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ABSTRACT

Electrolyzed water (EW) has recently attracted much attention due to its efficacy against a broad spectrum of microorganisms. In this study, we investigated the impact of two EW treatments (40 and 400 mg/L free chlorine) on grape mycobiota using culture-dependent and -independent approaches. Moreover, the effect of yeast inoculation on treated and non-treated grapes was also considered. At the end of the fermentation, the wines produced were subjected to chemical and aroma analyses. The results revealed a decrease of about 0.5 log CFU/mL of the total yeast population on grapes surface independently of the dose of EW applied. Yeast inoculation and EW treatments shortened the time needed by *Saccharomyces cerevisiae* to dominate apiculate yeasts, particularly, 2 days for inoculated and 7 days for spontaneous fermentations. A decrease of acetic acid (about 55%) was also observed compared to untreated spontaneous fermentation. In addition, aroma analysis highlighted a positive contribution of inoculated yeast on the wine aromas, since they had approximately 50 % higher pleasant esters compared to spontaneous fermented wines.

Industrial Relevance: Sulfur dioxide is widely used in crushed grapes prior to fermentation due to its antimicrobial and antioxidant activity. However, legislative rules, health risks and negative consumer perception related to its presence and use have resulted in a need to find new sanitizers able to reduce its use. The effectiveness of EW to reduce yeast species able to produce high levels of undesirable compounds was demonstrated. This research introduced an innovative antimicrobial agent, which could assist in the first step of wine production to reduce the use of SO_2.

Keywords: *Electrolyzed water*; *Grape*; *Wine*; *Yeast dynamics*; *Sanitization*; *Innovative treatment*

1. Introduction

In wine, like in other fermented beverages, fermentations occur under conditions in which microbial activities, either from inoculated or environmental yeasts and bacteria, have a substantial role in the quality characteristics of the final product (Bokulich, Ohta, Richardson, & Mills, 2013). The adoption of fermentation practices, which limit spoilage by controlling the growth of desirable microorganisms is fundamental in order to enhance wine quality and safety (Du Toit & Pretorius, 2000). Sulfur dioxide (SO_2) is an antimicrobial agent commonly used in crushed grapes to inhibit the growth of spoilage microorganisms, including apiculate yeasts, acetic and lactic acid bacteria, and to minimize the oxidation of phenolic compounds (Boulton, Singleton, Bisson, & Kunkee, 1996).

In spite of these advantages, the resulting sulfites from the addition of SO_2 have been related to headaches, allergic reactions and breathing difficulties in asthma patients (Santos, Nunes, Saraiva, & Coimbra, 2012; Vally, Misso, & Madan, 2009). This negative impact of SO_2 led the International Organization of Vine and Wine (OIV) to reduce the maximum concentration limit to 150 mg/L and 200 mg/L (European Union Regulation: No 606/2009) for the red and white wines, respectively. In Europe, wine producers must indicate the presence of sulfites on the bottle when this exceeds 10 mg/L, due to restrictions applied by law (European Union Regulation: No 1991/2004). In addition to these legislative rules, mainstream consumers have become more health-conscious in the last decade, and focus their attention on healthy and natural products free of substances that are considered negative, such as chemical preservatives (Bech-Larsen & Scholderer, 2007).

The addition of SO_2 in winemaking industry is a complex subject, because many compounds bound with SO_2 by reducing its effectiveness against microbial proliferation and oxidation. In this context, the use of moderate levels of SO_2 prior to fermentation does not ensure an antiseptic protection, since the added SO_2 binds rapidly with the abundant grape sugars and as a consequence the percentage of free SO_2 declines (Ribéreau Gayon et al., 2016). Thus, there is an increasing interest in the search of innovative technologies able to reduce the levels of SO_2 in this stage of vinification. Further, the chance of a

possible replacement of this additive could be particularly important in 'sulphite free' wines production (i.e. without SO_2 addition).

To this regard, the use of EW as sanitization agent is growing in popularity in the last decades due to the high antimicrobial activity against a wide spectrum of microorganisms (Hricova, Stephan, & Zweifel, 2008) and its simple generation by electrolysis from potable water and asalt (KCl) solutiononly (Buck, Iersel, Oetting, & Hung, 2002). EW can be produced on site with low production costs, while the treated water could be recycled during the harvest season by adding pure EW, favouring a wider implementation of this technology on an industrial scale. Concerning these positive aspects, in 2011 the Food and Drug Administration (FDA) declared EW to be considered as *Generally Recognized As Safe* (GRAS) substance to wash or to assist in peeling of fruit and vegetables, since it meets the requirements specified in 21CFR173.315 (FDA 2011).

Since that time, the application of EW in food industry has increased significantly (Jermann, Koutchma, Margas, Leadley, & Ros-Polski, 2015). Several studies investigated the antimicrobial effect of electrolyzed water in a wide variety of post-harvest fruits and vegetables. Despite this extensive use of EW in food industry, little is known about the application of EW in winemaking industry, except for few studies about the decay of *Botrytis cinerea* and the treatment effectiveness during the storage of post-harvest table grapes (Guentzel, Lam, Callan, Emmons, & Dunham, 2010; Kim, Chung, Kang, Chung, & Choi, 2003).

