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CICLO: XXX

OZONE AND ELECTROLYZED WATER TREATMENTS OF POST HARVESTED WINE GRAPES

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Abstract

Oggi il mondo alimentare, come quello enologico, dà sempre più importanza all'eco-compatibilità della filiera produttiva, con l'obiettivo di produrre alimenti e vini sempre più salubri in assenza di molecole dannose per l'uomo, garantendo comunque un alto livello di qualità. In questo contesto, l'uso dell'ozono (gassoso o in acqua) e dell'acqua elettrolizzata si sta diffondendo sempre di più nelle filiere alimentari. Infatti, questi due agenti hanno un'elevata attività antimicrobica ad ampio spettro d'azione che non lascia residui. Per questi motivi, negli ultimi anni, anche la ricerca enologica ha studiato il modo di sfruttare questi due sanitizzanti durante il processo di vinificazione. Oltre alla loro applicazione come sanitizzanti di attrezzature, vasche e macchine enologiche, un modo innovativo di sfruttare queste tecnologie è quello di usarle direttamente sulle uve in post-raccolta. Naturalmente la concentrazione dei principi attivi, il formulato utilizzato, le condizioni ambientali (temperatura, umidità), lo stato sanitario dell'uva, nonché i tempi di contatto possono avere un influenza sul risultato finale e per questo devono essere ottimizzati al fine di rendere il più efficace possibile il trattamento.

Parole chiave: ozono; acqua elettrolizzata; sanitificazione delle uve; processi eco-compatibili

Abstract

Today, the food world, like the oenological one, gives many importance to the eco-compatibility of the production chain, with the aim of producing safe foods and wines in the absence of harmful molecules, by ensuring a high level of quality. In this context, the use of ozone (gaseous or in aqueous form) and electrolyzed water is spreading more and more in the food chains. In fact, these two agents have an high anti-microbial activity with a high spectrum of activity without leaving residues. For these reasons, in recent years, oenological research has studied how to take advantage of these two sanitizers during the winemaking process. In addition to their application as sanitizers for equipment, tanks and oenological machines, an innovative way of exploiting these technologies is to use them directly on post-harvest grapes. Surely, the concentration of the active ingredients, the formulation used, the environmental conditions (temperature, humidity), the phytosanitary conditions of the grapes, as well as the contact times may have an influence on the final result of the treatments, for this reason they have to be carefully studied in order to maximize the antimicrobial efficiency.

Keywords: ozone; electrolyzed water; sanitation of the grapes; eco-compatible processes.

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Introduction

One of the important innovations in enology dates back to the end of the 17th century, when sulfur dioxide (SO_2) was introduced in the winemaking process (Ribéreau Gayon et al., 2006). Nowadays, its particular activities make it an oenological additive (European Union Regulation: 1148/2012), almost indispensable for obtaining high quality wines with a long shelf life. An excellent antiseptic action, a fast antioxidant property, rapid ability to inactivate oxidase enzymes and a fast ability to bind with various compounds, including acetaldehyde, are the major properties of SO_2, which make this compound difficult to replace during the winemaking (Ribéreau Gayon et al., 2006). SO_2 is a toxic additive for man, and for this reason, it is under accusation and observation by public opinion (Vally et al., 2003). The SO_2 has been transposed as a preservative (E220) by the World Health Organization, which does include a ADI (Acceptable Daily Intake) of 0.7 mg/kg body weight and a LD50 (50% Lethal Dose) of 1.5 g/kg body weight. In Directive 89/2003/EC, SO_2 is defined as an allergen, while, Directive 2007/68/EC establishes the obligation to indicate on the label the presence of SO_2 and/or of sulphites, in concentrations above 10 mg/kg or 10 mg/L expressed as SO_2. In addition to legislative restrictions, the effects of SO_2 present in wine on human health are being studied (Vally et al., 2001; Vally et al., 2009; Santos et al., 2012;). Although not all studies have shown a real negative effect of the SO_2 on human health, surely, these studies have a public awareness-raising effect, which are looking for more and more “sulphites free” wines or in any case to reduced levels of added SO_2.

It is important to note, that during grape crushing, it occurs one of the major additions of SO_2 during all winemaking process. In addition, it is not possible to use optimally the SO_2 properties, because it quickly binds to the many compounds present in the must (sugars, acetaldehyde, etc.) (Cravero et al., 2016). So, before the fermentation starts there is always a great addition of sulfur in wine in respect to what is needed and this is the critical and ideal point on which to work to reduce or eliminate the use of sulfur in the winemaking process.

The grape microbiota

One of the critical points of winemaking is the alcoholic fermentation, where before its beginning, it is good practice to add SO_2 for limiting the presence of spoilage yeasts present on the grapes surface (Boulton et al., 1996). Grape berry microbiota is a complex ecosystem, formed by yeasts, bacteria and moulds, influenced by several factors, including geographical area, climatic conditions, diseases, agronomical and viticultural practices, phytosanitary conditions of grape berries (Barata, et al., 2012). Non-*Saccharomyces* are the main yeast species colonizing the grape berries, the main one are: *Hanseniaspora* spp., *Torulaspora delbruekii*, *Issatchenkian terricola*, *Cryptococcus carnescens Aureobasidium pullulans, Metschnikowian spp.* (Alessandria et al.,2015; Prakitchaiwattana et al., 2004). These yeasts are the first to colonize the must, indeed if not controlled immediately they may affect the quality of the wine (Prakitchaiwattana et al., 2004). In the last years, some of these yeasts, like *Strarmerella bacillaris, T. delbruekii, Metschnikowian* spp., and *Lachancea thermotolerans* are used in combination with *S. cerevisiae* to improve and regulate some aspects of the wines as ethanol, acetic acid, total acidity, aromatic complexity, etc. (Contreras et al., 2015; Gobbi et

al., 2013; Englezos et al., 2015, 2016). On the other hand, the apiculate yeasts and *Brettanomyces bruxellensis* are the main dangerous yeasts present on the grapes surface (Campolongo et. al., 2010; Jolly et al., 2013). In fact, apiculate yeasts are known as major producers of acetic acid, especially in the early stages of alcoholic fermentation (Romano et al., 1993; Comi et al., 2001). While, thanks its metabolic abilities, *B. bruxellensis* is considered the most spoilage wine yeast, indeed, *B. bruxellensis* is capable to produce high levels of volatile phenols and in some cases, even of acetic acid and derivatives of amino acids such as 2-acetyl-1,4,5,6-tetrahydropyridine responsible of the ''mousy'' off aroma (Cocolin et al., 2004; Suarez et al., 2007). The ability to produce volatile phenols is guaranteed by the subsequent use of two enzymes capable of transforming the hydroxycinnamic acids into 4-vinylphenols and 4-ethylphenols (Benito et al., 2009). The high level of these phenols produce the so-called off flavor of the wine, disagreeable aromas often described as: pharmaceutical, horse-like, barnyard-like, horse blanket, wet dog, tobacco, varnish, leathery and perhaps mousey descriptors (Campolongo et al., 2014).

Besides being a very harmful yeast, *B. bruxellensis* has good resistance to high levels of SO_2, indeed, in the last years several study searched alternative methods to control this yeast on the grapes and on the must like ultraviolet radiation (UV-C) and low electric current (LEC). The UV-C acts rearrangement of the microorganism's nucleic acid inhibiting the reproduce, while, the LEC influence the cell membrane inducing the break (Santos et al., 2012). Several works have showed the efficiency of these two techniques on the control of *B. bruxellenisis* in enology, although further investigations are necessary to ensure satisfactory results (Lustarto et al., 2010; Fredericks et al.,2011).

Today, to improve the knowledge of the grape berry microbiota is used a multiphasic approach employing together at the culture-dependent (traditional plate counts) also the -independent techniques, based on DNA and RNA amplification (PCR-denaturing gradient gel electrophoresis [DGGE] and reverse transcription PCR [RT-PCR]-DGGE). This technique is based on the amplification and observation of key molecular loci, like ribosomal RNAs (rRNAs), so as to compare the samples with known individuals (Head et al.,1998). Furthermore, it enables to directly sample DNA and/or RNA from the environment without having to pass for the culture. Therefore, it permits to avoid the risk of error due to the enrichment medium, that can disadvantage some species of microorganisms and select others, thus distorting the result. Farther, the (PCR)-DGGE allows to identify non-cultivable microorganisms giving the real composition of the studied ecology (Cocolin et al., 2000). For these reasons in my works, I have always used a multiphasic approach, to improve the truth of the results.

Electrolyzed water and ozone: alternative sanitizing

The current challenge in enology is to decrease or to eliminate the SO_2 in wines, without diminishing its quality and its shelf life. To this end, it is important to find alternative ways to the addition of SO_2 during the fermentation process, in order to reduce the possible damage caused by the wild yeast present on the grape surface. Electrolyzed water and ozone are potential eco-friendly sanitizers to be used directly on post-harvest grapes before fermentation.

<u>Electrolyzed water</u>

Electrolyzed water (EW) is an important sanitizer used in the food industry thanks to its positives quality. In fact, EW is commonly used as food-processing surface sanitizer, and also directly on vegetables and fruit surface for reducing the microbial contamination (Mahmoun, 2007; Jermann et al., 2015). The production of EW is relatively simple, dissolving KCl salt in tap water in an electrolyzed cell, where, thanks to a selective membrane is formed EW (Bucket al., 2002). This feature provides important advantages: production of EW continuously and directly at the workplace, as well as, the water used for the treatment could be recycled and reused during the harvest season by adding new salt, saving production costs. The main feature that has favored a rapid use of EW in the food industry is its broad spectrum of action against various microorganisms thanks to three combined actions: hydrogen ions, oxidation-reduction potential and free chlorine (Koseki et al., 2007). Several studies have demonstrated its ability to eco-sanitize fruit and vegetable surfaces on post-harvest and showing a high ability of the treatment to reduce a microbial load on different processed foods (Hricova et al., 2008; Jemni et al., 2014). On the other hand, few studies have been carried out in order to investigate its possible application in winemaking, except for the decay of *Botrytis cinerea* and the treatment effectiveness during the storage of post-harvest table grapes (Guentzel et al., 2010; Kim et al., 2003).

Ozone

In recent years, the use of ozone in the food industry has gained attention as hygienic agent thanks to its innumerable proprieties without any kind of contraindication (Guzel-Seydim et al., 2004). Ozone is a gas produced

by an ozonizer that take the molecules of oxygen present on the air and subjected them to high-voltage electric discharge (Khadre et al., 2001). One of the benefits of ozone is that it can be directly used in gaseous form or dissolved in tap water and then used in liquid form, this property allows also to use the ozone continuously and directly on the workplace (Jermann et al., 2015). Again, during the harvest period, aqueous ozone after its use can be retrieved, reactivated and reused, without wasting tap water, saving work costs. The ozone is a very efficient antiseptic because it is a powerful oxidant, able to attack several cellular constituents of the microorganisms present on vegetable and fruit surfaces (Khadre et al., 2001). Just because it's a powerful antioxidant, ozone is very efficient against numerous microorganisms including molds, bacteria and yeasts (Perry et al., 2011). Another benefit of ozone is its low stability, in fact it degrades quickly producing only oxygen, allowing ozone to leave no residues on the treated surface (Jermann et al., 2015). Thanks to these properties, ozone is one of the most commonly sanitizers used directly on the vegetable and fruit surfaces (Horvitz et al., 2014). Indeed, different studies used ozone as an eco-friendly approach to sanitize fruits and vegetables (Oztekin et al., 2006; Boonkorn et al., 2012; Sengun, 2014). The use ozone on grapes, especially on wine grapes, are few. To mention the use of aqueous ozone on table grapes as possible sanitizer against *Botrytis cinerea* contamination during the storage, to ensure the quality of the berries (Smilanick et al., 2002), and a study on post-harvest wine grapes using gaseous ozone, highlighting an increase of the skin hardness with a consequently enhanced extraction of phenolic compounds (Laureano et al., 2016).

