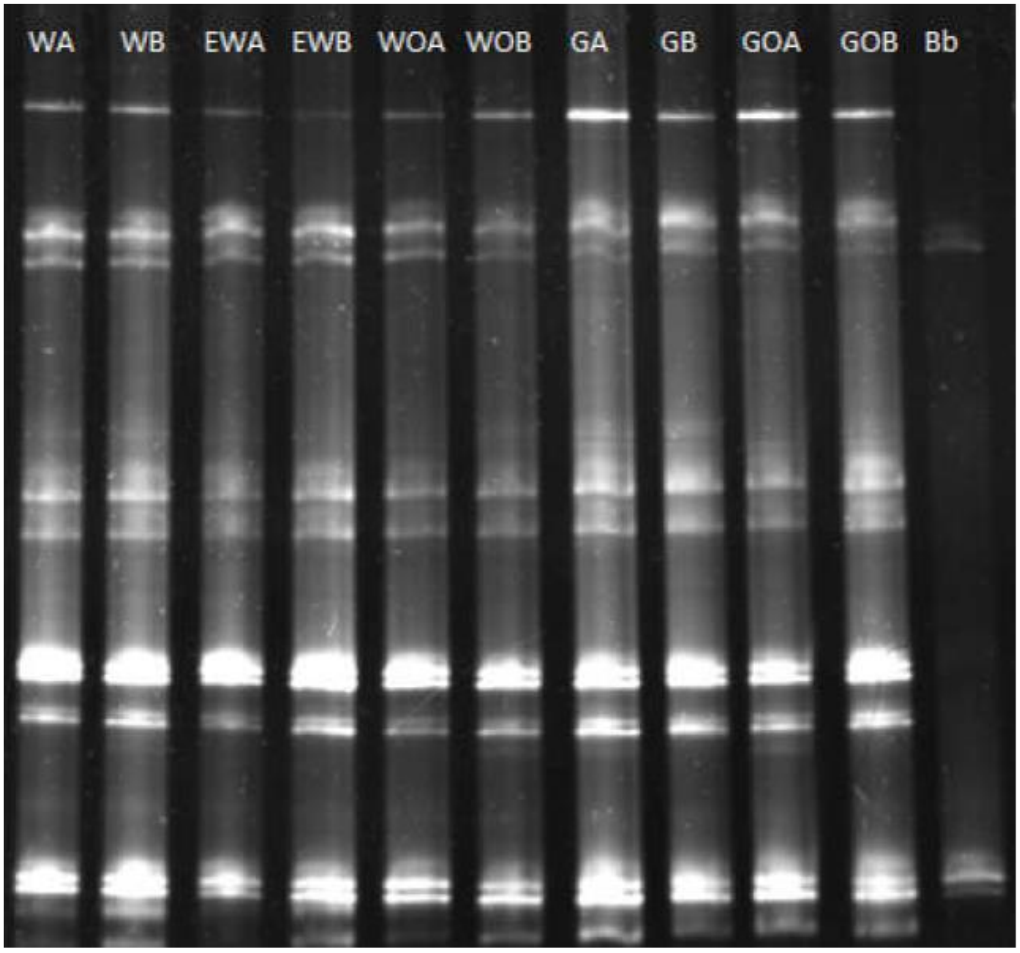
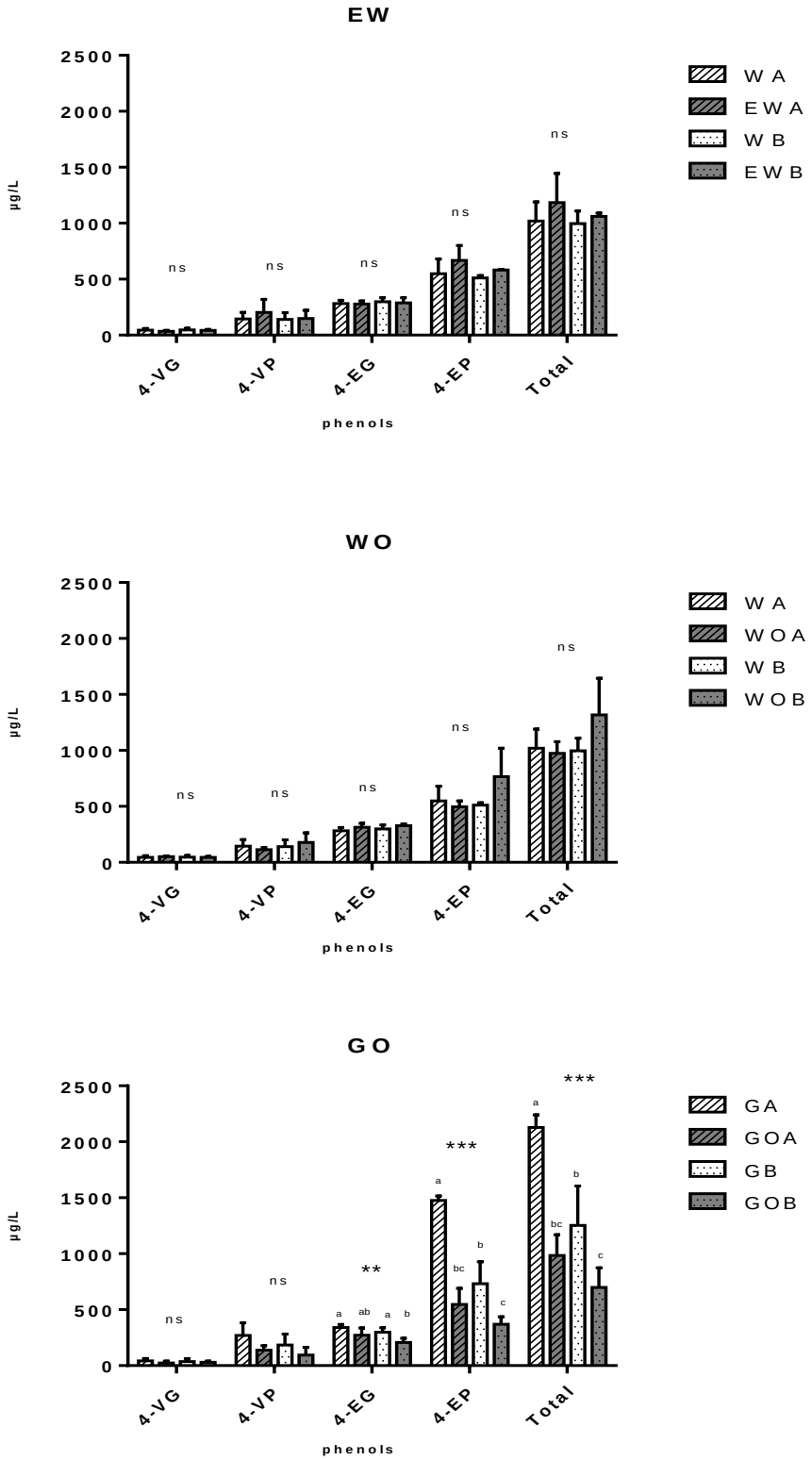


Fig. 3



691 **Fig. 4**


EW
2500
2000
1500
1000
500
0
µg/L
ns
ns
ns
ns
ns
4-VG
4-VP
4-EG
4-EP
Total
phenols
W A
EW A
W B
EW B
WO
2500
2000
1500
1000
500
0
µg/L
ns
ns
ns
ns
ns
4-VG
4-VP
4-EG
4-EP
Total
phenols
W A
WO A
W B
WO B
GO
2500
2000
1500
1000
500
0
µg/L
ns
ns
**
***
***
a
ab
a
b
a
bc
b
c
a
bc
b
c
4-VG
4-VP
4-EG
4-EP
Total
phenols
GA
GOA
GB
GOB

692