Information regarding the efficiency of EW to reduce or replace SO_2 in the first steps of the fermentation process against spoilage yeasts is needed to aid the development of alternative products with minimal environmental and health impact. Thus, the impact of grape EW treatments and yeast inoculation on wine fermentations was studied. Culture-dependent (traditional plate counts) and culture-independent (PCR-denaturing gradient gel electrophoresis [DGGE] and reverse transcription PCR [RT-PCR]-DGGE) techniques were used to depict yeast dynamics over the course of fermentation. Furthermore, two series of fermentations (spontaneous and inoculated with *Saccharomyces cerevisiae*) were investigated to assess the cumulative effects of inoculation and EW sanitization on yeast population dynamics and wine aroma profile.

2. Materials and Methods

2.1. Grape samples

Wine grapes (*Vitis vinifera* L. Cultivar Barbera), grown in Asti province (Piedmont, Italy), were harvested in good phytosanitary conditions. Immediately after harvesting, about 36 kg of grapes were transported to the laboratory. The main stalk was removed and the berries were kept in clusters of 3 to 5 berries with the pedicel attached.

2.2. Preparation of EW solutions and grapes treatment

Concentrated EW solution was generated by using EVA SYSTEM® 100equipment(Industrie De Nora S.p.A, Milan, Italy), following the manufacturer's instructions. An aqueous solution of40 g/L of potassium chloride (KCl) was prepared to obtain by electrolysis an EW solution of approximately 4000 mg/L of free chlorine, pH 9.0. This stock solution of EW was diluted with sterile deionized water (for avoid external contamination) to obtain the two working solutions with concentrations of 40 and 400 mg/L of free chlorine (pH 9.0 and 1% residual KCl). All EW solutions were freshly prepared before use. The amount of free chlorine, as well as the pH were verified prior to use according to the methods described by Laureano et al. (2016). About 2 kg ($\pm$ 100 g) of berries were placed in a single layer into perforated boxes (50x30x15cm) and subsequently sprayed with 100 mL of working EW solution, using a hand spray bottle according to the following treatments, in six plicate: A, not treated with EW (Control); B, treated with EW containing 40 mg/L of free chlorine; and C, with EW containing 400 mg/L of free chlorine. After treatment each lot of grapes were crushed originating must, which was subjected to fermentation according the experimental plan (Fig. 1). For each treatment applied (A, B and C) two different sets of laboratory fermentations were performed: one trial was conducted by indigenous yeasts present on grape berries (sample codes Control SA, treatment SB and SC), while in the second trial a commercial active dry yeast was inoculated (sample codes Control IA, treatment IB and IC). Each fermentation was performed in triplicate.

2.3. Grape sampling

A set of about 30 berries, before and after treatments from each perforated box were sampled randomly and placed in a stomacher bag. After manual crushing, the resulting juice was subjected to microbiological analysis. Aliquots of one mL each, in duplicate, were centrifuged for 10 min at 14.000 rpm and the supernatant was removed. Pellets to be used for DNA extraction were immediately frozen at -20°C, while those

destined to RNA analysis were covered with 200 μL of RNA later (Ambion, Milan, Italy) prior to freezing.

2.4. Must fermentations

After each treatment, berries from each perforated box (about 2 kg ± 100 g) with the pedicel attached were aseptically collected in sterile plastic bags, immediately crushed and the juice with skins was transferred to sterile 2.5 L glass bottles contained approximately 1.7 L of grape must. The mean values of standard chemical parameters of the musts obtained were: 21.9 °Brix, pH 3.14 and titratable acidity of 9.51 g/L (expressed as tartaric acid). Inoculated fermentations were performed inoculating *S. cerevisiae* (Lalvin EC1118®, Lallemand, Montreal, Canada), according to manufacturer's instructions, at an initial cell concentration of 2.0×10^6 cells/mL. The bottles were closed with a sterile Müller valve containing sterile vaseline oil, in order to allow the CO_2 formed during the fermentation progress to escape from the system. Fermentations were carried out for 14 days, under static conditions at 25 ± 1 °C. Samples of the fermented musts were collected aseptically at the beginning (immediately following crushing), and after 2, 5, 7 and 14 days of fermentation. Aliquots for DNA and RNA extractions were taken only from the spontaneously fermented musts and stored at -20 °C until further processing.

2.5. Microbiological analyses

Samples were serially diluted in quarter strength Ringer's solution (Oxoid, Milan, Italy), then plated for cultivation and subsequent enumeration in two different microbiological media: the non-selective Wallerstein laboratory nutrient medium agar (WLN) (Biogenetics, Milan, Italy) and the selective medium Lysine medium agar (Oxoid, Milan, Italy). The latter was used to count the non-*Saccharomyces* yeast species, since it is a medium containing glucose, vitamins, inorganic salts, and L-lysine as the sole nitrogen source, which cannot be assimilated by the *Saccharomyces* spp. (Angelo & Siebert, 1987). Plates were incubated for 5 days at 30°C and colonies were counted on the basis of the colour and morphology as described previously by Urso et al. (2008). Five isolates of each colony morphotype were picked and purified by streaking on WLN medium. All of them were stored in YPD broth (10 g/L yeast extract, 20 g/L bacteriological peptone and 20 g/L dextrose; Biogenetics, Milan, Italy) with glycerol (30%) (Sigma, Milan, Italy) at −20°C for further analysis.