Considering the interesting properties of the EW and ozone, future studies are needed in order to better understand if they can be used as eco-friendly sanitizing directly on post-harvest wine grapes in order to eliminate or to

reduce the use of sulfur dioxide in the crushing/fermentation step of winemaking.

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Aims of the PhD thesis

The goal of this PhD project was to investigate the potential use of electrolyzed water and ozone (in gaseous and aqueous form) on post-harvest wine grapes. For this purpose, the objectives of the study were:

- to understand the impact of ozone and electrolyzed water treatments on wild yeasts present on the berry surface;
- to study the evolution of yeasts during spontaneous and inoculated fermentations using treated and untreated grapes;
- to monitor the chemical parameters of the wines produced by treated and untreated grapes treated with ozone and electrolyzed water, after spontaneous and inoculated fermentations;
- to evaluate the efficiency of treatments against *Brettanomyces bruxellensis* inoculated on the wine grapes, to study their evolution during the fermentation and measure chemical parameters connected to their spoilage activity in the wines produced.

List of publications

The following publications constitute the basis for this thesis. Each paper is published or submitted in a ISI-indexed journal.

I. Cravero, F., Englezos, V., Torchio, F., Giacosa, G., Río Segade, S., Gerbi, V., Rantsiou, K., Rolle, L., Cocolin, L. (2016). Post-harvest control of wine-grape mycobiota using electrolyzed water. *Innovative Food Science and Emerging Technology,* 35, 21-28.

II. Cravero, F., Englezos, V., Rantsiou, K., Torchio, F., Giacosa, S., Río Segade, S., Gerbi, V., Rolle, L., Cocolin, L. (2016). Ozone treatments of post harvested wine grapes: Impact on fermentative yeasts and wine chemical properties. *Food Research International*, 87, 134-141.

III. Cravero, F., Englezos, V., Rantsiou, K., Torchio, F., Giacosa, S., Río Segade, S., Gerbi, V., Rolle, L., Cocolin, L. (2017). Control of *Brettanomyces bruxellensis* on wine grapes by post-harvest treatments with electrolyzed water, ozonated water and gaseous ozone. *Innovative Food Science and Emerging Technology, submitted in revised form.*

Post-harvest control of wine-grape mycobiota using electrolyzed water

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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 SO2.

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

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 et al., 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 et al., 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 et al., 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 et al., 2012; Vally et al., 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 et al., 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 et al., 2008) and its simple generation by electrolysis from potable water and salt (KCl) solution only (Buck et al., 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 et al., 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 et al., 2010; Kim et al., 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.

Materials and methods

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.

Preparation of EW solutions and grapes treatment

Concentrated EW solution was generated by using EVA SYSTEM® 100 equipment (Industrie De Nora S.p.A, Milan, Italy), following the manufacturer's instructions. An aqueous solution of 40 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 (± 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.

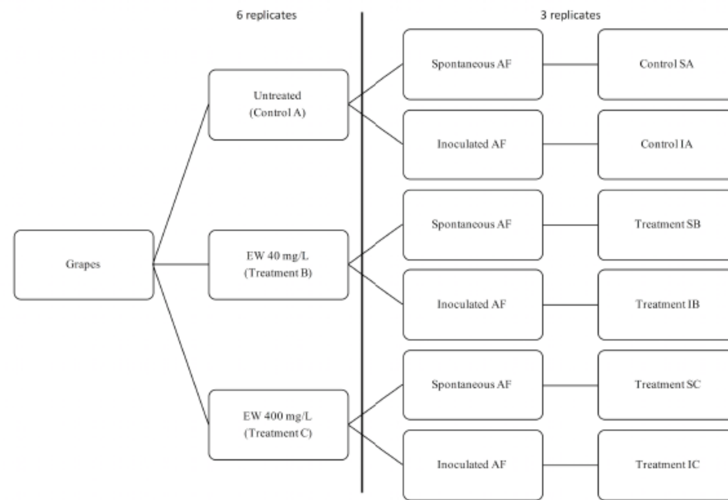

Fig. 1 Experimental procedure and sample codes of spontaneous and inoculated fermentation wines produced using treated and untreated grapes. AF = alcoholic fermentation.

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.

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 x 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.

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 et al., 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.

Molecular analysis

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 et al., 1997).

Genotypic characterization of *S. cerevisiae* isolates

Molecular identification and characterization of 225 putative colonies of *Saccharomyces* spp. (5 from 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 et al., (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 et al., 1992).

Direct extraction of nucleic acid from grapes and must samples

Total DNA and RNA were extracted from the pelleted cells by using the MasterPure™ 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). Both DNA 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.

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).

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).

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 (Rantsiou et 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 et al., (2015).

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.

Results and Discussion

Yeast colonization 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 et al., 1993; Milanović et al., 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 morphotypes, 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 et al., 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).

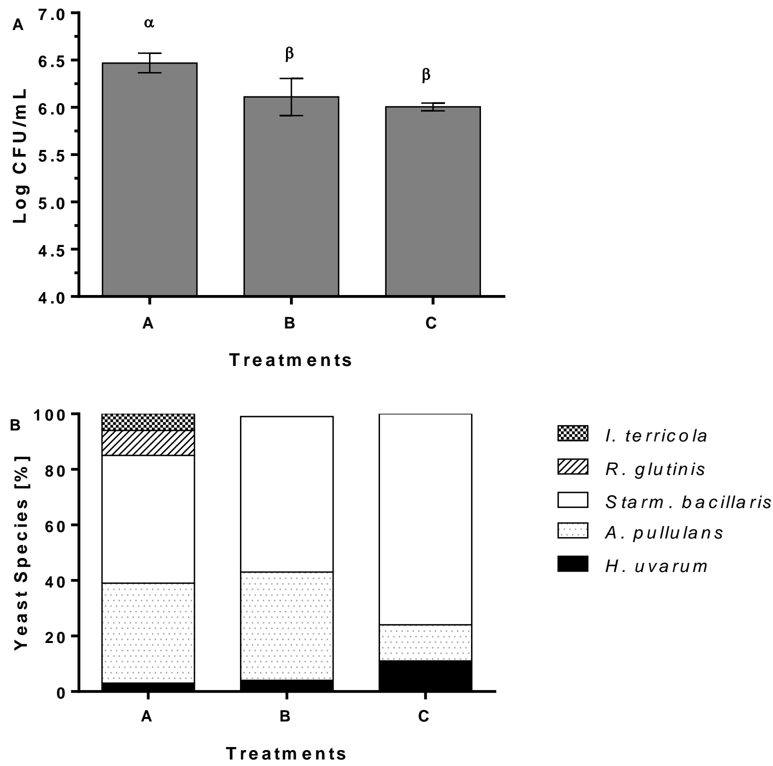

Fig. 2 Total yeast count (A) and yeast species heterogeneity (B) registered on the grapes before (A) and after EW treatments (B and C). Data are the mean (± SD) of six biological replicates from four clusters of grape berries for each treatment applied. The different Greek letters in each column indicated significant differences according to ANOVA and Duncan test (P < 0.001).

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 et al., 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 et al., 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 et al., 2012; Nissen et al., 2003).

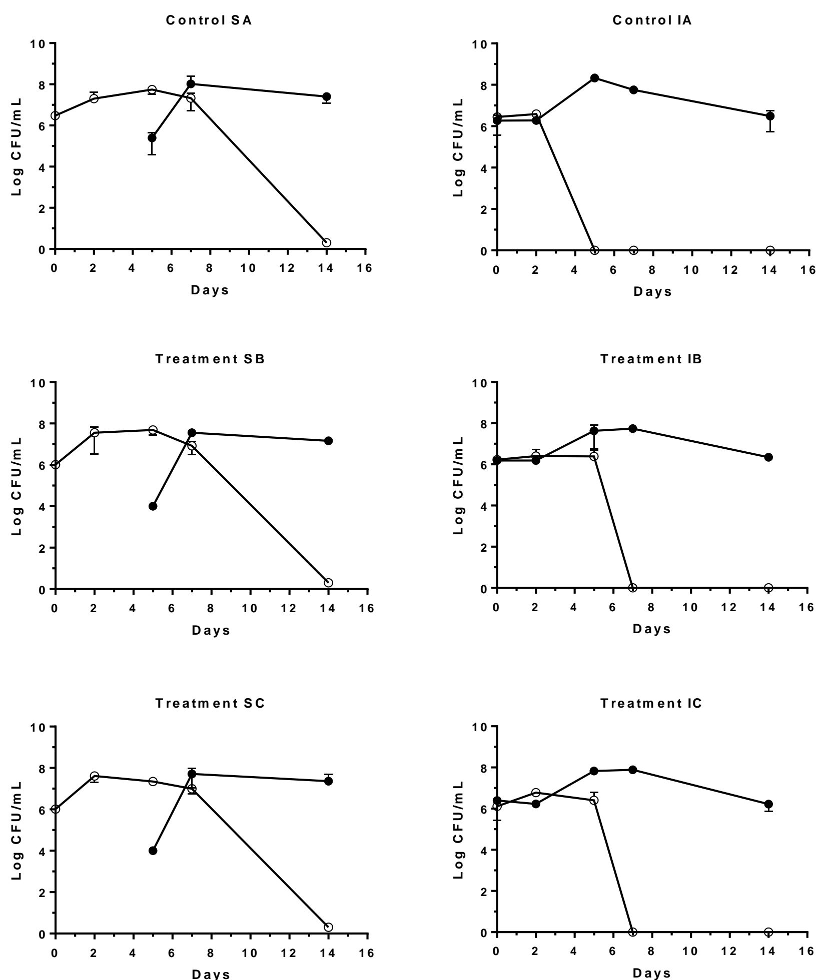

Fig. 3 Colony forming unit analysis of *S. cerevisiae* [●] and non-*Saccharomyces* [○] during the alcoholic fermentation of spontaneous (Control SA, treatment SB and SC) and

inoculated fermented musts (Control IA, treatment IB and IC). *S. cerevisiae* counts were determined by RFLP and partial 26 rRNA gene sequence analysis, while non-*Saccharomyces* on Lysine medium. Reported values represent the average values (± SD) of three independent experiments.

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 et al., 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 et al., 2008; Bokulich et al., 2015). Both yeast inoculum and EW treatments kept non-*Saccharomyces* populations at low levels.

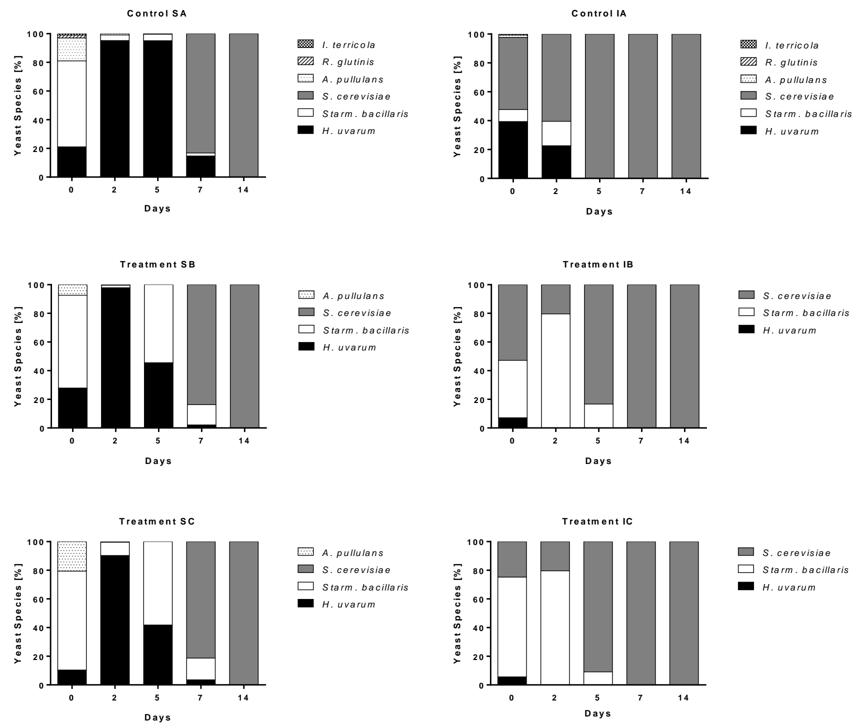

Fig.4 Yeast species heterogeneity of spontaneous (Control SA, treatment SB and SC) and inoculated (Control IA, treatment IB and IC) alcoholic fermentations.