2.6. Molecular analysis

2.6.1. DNA extraction from pure cultures

Genomic DNA of each isolate was extracted from one-millilitre of an overnight culture in YPD broth, following the protocols described by Alessandria et al. (2015). Extracted DNA was quantified by using a Nanodrop Spectrophotometer (ND-100, Thermo Fisher Scientific, Milan, Italy) and standardized at 50 ng/μL. The isolates were identified by Restriction Fragment Length Polymorphism (RFLP) analysis of the 5.8S ITS rDNA region (Alessandria et al., 2015) using the restriction enzymes, *Hae*III, *Hinf*Iand *Cfo*I (Promega, Milan, Italy). Confirmation of the identification was obtained by sequencing the D1–D2 loop of the 26S rRNA gene, as previously described (Kurtzman & Robnett, 1997).

2.6.2. Genotypic characterization of S. cerevisiae *isolates*

Molecular identification and characterization of 225 putative colonies of *Saccharomyces* spp. (5from each sampling point, 25 for each fermentation) isolated from the inoculated wines was performed by the interdelta PCR, according to the protocols described by Charpentier, Colin, Alais, & Legras(2009). The molecular profile of each isolate was subjected to cluster analysis, using the computer software package Bionumerics, version 4.0 (Applied Maths, Kortrijk, Belgium). Un weighted Pair Group Method using Arithmetic Averages (UPGMA) and the Pearson's coefficient were used to calculate dendrograms and group together genetically similar isolates (Vauterin&Vauterin, 1992).

2.6.3. Direct extraction of nucleic acid from grapes and must samples

Total DNA and RNA were extracted from the pelleted cells by using the MasterPure[TM] Complete DNA and RNA Purification Kit (Epicentre, Madison, WI, USA), according to the manufacturer's protocol. The co-precipitated DNA in the resuspended RNA was eliminated by DNase I treatment (Turbo DNase, Ambion, Milan, Italy). BothDNA and RNA concentrations were determined with the aforementioned spectrophotometer. RNA was stained with ethidium bromide in 0.8% (w/v) agarose gel to check the integrity. Lack of genomic DNA contamination in the RNA samples was checked by PCR amplification.

2.6.4. PCR and reverse transcriptase (RT) amplification

PCR and RT-PCR protocols were as previously described by Rantsiou et al. (2013). For cDNA synthesis, about 500 ng of total RNA was used as template using M-MLV reverse transcriptase (Promega, Milan, Italy).

2.6.5. DGGE analysis and identification by sequencing

PCR products obtained from grapes and fermented musts were analysed by DGGE using a D-Code apparatus (Bio-Rad, Hercules, CA, USA)as described by Rantsiou et al. (2013).The DGGE bands of interest were excised directly from the gels by using a sterile tip and amplified with NL1 (without the GC clamp) and LS2 primers and sent for sequencing (MWG Biotech, Ebersberg, Germany).The resultant sequences were aligned with those present in GenBank using the BLASTN tool from the NCBI web site (http://blast.ncbi.nlm.nih.gov).

2.7.Chemical analyses

Ethanol, glycerol, organic acids (malic and acetic acids) and reducing sugars (fructose and glucose) concentrations in the initial must and in the final wines were determined by High-Performance Liquid Chromatography (HPLC) as described previously (Rantsiouet al., 2013). Volatile compounds were extracted and then quantified by means of Head Space Solid Phase Micro-Extraction (HS-SPME), coupled with Gas Chromatography-Mass Spectrometry (GC-MS) by using the protocols reported by Rolle, Torchio, Giacosa, & RíoSegade (2015).

2.8.Statistical analysis

Statistical analysis was performed using the statistical software package IBM SPSS Statistics (version 21.0, IBM Corp., Armonk, NY, USA). The Duncan test at $P < 0.05$ was used to establish significant differences by one-way analysis of variance (ANOVA). Principal Component Analysis (PCA) was used to evaluate the fermentation performance in terms of aromatic composition.

3. Results and discussion

3.1.Yeast colonisation on the grape berry surface

The yeast population present on the grape berry surface ranges from 5.0 to 6.0 log colony forming units (CFU)/mL, in agreement with the values reported in literature for

mature grapes (Fleet & Heard, 1993; Milanović, Comitini, & Ciani, 2013). As seen in Figure 2 (panel A), the viable yeast counts on grapes decreased from 6.47 ± 0.12 to 6.11 ± 0.24 and 6.01 ± 0.05 log CFU/mL, after treatment with EW with 40 and 400 mg/L of free chlorine, respectively, which corresponds to a reduction of about 0.5 log CFU/mL. The differences between untreated and treated grapes were significant according to the Duncan test ($P < 0.05$). The increase of the concentration of free chlorine in the EW from 40 to 400 mg/L, did not result in a significant decrease of the yeast communities, therefore, a low dose treatment was already effective.