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 et al., 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 et al., 2003).

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.

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), either spontaneously 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 et al.,2001; Romano et al., 2003).

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	***

All data are expressed as average value ± standard deviation (n = 3). Different Latin letters within the same column indicate significant differences among the different treatments applied, according to the Duncan test ($p< 0.05$). nd = not detected.

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

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 18 esters, 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 et al., 2001). Sensory analysis immediately after the end of fermentation did not reveal wine faults (data not shown).

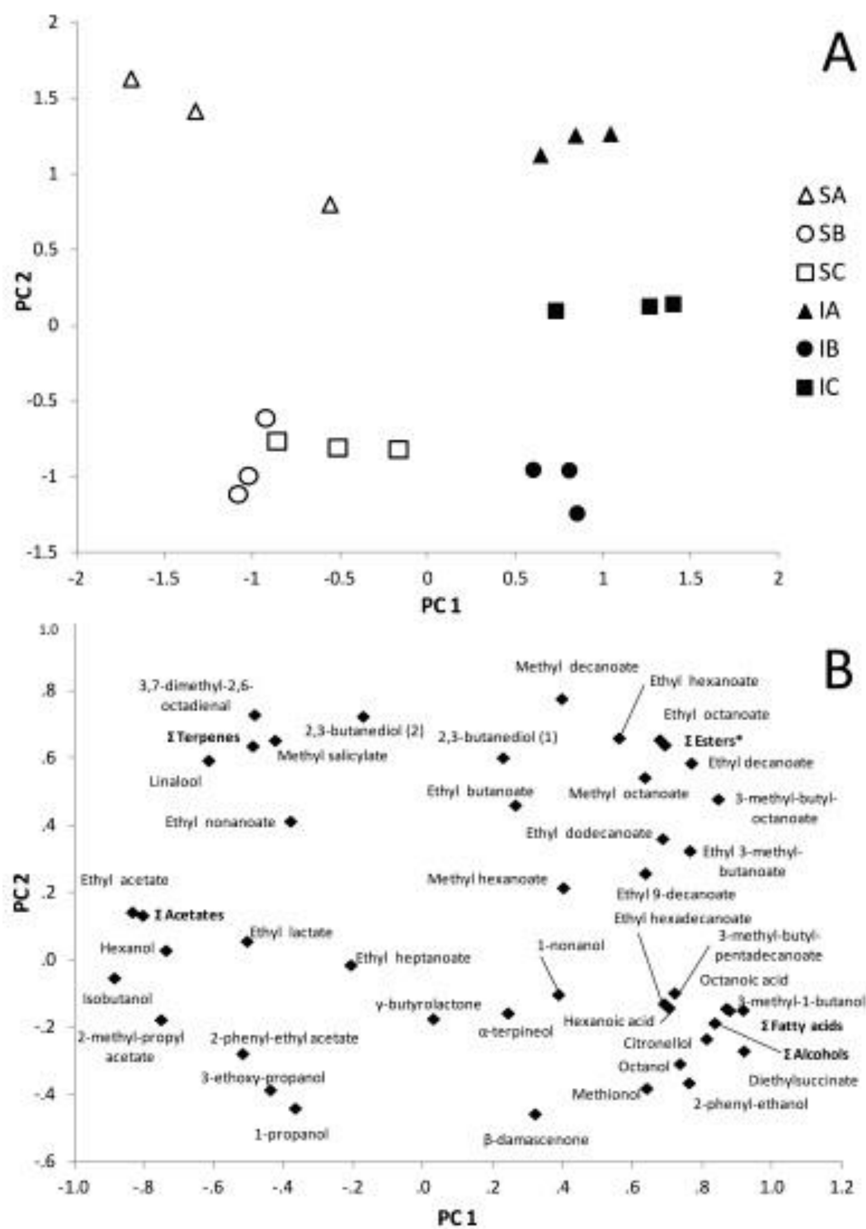

Fig. 5 Score plot (A) and loading plot (B) of the first and second principal components (PC) after PCA of the volatile compounds identified in the Barbera wines. Control SA

(Δ), Treatment SB (○), Treatment SC (□), Control IA (▲), Treatment IB (●) and Treatment IC (■). * Esters: esters without acetates.

Conclusion

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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Paper II

Ozone treatments of post harvested wine grapes: impact on fermentative yeasts and wine chemical properties

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Abstract

Ozone represents a potent antimicrobial compound that is already proposed as a possible sanitizing agent, especially for surface decontamination of fruits and vegetables. The main objective of this study was to evaluate the effect of ozone, either in aqueous or gaseous form, on wine grape mycobiota and its impact during spontaneous and inoculated fermentations. Gaseous (32±1 μL/L, 12 and 24 h) and aqueous (5±0.25 mg/L, 6 and 12 min) ozone were tested as sanitizing treatments. A multiphasic approach was used employing culture-dependent (traditional plate counts) and -independent techniques, based on DNA and RNA amplification (PCR-denaturing gradient gel electrophoresis [DGGE] and reverse transcription PCR [RT-PCR]-DGGE), respectively. Microbiological analysis data highlighted a reduction of more than 0.5 Log CFU/mL of the total yeasts present on grape berry surfaces after ozone treatments, mainly due to the reduction of apiculate yeasts. The chemical analysis of the wines, produced from the treated grapes, showed higher acetic acid content in the untreated spontaneous fermentations (0.52 g/L) compared to the treated (ranged from 0.16 to 0.38 g/L), while all fermentation-inoculated wines contained higher amounts of pleasant volatile compounds.

Keywords: Aqueous ozone; Gaseous ozone; Innovative sanitizing; Mycobiota; Wine grapes; Wines

Grape berry microbiota is a complex ecosystem, formed by yeasts, bacteria and moulds that can have an impact on wine composition and quality. This microbial ecosystem can be influenced by several factors, including geographical area, climatic conditions, diseases, agronomical and viticultural practices, phytosanitary conditions of grape berries (Barata et al., 2012). The main yeast species present on grape berries are non-*Saccharomyces*, namely *Aureobasidium pullulans*, *Hanseniaspora* spp., *Torulaspora delbruekii*, *Metschnikowian* spp., *Issatchenkian terricola*, *Cryptococcus carnescens* (Alessandria et al., 2015; Prakitchaiwattana et al., 2004). These yeasts spontaneously initiate the alcoholic fermentation in non-inoculated grape musts, modifying positively or negatively the chemical and sensorial properties of the wines.

In the last years, there is an increasing interest at winemaking industry towards the use of non-*Saccharomyces* yeasts like *T. delbruekii*, *Metschnikowia* spp., *Lachancea thermotolerans*, and *Starmerella bacillaris* (synonym *Candida zemplinina*) (Duarte et al., 2012) in combination with *S. cerevisiae* strains, to regulate the production of specific traits (ethanol, acetic acid, total acidity, aromatic complexity, etc.) in wines (Contreras et al., 2015; Gobbi et al., 2013; Englezos et al., 2015, 2016; Rantsiou et al., 2012; Renault et al., 2015). On the other hand, wild wine yeasts, like apiculate yeasts, are considered not suitable for wine production, due to their ability to produce relatively high levels of undesirable compounds, such as acetic acid and ethyl acetate (Jolly et al., 2013). In addition, on the grape surface, several studies have found

Brettanomyces spp. (Campolongo et al., 2010), which in concentrations higher than 10^4 cells/mL could produce high levels of acetic acid and undesirable volatile phenols (Kheir et al., 2013).

In vinification, the management of indigenous microbiota is generally carried out using sulfur dioxide (SO_2) thanks to its antiseptic proprieties. However, this practice is under reconsideration, since the use of high concentrations of SO_2 could have negative effects on human health and could alter the wine aroma quality due to its unpleasant odor (Vally et al., 2001). For this reason, it is fundamental to find alternative methods to achieve a microbial stabilization and to reduce the production of off-flavours (Santos et al., 2012). Several studies have investigated the capacity of ozone as an eco-friendly approach to sanitize fruits and vegetables surface (Boonkorn et al., 2012; Jermann et al., 2015; Oztekin et al., 2006; Sengun, 2014). Indeed, ozone has a broad-spectrum of disinfectant action, due to its high oxidizing potential and its ability to attack several cellular constituents (Khadre et al., 2001). Furthermore, ozone leaves no residues on treated surfaces protecting the environment and human health, and it can be used in aqueous or in gaseous form according to the required needs (Horvitz et al., 2014). Ozone has been used to treat post-harvest grapes. In fact, in different studies, ozone in aqueous form was proposed as possible sanitizer to ensure quality during storage of table grapes by reducing *Botrytis cinerea* contamination (Smilanick et al., 2002). Recent studies demonstrated the positive effect of ozone gas treatments on table and wine grapes because increased skin hardness could enhance the extraction of phenolic compounds (Laureano et al., 2016). However, no studies have been carried out with the aim of evaluating the effect of ozone on mycobiota of wine grapes and the wine produced.

The main objective of this study was to evaluate the effect of the ozone use (either in gaseous or aqueous form) in post-harvest, on the yeast ecology present on the grape surface and during fermentations (spontaneous and inoculated). Yeast populations, before and after treatments, as well as during fermentations, were monitored by culture-dependent (traditional plate counts) and -independent techniques based on DNA and RNA amplification (PCR-denaturing gradient gel electrophoresis [DGGE] and reverse transcription PCR [RT-PCR]-DGGE). Lastly, wines were subjected to chemical and aroma analyses.

Materials and methods

Grapes

Grapes of a local cultivar from the Piedmont wine region (Asti province, Italy), *Vitis vinifera* L. cultivar Barbera, were used in this study. The grapes in good phytosanitary condition (without signs of bird damage or damage/infection by *Botrytis cinerea* or other grape pathogens) were harvested and then subdivided in small clusters of 8-9 berries with the pedicels attached. Afterwards, they were placed in monolayers into perforated boxes (50x30x15 cm), forming batches of 2.0 ± 0.1 kg. The batches were divided as follows: untreated (A), treated with gaseous ozone (GO) and aqueous ozone (AO). Each treatment was performed using the methodology described below. For each test, six independent replicates were performed, which after the treatments were divided in two different fermentations: three inoculated replicates (I) and three spontaneous replicates (S) (Fig. 1).

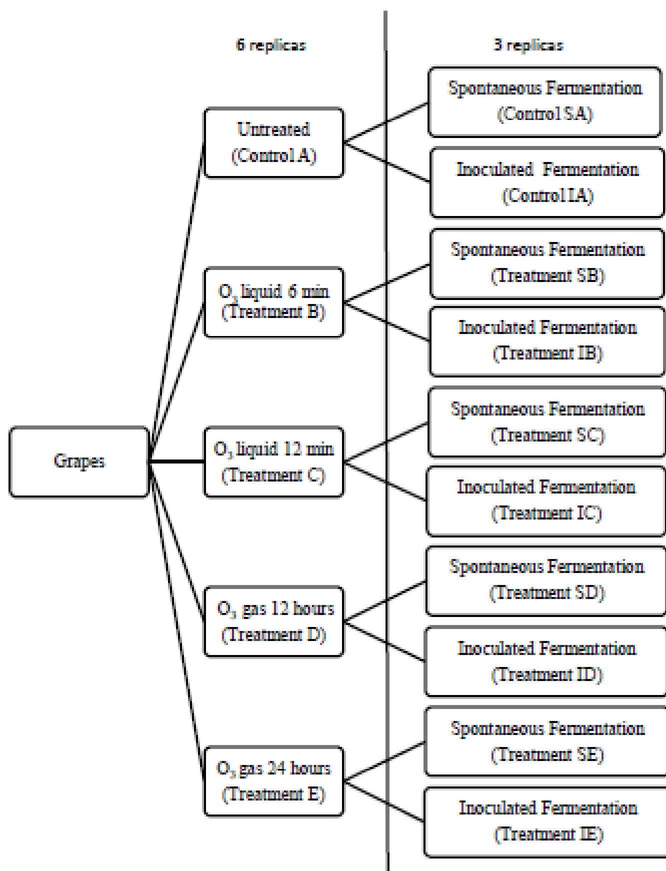


Fig.1. Experimental procedure and sample codes of untreated and treated grapes and spontaneous and inoculated fermentation.

Treatments with gaseous and aqueous ozone

Ozone was produced in gaseous and aqueous form using an ozone generator (Model C32-AG, Industrie De Nora SpA, MI, Italy), equipped with an oxygen concentrator, with a nominal production capacity of 32 g O_3/h (Laureano et al., 2016). The AO treatments were performed by applying water containing 5 ± 0.25 mg/L of ozone for 6 and 12 min, referred as treatments B and C, respectively. For each experiment, the clusters of berries were sprayed with the ozone solution through a nozzle

connected to a peristaltic pump (SP311, Velp Scientifica, Usmate, MB, Italy) to maintain constant flux. The treatment conditions were: flow of 200 mL/min (100 mL/min/kg of grape) and water temperature of 25°C.

The GO treatments were carried out in a saturated chamber with 32 ± 1 μL/L of gaseous ozone. Also in this case, two different application times were used: 12 and 24 h, referred as treatments D and E, respectively. The concentration of ozone was stable during the experiment by recirculation of ozone-enriched air in the chamber, and ozone was continuously monitored through a UV-photometric ozone analyzer BMT 964 (BMT Messtechnik Gmbh, GE) that controls the generator output. The thermohygrometric conditions were: temperature of 20 ± 1 ºC and relative humidity of 57±3 %.

Grape sampling and must fermentation

About 35 grape berries were randomly picked before and after ozone treatments from each of the six different perforated boxes, and placed in sterile stomacher bags. The berries were manually crushed and the obtained juice was subjected to microbiological analysis. After the ozone treatments, grapes from each replicate were placed in sterile plastic bags, crushed, and the grape mash (must and skins) was placed in 2.5 L sterile glass bottles, of closed with a sterile Müller valve containing sterile vaseline oil. The total volume (must and skins) of each trial was about 1.7 L. The chemical composition of the must was: 22.1°Brix, pH 3.18 and titratable acidity of 9.45 g/L (expressed as tartaric acid). For inoculated trials, the commercial *Saccharomyces cerevisiae* EC1118® (Lallemand Inc., Montreal, Canada) strain was rehydrated according to the manufacturer's instructions and then used at a concentration of 2.0 x 10^6

cells/mL. Wine fermentations were sampled aseptically at 0, 2, 5, 7 and 14 days. The fermentations were carried out under static conditions at 25 ± 1°C.

Microbiological analysis

Samples were serially diluted in Ringer solution (Oxoid, Milano, Italy), plated in duplicate on Wallerstein Laboratory Nutrient medium (WLN) (Biogenetics, Milano, Italy) and Lysine medium (Oxoid) and incubated for 3-5 days at 30°C. The colonies grown on WLN medium were firstly divided in groups, based on their color, aspect and shape as previously described (Cavazza et al., 2008). Afterwards, colonies (n= 5 to 6 from each sample) were streaked on WLM medium and then inoculated in 1 mL of YPD broth containing 1% (w/v) yeast extract, 2% (w/v) bacteriological peptone and 2% (w/v) dextrose (Biogenetics). After 24 h growth, the culture was supplemented with 30% of sterile glycerol (Sigma, Milano, Italy) and then stored at -20°C.

Molecular analysis

DNA extraction and identification of pure cultures

For DNA extraction, pure cultures of isolates were centrifuged at 14,000 rpm for 10 min at 4°C to precipitate the cells, and the DNA extraction was performed using a bead beater treatment as described by Cocolin et al., (2000). Molecular identification of the isolates was carried out by Restriction Fragment Length Polymorphism (RFLP) analysis of the ITS1-

5.8S ribosomal RNA (rRNA)-ITS2 region using the protocols described by Alessandria et al. (2015).

Interdelta-PCR to monitor *S. Cerevisiae* Lalvin EC1118® during inoculated fermentations

At each sampling point of the inoculated fermentation, 5 putative colonies of *S. cerevisiae* were isolated and then subjected to interdelta-PCR molecular fingerprinting analysis (Charpentier et al., 2009). The electrophoretic analysis was performed in 2.0% (w/v) agarose gels containing 0.5 mg/L ethidium bromide (Sigma) in 1X TBE buffer solution at 120 V for 120 min. The profiles obtained were processed by cluster analysis using the computer software package Bionumerics, version 4.0 (AppliedMaths, Kortrijk, Belgium). Genetic similarity of isolates was determined using the Unweighted Pair Group Method using Arithmetic Averages (UPGMA) and the Pearson's coefficient.

DNA and RNA extraction from grape and must samples

For each grape treatment, before and after treatments as well as during spontaneous fermentations (0, 2, 5, 7 and 14 days), 1 mL of grape juice was collected for both DNA and RNA extraction. Samples were centrifuged at 14,000 rpm for 10 min, and the precipitate was then stored at -20°C, until analysis. In the tubes containing the pellets for RNA analysis, 200 µL of RNA later (Ambion, Milano, Italy) were added before storage. The pellets were subjected to nucleic acid extraction by using the MasterPure™ Complete DNA and RNA Purification kit (Epicentre, Madison, WI, USA) as described by Rantsiou et al. (2013). The extracted

DNA was quantified and then standardized at 100 ng/µL using the Nanodrop ND-1000 spectrophotometer (Celbio, Milano, Italy). DNA in the RNA samples was digested using Turbo DNase (Ambion), following manufacturer instructions.

PCR and (RT)-PCR amplification

Reverse transcription (RT) reactions were carried out as follows: 500 ng of RNA were mixed with 100 µM primer LS2 in a total volume of 10 µL, and denatured for 5 min at 75°C.The tubes were then placed in ice. The reverse transcription was performed in 25 µL containing 50 Mm Tris-HCl (pH 8.3), 75 Mm KCl, 3 mM $MgCl_2$, 10 Mm DTT, 2 Mm dNTPs, 4 mM primer, 200 units of M-MLV reverse transcriptase (Promega, Milano, Italy) and 0.48-0.96 units of RNasinribonuclease inhibitor. The reaction was performed at 42°C for 1 h, and 1 µL of RT reaction was used for the regular PCR reaction. DNA and cDNA template of the grape and must samples were amplified with NL1 (with the GC clamp) and LS2 yeast primers, as reported by Rantsiouet al. (2013).

Denaturing gradient gel electrophoresis (DGGE)

For the DGGE analysis, the D-Code universal mutation detection system (Bio-Rad, Milano, Italy) was used as previously described (Cocolin et al., 2000). The amplified product was loaded in a 0.8 mm thick polyacrylamide gel [8% (w/v) acrylamide-bisacrylamide 37.5: 1] with a denaturing gradient of 30 to 50%.

Sequencing and identification of the DGGE bands obtained from DNA and cDNA

The DGGE bands of interest were excised from the gels with sterile pipette tips, put into 40 μL ultra pure sterile water, and put overnight at 4°C. The identification of each band was carried out by sequencing and subsequent alignment of the resulting sequence in GenBank using the BLAST program (http://blast.ncbi.nlm.nih.gov), as described by Rantsiou et al. (2013).

Chemical analysis

Standard chemical parameters determination

The chemical composition of the wines was determined 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 (Giordano et al., 2009). The chemical compounds quantified were: residual sugars (glucose and fructose), organic acids (tartaric, malic, lactic, citric, succinic and acetic acid), ethanol and glycerol. Total acidity was determined according to the methods proposed by the International Organization of Vine and Wine (OIV, 2008).

Volatile compounds determination

The main families of chemical compounds determined were: alcohols, esters, fatty acids, terpenes, and C13-norisoprenoids. The volatile aroma

compounds of wines were detected by Head Space Solid Phase Micro-Extraction (HS-SPME) coupled with Gas Chromatography-Mass Spectrometry (GC-MS). The instrumentation and experimental conditions used were described by Rolle et al., (2015). Five mL of each wine sample were placed into a 20-mL glass vial containing 5 mL of water, 2 g of NaCl and 1-heptanol as internal standard (IS). The 50/30 µm DVB/CAR/PDMS fiber (Supelco, Bellefonte, PA, USA) was used for the extraction of volatile compounds. Volatile compounds were identified according to pure standards and/or the NIST database (http://webbook.nist.gov/chemistry/). Isobutanol, Isoamylic alcohol, 3-methyl-butanol acetate, 1-octanol, 2,3-butanediol isomers, 2-ethyl hexanol, 2-phenylethanol, citronellol, diethyl succinate, ethyl acetate, ethyl decanoate, ethyl dodecanoate, ethyl heptanoate, ethyl hexanoate, ethyl nonanoate, ethyl octanoate, ethyl phenylacetate, hexanal, hexanoic acid, hexyl acetate, linalool, methyl decanoate, methyl salicylate, nerol, octanoic acid, α-terpineol, β-damascenone were used as pure standards (Sigma).

Statistical analysis

The results of the yeast counts from the grape surface and the values of standard chemical parameters were subjected to one-way Analysis of Variance (ANOVA). The Duncan test for microbiological and chemical analyses at a confidence level of 95% was used to identify statistical differences between trials. In order to understand the diversity of wines, the contents of volatile compounds were subjected to Principal Component Analysis (PCA). Statistical analyses were performed using the software package IBM SPSS Statistics (version 21.0, IBM Corp., Armonk, NY, USA).

Yeast counts and biodiversity on the grapes surface

Total yeast counts on the grapes surface before and after treatments are reported in Fig. 2 (panel A). The counts were performed on WLN medium. The plate counts highlighted a significant difference between the treated and untreated grapes, while no significant differences were registered between the treatments. Indeed, both gaseous and aqueous ozone reduced yeast populations by about 0.5 Log CFU/mL. More specifically, cell counts on the untreated grapes were 5.0 Log CFU/mL, while after the treatments they comprised between 4.5 and 4.0 Log CFU/mL. The counts in the untreated grapes are in agreement with those reported in the literature for ripe grapes in good phytosanitary state (Milanovic et al., 2013). The effect of ozone on fruit surfaces was previously investigated by Oztekin et al. (2006). The results obtained here are in agreement with those already published, underlining an impact of ozone in reducing yeast populations (Fig. 2 A). It is worth noticing that the decrease of yeast populations in treated samples (both gaseous and aqueous ozone) was related to the reduction of apiculate yeasts number. This result is interesting because several studies have highlighted the negative impact of this species on wine composition due to their ability to produce high contents of acetic acid (Jolly et al., 2013).
Yeast species biodiversity identified on WLN medium before and after ozone treatments is shown in Fig. 2B. Untreated grapes mycobiota, was characterized by the presence of *H. uvarum* (46%), *Starm. bacillaris* (39%), *A. pullulans* (3%), *Rhodotorula glutinis* (7%) and *I. terricola* (4%).