Five yeast colonies with different morphotypes on WLN medium, from treated and untreated grapes, were picked, isolated and identified. Using PCR-RFLP analysis of the rRNA operon ITS region and sequencing of the partial 26S rRNA gene, these yeasts were identified as *Hanseniaspora uvarum*, *Aerobasidium pullulans*, *Starmerella bacillaris* (synonym *Candida zemplinina*), *Rhodotorula glutinis* and *Issatchenkia terricola*. A higher diversity of non-*Saccharomyces* yeast species was found in the untreated grapes compared with those treated with 40 and 400 mg/L offree chlorine (5 morphotypesvs 3 morphotypes, Fig. 2, panel B). *R. glutinis* and *I .terricola* were the species mostly affected by EW treatments. *Starm. bacillaris* was the dominant species in the treated grapes, followed by *H. uvarum* and *A. pullulans*. The presence of fermentative yeasts such as *H. uvarum* and *Starm. bacillaris* on the grape berry surface may be explained by the sugar leach or diffusion from the inner tissues of the grapes to the surface, which occurs in the mature grapes (Fleet, 2003). The lack of identification of *S. cerevisiae* by plate counts on the grape surface confirms the low presence of this species on wine grapes (Martini, Ciani, & Scorzetti, 1996), since it generally occurs at populations less than $10 - 100$ CFU/g on undamaged grapes (Fleet, 2003), and is greatly associated with winery environment (Fleet, 2003).

3.2. Sanitization impact on yeast diversity (or population dynamics)

Figure 3 shows the growth dynamics of non-*Saccharomyces* and *Saccharomyces* yeasts during fermentation for each of the treatments investigated. Important differences in kinetic patterns were observed between spontaneous and inoculated fermentations. Independently of the treatment, in spontaneously fermented wines a first phase dominated by non-*Saccharomyces* yeasts was followed by a second one with a robust proliferation of indigenous *S. cerevisiae* strains on the fifth day. Non-*Saccharomyces* yeasts grew well during early stages of fermentation by reaching the stationary phase ($7.1 - 7.5$ log

CFU/mL) in two days, afterwards remained quite stable for 3 days, while no viable cells were registered at day 14 of fermentation. This sharp decline was observed when ethanol started to increase (5.5 – 7.5 % vol.). This result agrees with previous studies, which demonstrated a higher reduction of non-*Saccharomyces* viable cell population at medium-high ethanol concentrations (Fleet & Heard, 1993). *S. cerevisiae* cells were found from day 5 (4.1 – 5.5 log CFU/mL) and became predominant (8.1 – 8.5 log CFU/mL) in only two days, remaining at these values until the end of the process. The increasing levels of ethanol throughout the fermentation progress influenced greatly the *S. cerevisiae* dominance, as demonstrated by others (Bisson & Walker, 2014).

Concerning the inoculated musts, *S. cerevisiae* governed the fermentations reaching a maximum population of 8.1 log CFU/mL at day 5, while non-*Saccharomyces* exhibited a moderate increase (from 6.1 to 6.8 log CFU/mL), except for untreated musts where counts remained stable for two days and thereafter a remarkable drop (<10 CFU/mL) in viable cells was recorded at day 5. It is worth noticing that non-*Saccharomyces* populations became undetectable on WLN medium sooner in the must obtained from untreated grapes (4 days versus 7 days). Non-*Saccharomyces* populations were strongly affected by starter yeast inoculation, probably due to the high competition with *S. cerevisiae* for nutrients or/and the presence of cell-to-cell contact mechanisms (Medina, Boido, Dellacassa, & Carrau, 2012; Nissen, Nielsen, & Arneborg, 2003).

Yeast species diversity and population development in spontaneous and inoculated musts at different stages of alcoholic fermentation was depicted by RFLP analysis and partial 26 rRNA gene sequence analysis. The dynamics of yeast species is shown in Figure 4. A total of 6 yeast species belonging to 6 different genera were identified in the untreated samples, while a total of 4 species belonging to 4 different genera were observed in the treated samples, with no differences observed between the two EW treatments applied. In agreement with other ecological studies, spontaneously fermented wine plate counts revealed higher populations of non-*Saccharomyces* yeasts in the first fermentation days (Combina et al., 2005, Di Maro, Ercolini, & Coppola, 2007). As expected, in the control spontaneously fermented wine (Control SA), a great percentage and high diversity of non-*Saccharomyces* was found at the beginning of the fermentation: *H. uvarum* (21 %), *Starm. bacillaris* 60 %), *A. pullulans* (16 %), *R. glutinis* (2%) and *I. terricola* (1 %). *H. uvarum* increased its population during the initial stages of the alcoholic fermentations, reaching 90 % of the total yeast population at days 2 and 5. In contrast, *Starm. bacillaris* population decreased rapidly to 10 % and remained at this level for seven days.

Indigenous *S. cerevisiae* populations were identified for the first time on the fifth day and dominated until the end of the fermentation.

Treatments SB and SC affected the proportion of yeast species at the beginning of the fermentation with respect to the control (SA), since *R. glutinis* and *I. terricola* decreased sharply to undetectable levels by plating after treatments. SB and SC samples showed similar yeast heterogeneity: *H. uvarum* increased to 90 - 95 % (day 2) and decreased by the end of fermentation. In contrast, *Starm. bacillaris* population increased throughout the fermentation with a peak of total yeast counts on day 5 and decreased thereafter. *S. cerevisiae* was detected from day 5 representing 1 % of the yeast community, and completely dominated at the end of the monitored period (80 – 100%). From these results it can be hypothesized that EW treatments alter the trend of the CFU counts, favouring the growth of *Starm. bacillaris* against *H. uvarum* in spontaneous fermentations.