Yeast biodiversity was reduced in treated grapes as follows: by using AO for 6 and 12 min (treatments B and C, respectively) the species isolated were *H. uvarum* (38- 57%), *Starm. bacillaris* (40-59%) and *A. pullulans* (3%), while with GO, for both 12 h (treatment D) and 24 h (treatment E), yeast populations included *H. uvarum* (49-67%) and *Starm. bacillaris* (33-51%). These evidences show the ability of the two types of to modify the yeast ecology on the surface of the grapes by reducing species biodiversity. In fact, after the AO treatments, *I. terricola* and the *R. glutinis* were not detected on WLN plates, while in addition to this, after the GO treatments also *A. pullulans* was not detected. The reduction of 0.5 Log CFU/mL obtained by the plate counts is due to the decrease of the apiculate yeasts, even if the percentages relative increased. The alteration of population size after treatments may be explained by the different sensitivity of the yeast species to ozone. As already been described in previous studies, ozone shows a different efficacy against the microorganisms in function of multiple factors like species and strain sensitivity, density of microbiota treated, form used of the ozone (gaseous or in solution) and method of measuring antimicrobial efficacy (Guzzon et al., 2013; Khadre et al., 2001).

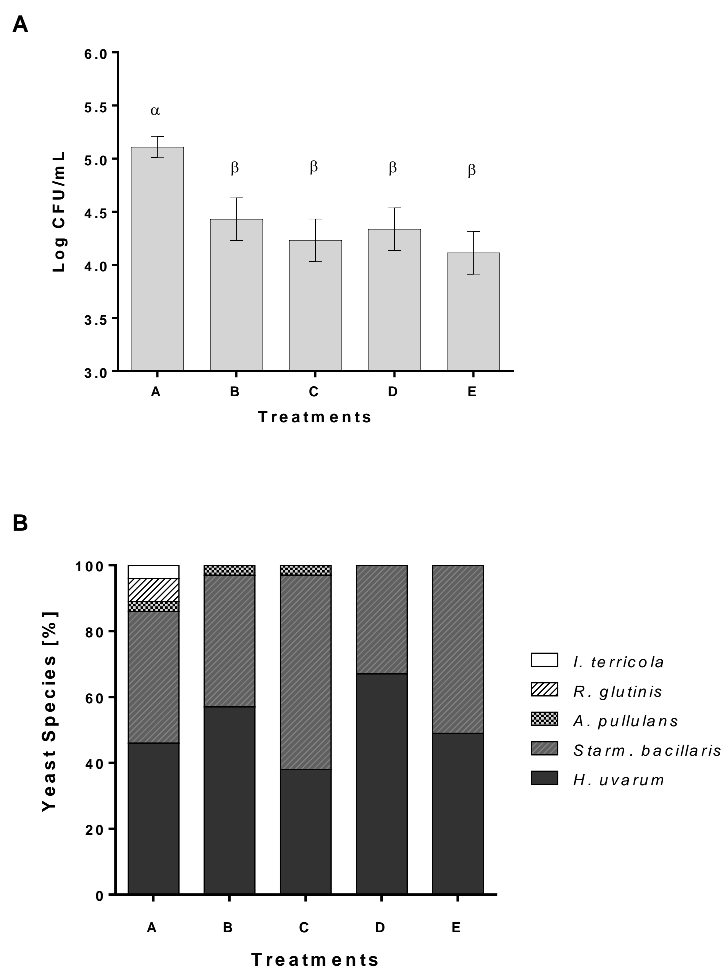
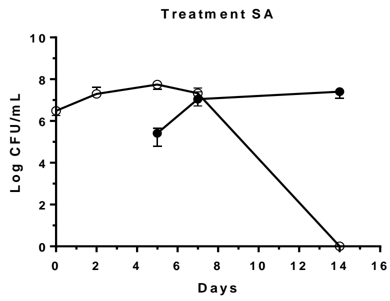


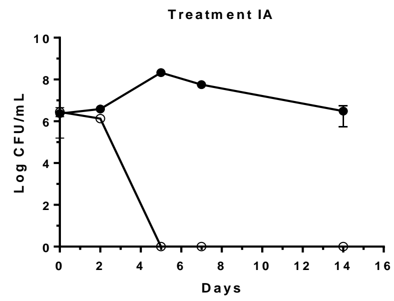
Fig.2. Total yeast counts (panel A) and yeast species biodiversity (panel B) on grapes surface before and after treatments registered on WLN medium. Data are the mean (± SD) of six biological replicates from each treatment applied. The different letters above each column indicate significant differences according to ANOVA and Duncan test ($p < 0.001$). **A**: untreated control; **B**: aqueous ozone for 6 minutes at 5 mg/L; **C**: aqueous ozone for 12 minutes at 5 mg/L; **D**: gaseous ozone for 12 hours at 32 μL/L; **E**: gaseous ozone for 24 hours at 32 μL/L.

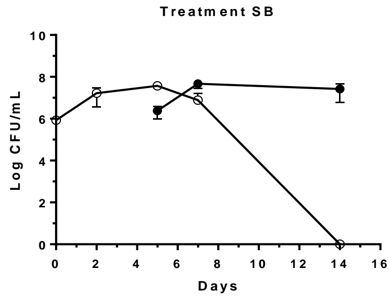
Spontaneous and inoculated fermentations: counts and yeast biodiversity

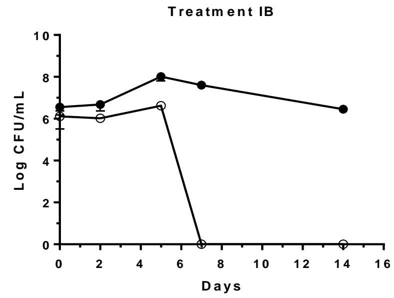
The growth dynamics of non-*Saccharomyces* and *S. cerevisiae* yeasts during spontaneous and inoculated fermentations are shown in Fig. 3. In spontaneous fermentations, the initial population of non-*Saccharomyces* was higher in the control untreated trial (SA), with about 6.5 Log CFU/mL, compared to the must originated from treated grapes, which was about 6.0 Log CFU/mL. This may be ascribable to the reduction of yeasts on the surface of the grapes because of ozone treatment. During fermentation, non-*Saccharomyces* yeasts showed comparable count trends. Afterwards, their population increased until day 7 and reaching undetectable levels on Lysine medium at the end of the monitored period. This sharp decreases is most likely correlated to increasing ethanol content due to *S. cerevisiae* activity (Fleet, 2003). For treatments SA, SB and SC, *S. cerevisiae* cells appeared on day 5 of fermentation, with a population range of about 5.0 - 6.0 Log CFU/mL. Afterwards, their population increased until day 5 and remained constant at 7.0 Log CFU/mL until day 14. For treatments SD and SE, *S. cerevisiae* was detected only at day 7 with counts of about 6.0 Log CFU/mL and then increased at 7.0 Log CFU/mL at the end of the fermentation. In the inoculated fermentations, *S. cerevisiae* population was about 6.0 Log CFU/mL. The results of interdelta-PCR and subsequent cluster analysis using a similarity coefficient of 90% demonstrated a clear dominance of *S. cerevisiae* Lalvin EC1118® during wine fermentations (data not shown). *S. cerevisiae* populations in IA, IB, ID and IE fermentations showed the same trend, increasing until day 5 (>8.0 Log CFU/mL), remaining stable until day 7, and then decreasing (to about 6.0 Log CFU/mL) by the end of the period monitored. Differently, in the IC

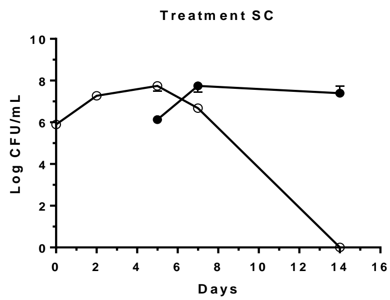
fermentation *S. cerevisiae* cells steadily increased until day 7, reaching 8.0 Log CFU/mL, and declined to 7.0 Log CFU/mL at the end of the fermentation. Regarding non-*Saccharomyces* yeasts, for the treatment IA, the cells decreased slightly during the first two days and they disappeared at day 5. For treatments IB, IC, ID and IE, non-*Saccharomyces* population remained fairly stable at around 6.0-7.0 Log CFU/mL until day 5 and disappeared at day 7. The early death of non-*Saccharomyces* in the inoculated fermentations could be explained by the relative high competition for nutrients at the beginning of fermentations and cell contact mechanisms with the yeast starter (Medina et al., 2012; Nissen et al., 2003).

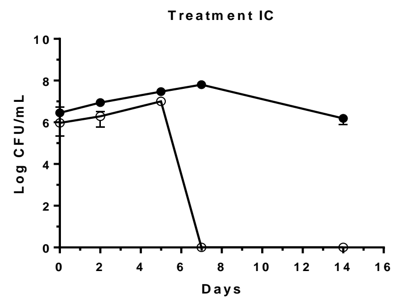

Treatment SA
Log CFU/mL
Days

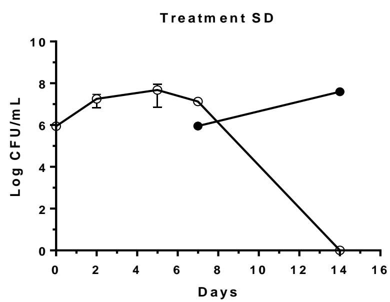

Treatment IA
Log CFU/mL
Days

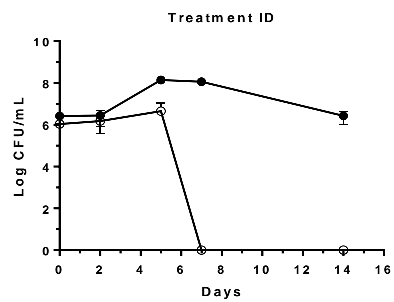

Treatment SB
Log CFU/mL
Days

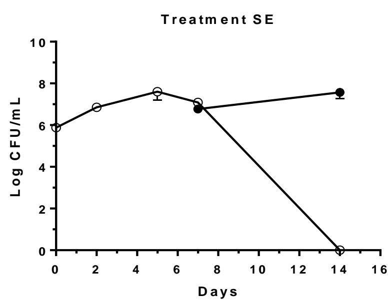

Treatment IB
Log CFU/mL
Days


Treatment SC
Log CFU/mL
Days


Treatment IC
Log CFU/mL
Days


Treatment SD
Log CFU/mL
Days


Treatment ID
Log CFU/mL
Days


Treatment SE
Log CFU/mL
Days

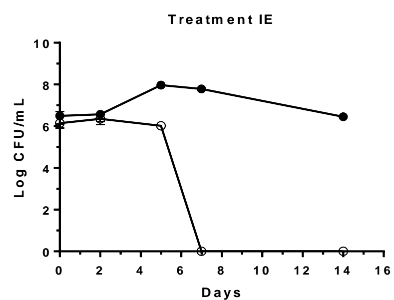

Treatment IE
Log CFU/mL
Days

Fig.3. Colony forming unit counts for millilitre (CFU/mL) of *S. cerevisiae* [●] and non-*Saccharomyces* yeasts [○] during spontaneous (**S**) and inoculated fermentations (**I**). *S. cerevisiae* counts were determined on WLN medium and identified through RFLP analysis of the ITS1-5.8S ribosomal RNA (rRNA)-ITS2, while non-*Saccharomyces* on Lysine medium. The counts were reported as mean (± SD) of three independent experiments. **A**: untreated control; **B**: aqueous ozone for 6 minutes at 5 mg/L; **C**: aqueous ozone for 12 minutes at 5 mg/L; **D**: gaseous ozone for 12 hours at 32 μL/L; **E**: gaseous ozone for 24 hours at 32 μL/L.