In the control inoculated fermentation (Control IA), *S. cerevisiae* was the major species during the first two days, with the presence of *H. uvarum* and *Starm. bacillaris* as secondary species (40 – 45 % of the overall population). Afterwards, *S. cerevisiae* dominated throughout the rest of fermentation, since it was the only species detected. In treatments IB and IC, despite the *S. cerevisiae* inoculation, *Starm. bacillaris* population controlled the overall yeast communities two days after inoculation. *S. cerevisiae* dominated the middle – end stages of fermentation. From an oenological point of view, EW treatments in combination with yeast inoculation confirmed the results previously obtained using SO_2, by decreasing the proportion of non-*Saccharomyces* (especially apiculate yeasts) vs *S. cerevisiae* in a shorter time (Andorra, Landi, Mas, Guillamón, & Esteve-Zarzoso, 2008; Bokulich, Swadener, Sakamoto, Mills, & Bisson, 2015). Both yeast inoculum and EW treatments kept non-*Saccharomyces* populations at low levels.

3.3. PCR and RT-PCR–DGGE analysis

Both DNA and RNA were directly extracted from grapes and from the different stages of must fermentation in order to obtain a detailed picture of the differences in yeast communities between untreated and treated samples (data not shown). The profiles generated by the grapes and must were similar and mirror the CFU data, since higher yeast diversity was observed in the untreated grape samples. In both DNA and RNA profiles four bands were observed, belonging to *A. pullulans*, *Starm. bacillaris, R. glutinis* and *H. uvarum*. A band corresponding to *I. terricola* was not detected in the DGGE gels, most

likely due to the low number of CFU present ($< 10^4$ CFU/mL). As the fermentation progressed, a band belonging to *S. cerevisiae* became visible at day 5, once the corresponding *S. cerevisiae* achieved levels greater than $10^4 - 10^5$ CFU/mL. After this point, DGGE bands at both DNA and RNA profiles, belonging to *S. cerevisiae, Starm. bacillaris* and *H. uvarum*, were visible during the whole fermentation, even if the two last species were not detected (no colonies on WLN medium) by viable count at the end of the fermentation in agreement with previous findings (Cocolin & Mills, 2003). A band corresponding to *A. pullulans* disappeared from the PCR-DGGE and RT-PCR-DGGE profiles, when the relative population on WLN medium dropped below 10^4 CFU/mL. The present results underline the significance of applying multiphasic approach techniques rather than a single technique to get a better view of yeast communities that occur on wine grapes during fermentations (Alessandria et al., 2015; Cocolin & Mills, 2003).

3.4. Evaluation of dominance of inoculated S. cerevisiae Lalvin EC1118®

Two hundred and twenty-five (225) putative colonies of *S. cerevisiae* isolated from must samples in different fermentation stages were subjected to interdelta-PCR molecular fingerprinting analysis, in order to reveal the dominance of the inoculated *S. cerevisiae* starter over the indigenous *S. cerevisiae* populations. The resulting cluster analysis using a similarity coefficient of 90 % showed a dominance of the starter biotype for all the profiles analysed (data not shown). This indicates the general dominance of the starter strain in the inoculated fermentations and excludes the contribution of indigenous *S. cerevisiae* strains on the chemical and aromatic composition of the wines produced.

3.5. Chemical composition of Barbera wines

The chemical composition of the wines produced from each treatment and fermentation procedure (spontaneous and inoculated) applied in this study is reported in Table 1. Complete fermentation of sugars was observed after 7 and 14 days for the inoculated and spontaneously fermented musts, respectively (data not shown), independently of the treatment applied. Concerning glycerol production, no statistical differences were noticed and all the wines reached values ranging from 10.2 to 11.4 g/L. On the contrary, ethanol production was greatly affected by the treatments. Treatments SB and SC showed a significant reduction in ethanol up 1.0 % (v/v) compared to the other treatments. This could be explained by the relatively high populations of *Starm. bacillaris*

in these samples (SB and SC) at the middle stages of fermentation due to the capacity of this species to utilize sugars to produce biomass and by-products, rather than ethanol (Englezos et al., 2015). The most noticeable impact of the EW treatments was on acetic acid production. The musts from grapes treated with EW (40 and 400 mg/L free chlorine),eitherspontaneously fermented or inoculated with the commercial strain Lalvin EC1118®, produced wines with significantly lower contents of acetic acid compared to the control (SA). The most obvious explanation for the acetic acid reduction is the effect of EW treatments and yeast inoculum towards apiculate yeasts. They are considered high producers of this metabolite (0.6 - 3.4 g/L) and therefore are less attractive for wine production (Comi, Romano, Cocolin, & Fiore, 2001;Romano, Fiore, Paraggio, Caruso, & Capece, 2003).

3.6. *Effect* of EW treatments *on wine aroma*

In the Barbera wines, forty-five (45) volatile compounds were identified and listed into 5 chemical categories, including 18esters, 12 alcohols, 7 terpenes and C13-norisoprenoids, 6 acetates and 2 fatty acids. A PCA was performed on these data, in order to uncover possible correlations between chemical compounds and to identify singular compounds or aroma families able to distinguish the treatments applied in this study (Fig. 5, panel A and B). The resulting PCA explained about 70 % of the total variance for the first two principal components (Fig. 5, panel B). The first component (PC1) was correlated negatively with terpenes, C13-norisoprenoids and acetates, and positively with alcohols, acids and esters. The second principal component (PC2) was positively correlated with terpenes and esters, and negatively with alcohols and β-damascenone.

Wines produced by yeast inoculation were clearly grouped on the right part of the plot (Fig. 5, panel A) and could be easily differentiated from the other wines, mainly due to the relatively high presence of alcohols, fatty acids and esters. Spontaneously fermented wines were grouped on the left part of the PCA plot, mainly due to the relative abundance in unpleasant odour compounds like acetates, isobutanol and ethyl acetate. Isobutanol and ethyl acetate (harsh, nail polish, fusel) were significantly higher in the spontaneously fermented wines, probably due to the higher population levels of *H. uvarum*. This result is in agreement with a previous study, which identified these unpleasant volatile compounds as aromatic markers of this non-*Saccharomyces* yeast (Romano et al., 2003). On the other hand, treatments SB and SC produced wines with high amounts of 2-phenyl-ethyl-acetate

(rose like fragrance). It is worth noticing, that EW treatments increased the concentration of 2-phenyl ethanol (rose flavour), compared to the wines produced from untreated grapes.

Isoamyl alcohol (3-methyl-1-butanol, cheese marzipan) was greater produced in the inoculated than in spontaneous fermented wines. The general increase of this volatile compound, in the fermentations in which *S. cerevisiae* was inoculated immediately after crushing, is similar to the results found by Andorrà et al. (2010) and Suzzi et al. (2012). Interestingly, the wines produced by the control spontaneous fermentation were separated from the other wines, due to the higher concentration of terpenes, (linalool in particular), ethyl nonanoate and 2,3-butanediol (1,2). The increase of these pleasant compounds appears to be related to the potential ability of the non-*Saccharomyces* to produce and secrete extracellular enzymes (such as esterases, β −glucosidases etc.), capable of liberating aroma substances in the wine (Strauss, Jolly, Lambrechts, & van Resemburg,2001). Sensory analysis immediately after the end of fermentation did not reveal wine faults (data not shown).

4. Conclusions

To our knowledge, this is the first time that EW was used on postharvest wine grapes. In this study, EW treatment at two different concentration levels was used to assess its effectiveness on altering the yeast communities present on grape surface and during the fermentation period with and without inoculation of the commercial *S. cerevisiae* strain EC1118[®]. Both low and high dose EW treatments (40 or 400 mg/L free chlorine)in combination with *S. cerevisiae* inoculation led to a faster increase of the portion of *Saccharomyces* vs. apiculate yeasts compared to the untreated trials. The chemical data also suggested that the EW treatment, independently from the use of yeast starter, is associated with a reduction of acetic acid. Since all of the data presented here were obtained immediately at the end of the fermentation, future works will focus on the evolution of the fermentative aromas during aging.

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1   **Table 1** Chemical composition of the Barbera must and wines

Treatment	Residual sugars (g/L)	Malic acid (g/L)	Lactic acid (g/L)	Acetic acid (g/L)	Glycerol (g/L)	Ethanol (% v/v)
Must	216.6 ± 10.1	4.20 ± 0.53	nd	nd	nd	nd
Control SA	1.0 ± 0.5b	3.48 ± 0.14a	0.70 ± 0.11b	0.52 ± 0.04a	10.2 ± 0.5	12.7 ± 0.2b
Treatment SB	0.4 ± 0.0a	3.98 ± 0.06b	0.30 ± 0.05a	0.33 ± 0.16b	11.4 ± 1.9	12.3 ± 0.3a
Treatment SC	0.4 ± 0.0a	3.37 ± 0.43a	0.70 ± 0.35b	0.21 ± 0.00b	10.8 ± 1.0	12.0 ± 0.0a
Control IA	0.7 ± 0.2ab	3.90 ± 0.04b	0.30 ± 0.04a	0.28 ± 0.07b	10.8 ± 0.0	12.9 ± 0.1bc
Treatment IB	0.7 ± 0.1ab	3.82 ± 0.07b	0.35 ± 0.06a	0.24 ± 0.01b	10.7 ± 0.1	13.1 ± 0.1c
Treatment IC	0.8 ± 0.0b	4.00 ± 0.13b	0.30 ± 0.10a	0.21 ± 0.02b	10.5 ± 0.2	13.1 ± 0.0c
Sign.[a]	**	***	*	***	NS	***

2   All data are expressed as average value ± standard deviation (n = 3). Different Latin letters within the same columnindicate significant

3   differences among the different treatments applied, according to the Duncan test ($p < 0.05$).nd = not detected.

4   [a] Sig: *, **, *** and NS indicate significance at$p < 0.05$,$p < 0.01$, $p < 0.001$ and not significant respectively.