The yeast species biodiversity during spontaneous and inoculated fermentations is reported in Fig. 4. In all of the spontaneous fermentation, the biodiversity observed in D0 is the same obtained on the grapes surface after the treatments, data reported in fig. 2B. This result confirmed the reproducibility and reliability of the data obtained in this part of the work. At the beginning of fermentation (day 0), the yeast species present in the non-inoculated musts were *H. uvarum* and *Starm. bacillaris*, representing more than 75% of the isolated colonies. These non-*Saccharomyces* yeasts were present until day 7 for SA, SB, SD and SE treatments, while for the treatment SC only *Starm. bacillaris* was present up to the day 7 and *H. uvarum* populations disappeared after 5 days. *A. pullulans* was also isolated in SA, SB and SC musts at the beginning of the fermentation. In SA, SB and SC trials, *S. cerevisiae* appeared at day 5, while in the SD and SE trials it was isolated from day 7. *S. cerevisiae* dominated the fermentation from day 7 in the SA (75%), SB (85%) and SC (92%) trials, while it was the major population for SD and SE treatments only on day 14.

In the inoculated fermentations, *S. cerevisiae* was present since the beginning and dominated all fermentations until the end. At the beginning of the fermentation, for the IA treatment, the yeast ecology was

characterized by the presence of *Starm. bacillaris* (8%), *H. uvarum* (39%) and *A. pullulans* (2%), and of them only *H. uvarum* and *Starm. bacillaris* were present until day 2, disappearing at day 5. In the IB and IC trials, the only non-*Saccharomyces* yeast present until day 5 was *Starm. bacillaris*. In ID and IE trials, also *H. uvarum* was isolated in the first 2 days together with *Starm. bacillaris*.

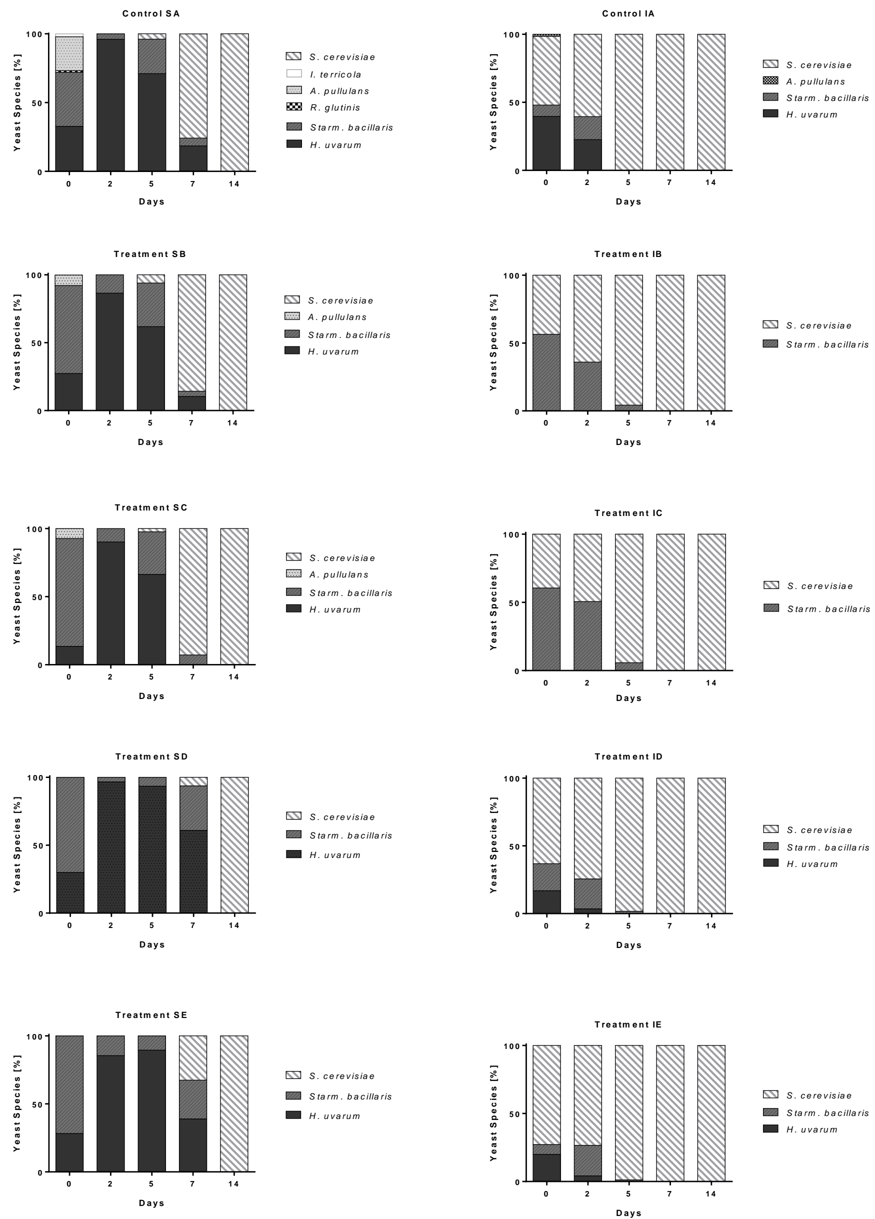


Fig.4. Yeast species diversity during spontaneous (**S**) and inoculated fermentations (**I**) of must obtained from treated and untreated grapes. **A**: untreated control; **B**: aqueous ozone for 6 minutes at 5 mg/L; **C**: aqueous ozone for 12 minutes at 5 mg/L; **D**: gaseous ozone for 12 hours at 32 μL/L; **E**: gaseous ozone for 24 hours at 32 μL/L.

PCR-DGGE analysis on the grapes surface and during spontaneous fermentations

The PCR-DGGE analysis, at both DNA and RNA level, didn't show differences between the replicates investigated for each treatment applied (data not shown). In the untreated grape samples, the profiles were in accordance with the traditional isolation based on the morphotypes on WLN medium and RFLP identification, and *H. uvarum, Starm. bacillaris*, *A. pullulans* and *R. glutinis* could be identified in the DGGE profiles. In samples B and C, *Starm. bacillaris* and *A. pullulans* were detected, while in the samples D and E only *Starm. bacillaris* could be detected. Also in this case the results were in agreement with plate counts. *I. terricola* in the sample A and *H. uvarum* in the samples B, C, D and E were not detected possibly because their counts were below the detection limit of the method, and therefore they were not visible in the DGGE profile (Prakitchaiwattana et al., 2004). During spontaneous fermentations, the presence of *Starm. bacillaris* and *H. uvarum* was observed until day 14. This result is in contrast with the plate counts on WLN and lysine media, in fact these non-*Saccharomyces* species disappeared at day 7. Since their signals could be observed also in the RT-PCR-DGGE gels, it can be speculated that the cells could have entered a viable but non-cultivable state (VBNC) as previously described by Cocolin et al., (2000). *S. cerevisiae* population appeared in the gels at day 5 in the trials SA, SB and SC, while, in the trials SD and SE, it was detected only at day 7, confirming again the counts on WLN medium.

Chemical analysis of the wines at the end of fermentations

The chemical composition of the wines produced from spontaneous and inoculated fermentations is shown in Table 1. The results obtained were comparable for all treatments applied. Less than 2.0 g/L of residual sugars were detected after 14 days of fermentation. However, a significant difference was observed for the acetic acid content. For the treatment SA, higher levels of this unpleasant compound were found, followed by the treatments with GO (SD and SE). For the treatments with AO (SB and SC), the resulting wines had the lowest contents of acetic acid. This reduction appears to be correlated to the decrease in the number of apiculate yeasts. Since these species are well known for their ability to produce high levels of acetic acid (Comi et al., 2001; Jolly et al., 2006; Romano et al., 1993). On the contrary, in all inoculated fermentations acid acetic was kept at low levels due to the domination of the starter culture over apiculate yeasts.

Table 1 Chemical analysis of Barbera wines produced from treated and untreated grapes: **S**: spontaneously fermented wines; **I**: inoculated wines; **A**: untreated control; **B**: aqueous ozone for 6 minutes at 5 mg/L; **C**: aqueous ozone for 12 minutes at 5 mg/L; **D**: gaseous ozone for 12 hours at 32 μL/L; **E**: gaseous ozone for 24 hours at 32 μL/L.

Test	Citric acid (g/L)	Tartaric acid (g/L)	Malic acid (g/L)	Lactic acid (g/L)	Succinic acid (g/L)	Acetic acid (g/L)	Glycerol (g/L)	Ethanol (% v/v)	Sugar (g/L)
must	0.30 ± 0.03	8.1 ± 0.2	4.2 ± 0.5	< 0.1	< 0.1	< 0.1	0.1 ± 0.1	< 0.1	216.6 ± 10.1
SA	0.31 ± 0.01	4.6 ± 0.2	3.5 ± 0.2 a	0.70 ± 0.10 c	1.04 ± 0.04 a	0.52 ± 0.05bc	10.2 ± 0.7	12.6 ± 0.1	< 2.0
SB	0.29 ± 0.02	4.4 ± 0.3	3.6 ± 0.2 ab	0.54 ± 0.11bc	1.09 ± 0.08 ab	0.16 ± 0.05a	10.3 ± 0.9	12.3 ± 0.3	< 2.0
SC	0.30 ± 0.04	4.3 ± 0.1	3.7 ± 0.2 ab	0.35 ± 0.12 ab	1.09 ± 0.04 abc	0.20 ± 0.01 a	9.9 ± 0.4	12.3 ± 0.2	< 2.0
SD	0.32 ± 0.01	4.4 ± 0.2	3.6 ± 0.1ab	0.50 ± 0.11ac	0.96 ± 0.04 a	0.38 ± 0.02ab	9.9 ± 0.2	12.5 ± 0.2	< 2.0
SE	0.32 ± 0.03	4.3 ± 0.1	3.5 ± 0.4 a	0.75 ± 0.30 c	1.12 ± 0.05 abc	0.35 ± 0.08ab	10.2 ± 0.1	12.6 ± 0.1	< 2.0
IA	0.30 ± 0.01	4.2 ± 0.1	3.9 ± 0.1 ab	0.30 ± 0.10 ab	1.33 ± 0.05 d	0.28 ± 0.09a	10.5 ± 0.1	12.8 ± 0.3	< 2.0
IB	0.30 ± 0.01	4.5 ± 0.2	4.0 ± 0.1 ab	0.20 ± 0.10a	1.28 ± 0.04 cd	0.25 ± 0.01 a	10.1 ± 0.2	12.7 ± 0.1	< 2.0
IC	0.30 ± 0.02	4.4 ± 0.2	4.1 ± 0.5 ab	0.10 ± 0.12a	1.38 ± 0.06 d	0.25 ± 0.05a	10.6 ± 0.4	12.7 ± 0.5	< 2.0
ID	0.31 ± 0.01	4.0 ± 0.1	4.4 ± 0.1 b	0.10± 0.12a	1.03 ± 0.18 a	0.31 ± 0.08a	9.3 ± 0.8	12.7 ± 0.1	< 2.0
IE	0.31 ± 0.01	4.0 ± 0.1	4.4 ± 0.1 ab	0.10 ± 0.13a	1.28 ± 0.08 bcd	0.27 ± 0.03a	10.2 ± 0.5	12.8 ± 0.3	< 2.0
Sign.	NS	NS	*	***	**	**	NS	NS	NS

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 Duncan test ($p < 0.05$). Sig: *, **, *** and NS indicate significance at $p < 0.05$, $p < 0.01$, $p < 0.001$ and not significant, respectively.

Volatile compounds of the wines at the end of fermentations

Volatile compounds were determined immediately after the end of the fermentation, and the results obtained revealed the effect of ozone treatments on wine aroma quality. The identified wine aroma compounds (alcohols, esters, fatty acids, terpenes, and C13-norisoprenoids) were quantified and then subjected to Principal Component Analysis (PCA), Fig. 5). Esters with acetic acid (acetates) were separated from the other esters to explain the differences reported in the PCA results.