5   and NS indicate significance at, $p < 0.01$, $p < 0.001$ and not significant respectively
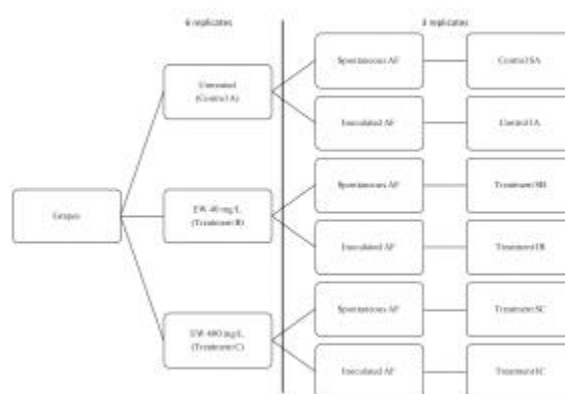
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12 **Fig. 1** Experimental procedure and sample codes of spontaneous and inoculated fermentation

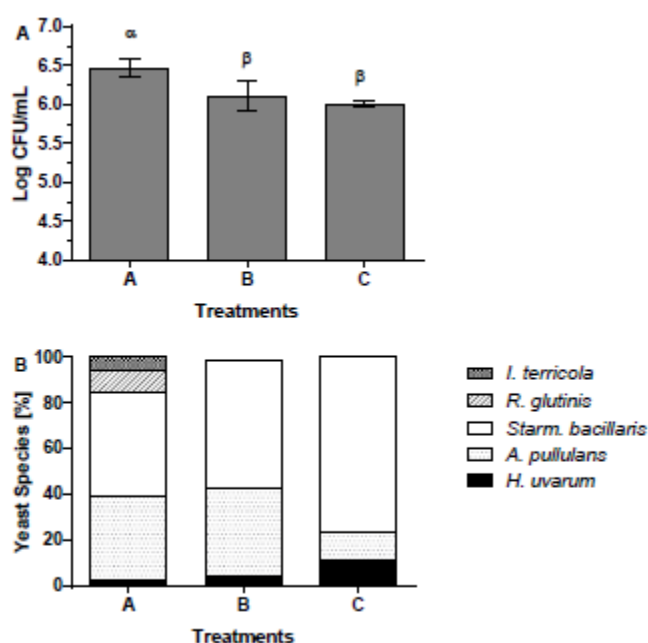13 wines produced using treated and untreated grapes. AF = alcoholic fermentation.

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17 **Fig. 2** Total yeast count (A) and yeast species heterogeneity (B) registered on the grapes

18 before (A) and after EW treatments (B and C). Data are the mean (± SD) of six biological

19 replicates from four clusters of grape berries for each treatment applied. The different Greek

20 letters in each column indicated significant differences according to ANOVA and Duncan

21 test (P < 0.001).

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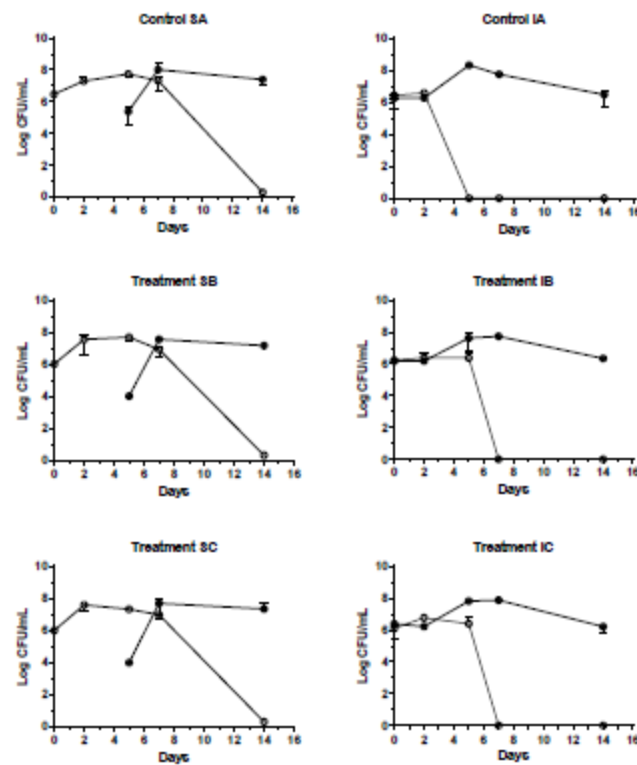
Fig.3 Colony forming unit of suspected *S. cerevisiae*[●] and non-*Saccharomyces* [○] during the alcoholic fermentation of spontaneous (Control SA, treatment SB and SC) and inoculated fermented musts (Control IA, treatment IB and IC). Identification of suspected *S. cerevisiae* colonies was determined by RFLP and partial 26 rRNA gene sequence analysis. Reported values represent the average values (± SD) of three independent experiments.
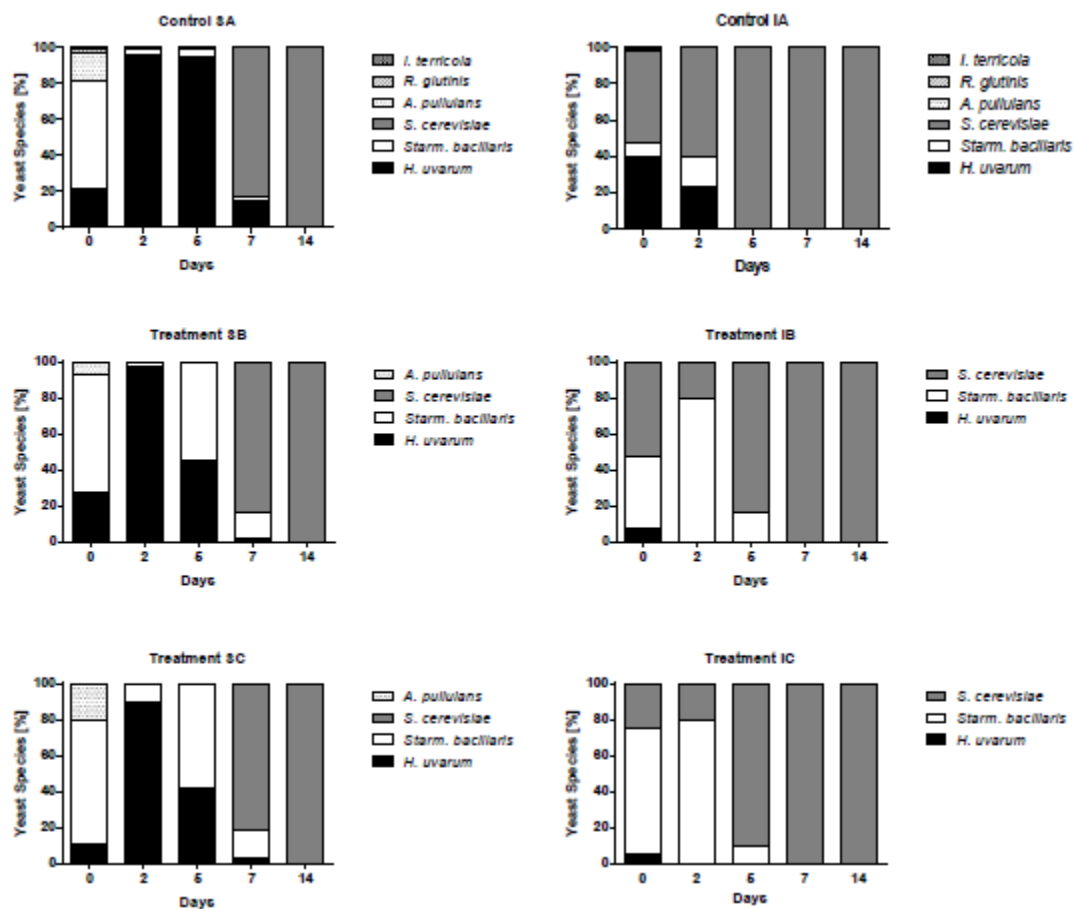
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33 **Fig.4** Yeast species heterogeneity of spontaneous (Control SA, treatment SB and SC) and