About 75% of the total variance was explained by the first two principal components. The correlation of the main aromatic families in the PCA plot was as follows: the first principal component (PC1) was correlated

positively with esters (without the contribution of acetates), alcohols and fatty acids, and negatively with acetates, terpenes and alcohols at low molecular mass (propanol, isobutanol). The second principal component (PC2) was correlated positively with terpenes and esters. Replicates of each trial were grouped together in the previous PCA. The PC1 was able to discriminate inoculated and spontaneous wines. In fact, as we can observe in the PCA plot, all spontaneous wines were grouped on the left part of the plot due to high quantities of acetates and terpenes. On the other hand, all inoculated wines were grouped on the right (high contents of esters, fatty acids and alcohols). It is interesting that spontaneously fermented wines from untreated grapes (SA) were differentiated from the other wines obtained from treated grapes without inoculation by the PC2. Particularly, the treatment SA was characterized by high concentrations of terpenes and ethyl acetate, while the other spontaneous wines were characterized by high presence of other acetates (2-methyl-propyl acetate and 2-ethyl-phenyl acetate) and β-damascenone.

Also inoculated wines were divided by the PC2 in two main groups. One group was composed of IA and ID, while the other group was formed by IB, IC and IE. The IA and ID wines were characterized by high concentrations of esters and alcohols. This result shows that the gaseous ozone treatments at 12 h reduced grapes mycobiota abut did not influence the wine aroma. In addition, the results obtained demonstrated that yeast inoculum had a greater influence on the final content of volatile compounds in the wines, compared to the ozone treatments previously applied on the grapes. Indeed, the inoculated wines were characterized by major concentrations of pleasant esters like methyl decanoate, methyl hexanoate and ethyl dodecanoate, which have fruit and flower fragrances.

The effect of the treatments is highlighted by the differences observed between SA and the other spontaneous fermentations, as it can be seen in PCA2. In fact, combining this result with the yeast species diversity at the beginning of the fermentation, we can see that ozone treatments altered the yeast population and as a consequence the chemical composition of the wines. In fact, SA wines were characterized by higher concentrations of ethyl acetate, and this can be correlated to the high cell populations of apiculate yeasts observed in these ferments (Romano et al., 2003).

Spontaneous fermentation, increased the content of important terpenes (linalool and nerol), especially in SA wines, due to a possible β-glycosidase activity of indigenous mycobiota (Fleet, 2008).

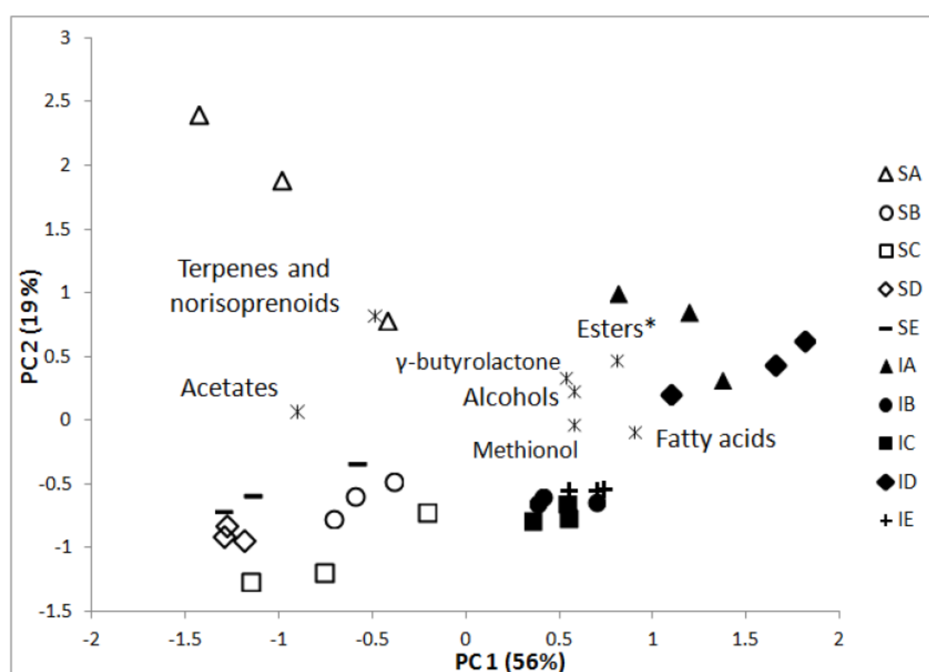


Fig. 5 Score plot of the first and second Principal Components for the volatile compounds and aromatic families identified in Barbera wines. **S**: spontaneously fermented wine; **I**: inoculated wine; **A**: untreated control; **B**: aqueous ozone for 6 minutes at 5 mg/L; **C**:

aqueous ozone for 12 minutes at 5 mg/L; **D**: gaseous ozone for 12 hours at 32 μL/L; **E**: gaseous ozone for 24 hours at 32 μL/L.

Conclusion

This study showed preliminary results of the effect of ozone treatment (either in aqueous or gaseous form) on yeast ecology of post-harvested wine grapes and during the fermentation process. The ability of the treatments to reduce and modify the yeast populations present on grape berry surfaces, and during the spontaneous and inoculated fermentations, was demonstrated. The results showed a selective antimicrobial property of the treatments (independently of the form, concentration and time of ozone treatment) on the population size of about 0.5 Log CFU/mL, mainly apiculate yeasts, and therefore decreasing the acetic acid content in the wines produced by spontaneous fermentation from treated grapes. This evidence demonstrates that the use of ozone treatments, also without inoculation of *S. cerevisiae*, could be considered a tool to control the population of undesirable yeasts in the first phase of the fermentation process and to produce wines with pleasant esters.

Acknowledgments

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

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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. The results showed that all the treatments reduced greatly the presence of this yeast. Particularly, GO treatments of 24 h and 12 h decreased its presence by about 2.1 and 1.6 Log, respectively, 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.

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 potential antimicrobial of the EW, WO and GO treatments against *B. bruxellensis* inoculated in post-harvest grapes.

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

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). 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 yeast cell populations, including *Brettanomyces spp.* (Barata et al., 2011; Guerzoni et al., 1987; Pretorius, 2000). 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 to produce in the wines some off-flavors due to the activity of two enzymes: cinnamate decarboxylase and vinyl phenol reductase (Suarez et al., 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 et al., 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 et al., 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 et al., 2003.; Romano et al., 2008; Snowdon, et al., 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 et al., 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. One possibility is that these yeasts arrive from the vineyard (Suarez et al., 2007). To this regard, Renouf et al., (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.

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 et al., 2010; Hricova et al., 2008; Smilanick et al., 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 et al., 2001; Jermann et al., 2015).

On 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 not still 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).

Materials and Methods

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 were 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 et al., 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.

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 EW and WO treatments, samples were steady 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 solution had a concentration of 400 mg/L of free chlorine, while the WO solution had an ozone concentration of 5.00 ± 0.25 mg/L. During treatments, the flow and the temperature were maintained constant at 200 mL/min and 25 °C, respectively. Control treatments were performed using tap water.

Two different times were used for the GO treatments (12 and 24 h) in a chamber saturated with gaseous ozone at a concentration of 32 ± 1 µL/L. The treatment was performed in controlled conditions of temperature (20 ± 1 ºC), relative humidity (57 ± 3 %) and at constant concentration of ozone, which was constantly monitored through a UV-photometric ozone analyzer BMT 964 (BMT Messtechnik Gmbh, Germany) that controls the generator output. Control treatments were performed in another chamber for 12 and 24 h in contact with air, using the abovementioned temperature and relative humidity conditions.

For each treatment, we have used three replicates and the experimental plan is summarized as follows: **WA**: treated with tap water for 6 min (control); **WB**: treated with tap water for 12 min (control); **EWA**: treated with electrolyzed water for 6 min; **EWB**: treated with electrolyzed water for 12 min; **WOA**: treated with ozonated water for 6 min; **WOB**: treated

with ozonated water for 12 min; **GA**: untreated for 12 h (control); **GB**: untreated for 24 h (control); **GOA**: treated with ozone gas for 12 h; **GOB**: treated with ozone gas for 24 h.

Laboratory-scale fermentations

For each trial, before and after treatments, about 30 berries were randomly picked up, placed in sterile bags, crushed and the must obtained was used for culture-dependent and -independent microbiological analyses. Afterwards, all remaining grape berries were crushed in sterile bags and the grape mash obtained (liquid, skins and seeds) was placed in a 2.5-L sterile glass bottle for the laboratory-scale fermentations. The bottles were equipped with sterile airlocks containing sterile vaseline oil, in order to let flow the carbon dioxide (CO_2) during the alcoholic fermentation while avoiding external contaminations. All musts were inoculated with the commercial *Saccharomyces cerevisiae* strain EC-1118 (Lallemand Inc., Montreal, Canada) strain was rehydrated according to the manufacturer's instructions and inoculated for obtain a density of around 2.0 x 10^6 cells/mL in order to standardize the fermentation process. Fermentations were performed under static conditions at 25 °C, and during the fermentation all bottles were shaken twice a day to soak the grape cap. Fermentations were monitored by microbiological analysis at 0, 4, 7, 17 and 20 days after the inoculum. Chemical analyses were performed after 7 days and at end (14 days) of fermentation.

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).

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).

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 et al., 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.

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).

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 et al., 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).

Chemical analyses

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.

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).

Statistical analysis

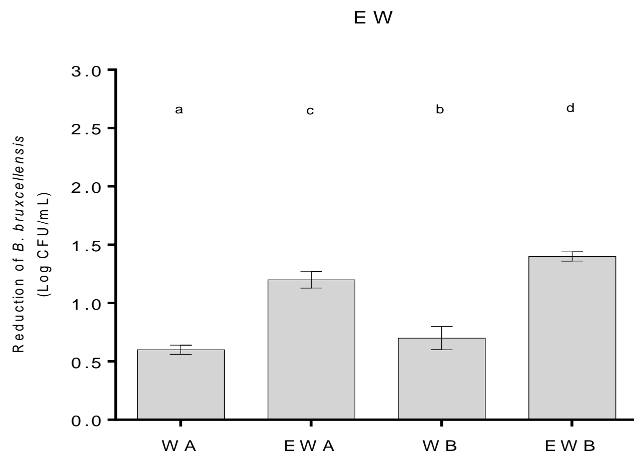
The microbiological and chemical results were submitted to one-way Analysis of Variance (ANOVA). To highlight statistical differences among treatments, we used the Duncan test with a confidence level of 95%. The statistical analyses were performed with the software package IBM SPSS Statistics (version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

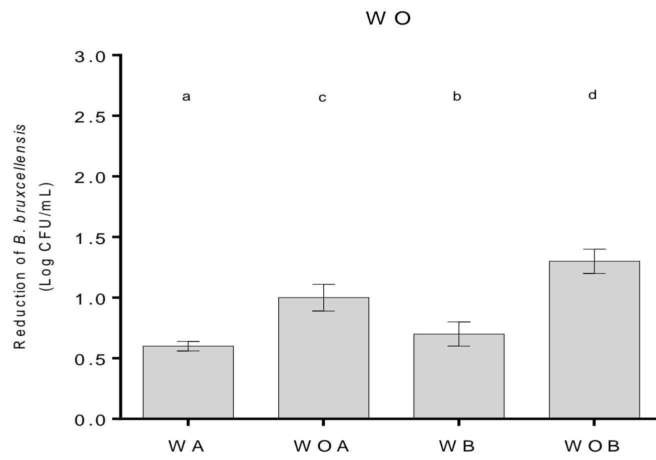
Results

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. Particularly, GOB and GOA treatments decreased its population by 2.1 and 1.6 Log, respectively. EW and WO treatments obtained comparable reductions, more precisely 1.2 Log for EWA, 1.4 Log for EWB, 1.0 and 1.3 Log for WOA and 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.