34 inoculated (Control IA, treatment IB and IC) alcoholic fermentations.
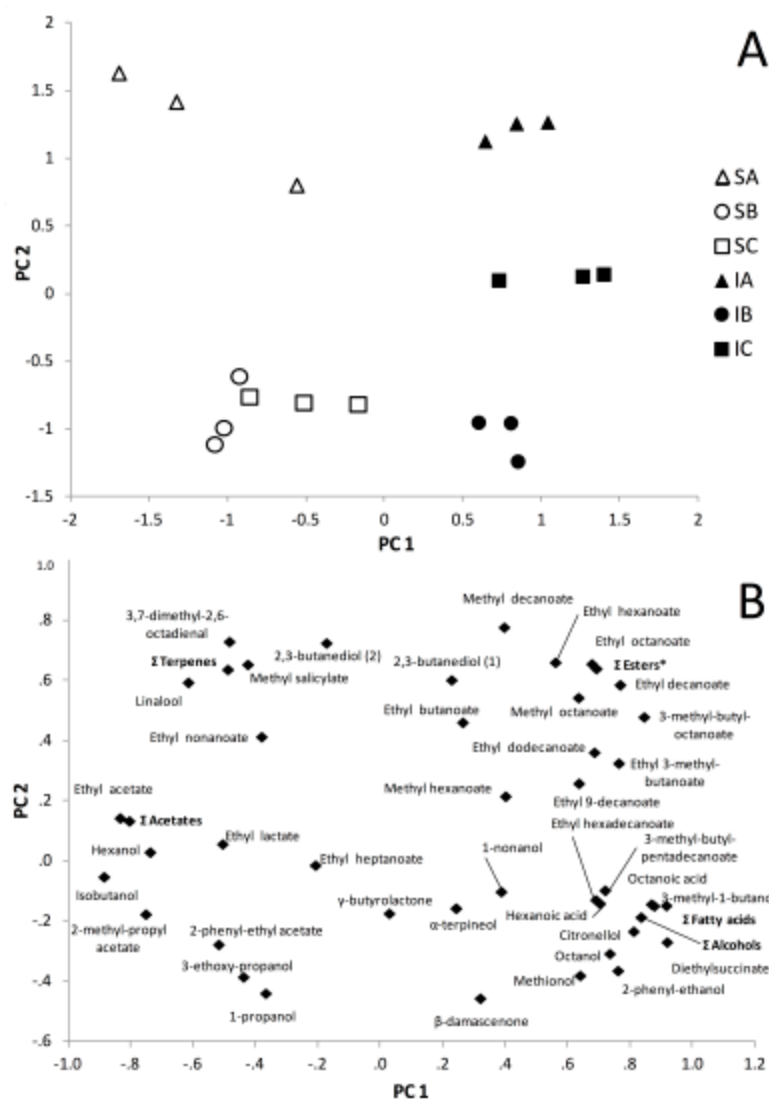
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Fig. 5 Score plot (A) and loading plot (B) of the first and second principal components (PC)
after PCA of the volatile compounds identified in the Barbera wines. Control SA (Δ),
Treatment SB (○), Treatment SC (□), Control IA (▲), Treatment IB (●) and Treatment IC
(■). * Esters: esters without acetates.