EW
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Reduction of B. bruxcellensis
(Log CFU/mL)
a
c
b
d
WA
EWA
WB
EWB


WO
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Reduction of B. bruxcellensis
(Log CFU/mL)
a
c
b
d
WA
WOA
WB
WOB

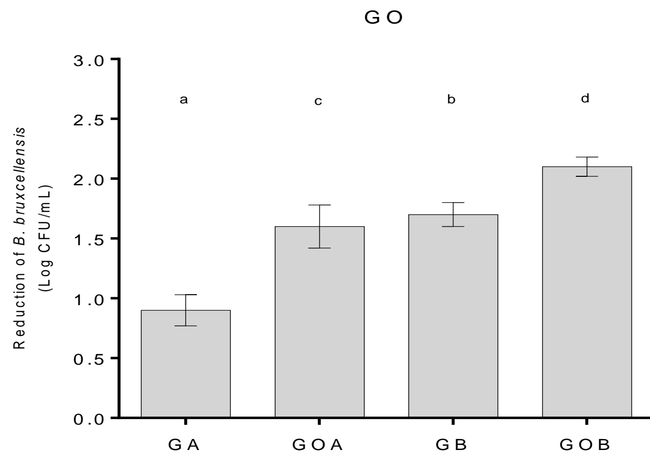

GO
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Reduction of B. bruxcellensis
(Log CFU/mL)
a
c
b
d
GA
GOA
GB
GOB

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 Duncan test ($p < 0.05$). **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.

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 et al., 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.

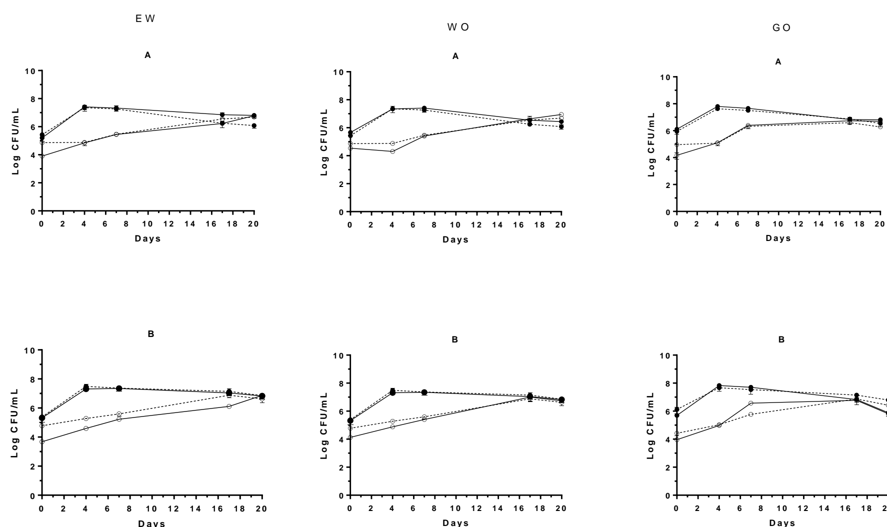


Fig. 2 Counts (CFU/mL) of *S. cerevisiae* Lalvin EC1118® [●] and *B. bruxellensis* DSM 7001 [○] in control (broken line) and treatments (solid line) of the **EW**, **WO** and **GO** trials. **A**: treatments of 6 min (EW and WO) and 12 h (GO); **B**: treatments of 12 min (EW and WO) and 24 h (GO). *B. 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).

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.

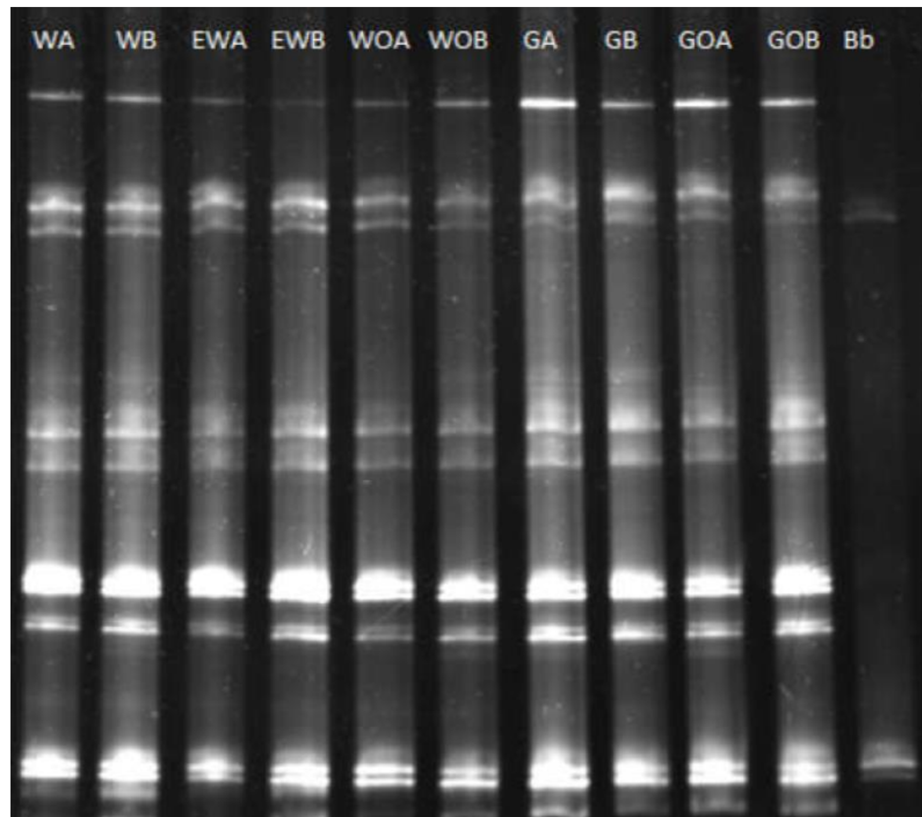


Fig. 3 RT-PCR-DGGE profile of the samples at the end of fermentation**. WA**: treated with water for 6 min; **WB**: treated with water for 12 min; **EWA**: treated with EW (400 mg/L of free chlorine) for 6 min; **EWB**: treated with EW (400 mg/L of free chlorine) for 12 min; **WOA**: treated with ozonated 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.

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 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.

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

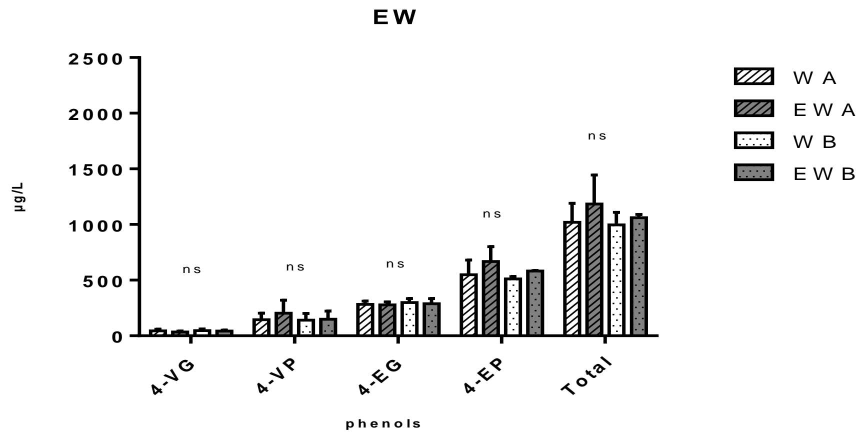
air for 24 h; **GOA**: treated with ozone gas (32 ± 1 μL/L) for 12 h; **GOB**: treated with ozone gas (32 ± 1 μL/L) for 24 h. All data are expressed as average value ± standard deviation (n = 3). Different Latin letters within the same column indicate significant differences among the treatments, according to the Duncan test ($p < 0.05$). 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

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.


EW
2500
2000
1500
1000
500
0
µg/L
ns
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4-EP
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phenols
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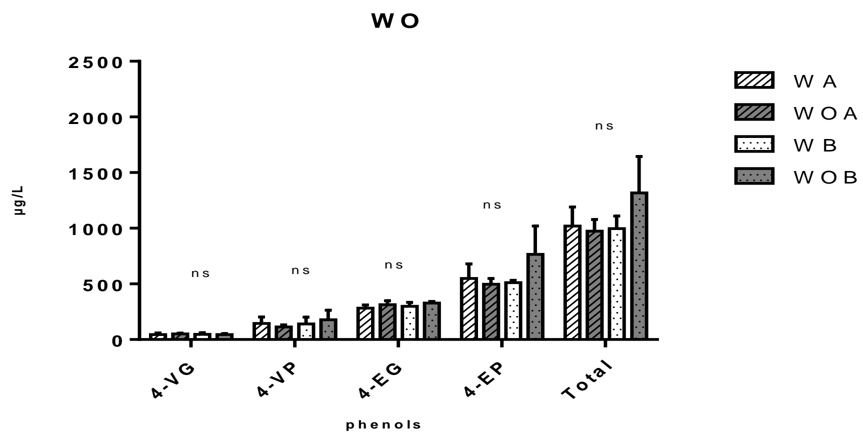
W O
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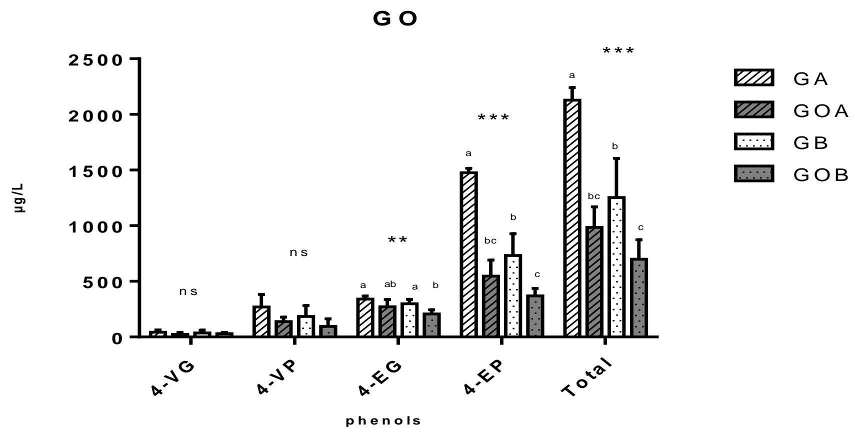
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4-VG
4-VP
4-EG
4-EP
Total
phenols
GA
GOA
GB
GOB

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 Duncan 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) 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. **4-VG**: 4-vinylguaiacol; **4-VP**: 4-vinylphenol; **4-EG**: 4-ethylguaiacol; **4-EP**: 4-ethylphenol.

Discussion

One possible approach to reduce the wine contamination by *B. bruxellensi*s is the use of electrolyzed water and ozone in post-harvest grapes wine thanks to their broad disinfectant action against microorganisms, eco-friendliness and easiness of on site application. In this contest, all treatments with EW and ozone had a significantly higher effect on yeast vitality respect the controls, even though controls have slightly reduced the charges of *Brettanomyces*. Particularly, the results showed greater efficacy of the treatments with gaseous ozone, where the longer treatment times influenced the yeast counts. In fact, in the GOB and GOA trials the decrease of the population yeast was 2.1 and 1.6 Log, respectively, while EW and WO treatments obtained reductions from 1.0 to 1.4 Log. The effectiveness of aqueous ozone on *B. bruxellensis* was already highlighted in another study (Guzzon et al., 2013), where it was shown that 5 mg/L of O_3 for 30 min were sufficient for a complete inactivation of a population with a concentration of 10^6 CFU/mL. The

results obtained here confirmed the low ozone tolerance of this yeast, although the ozone treatments used did not guarantee its complete elimination. This fact can be explained by the different treatment times and substrate used in the two different studies. Indeed, many studies have shown how the effectiveness of ozone is influenced by many factors including concentration, contact time, and substance on which it works (Khadre et al., 2001.; Jermannet et al., 2015). Furthermore, the high sensitivity of *B bruxellensis* to these antiseptics is confirmed by the decreases of the charges obtained in this study immediately after the treatments, which are all above 1.0 Log. In fact, other studies done on the use of EW and WO and GO on post-harvest grapes showed lower reductions in yeast charges, around of 0.5 Log (Cravero et al., 2016a, b). These two studies showed the antimicrobial property of EW, WO and GO on the population present on grapes surface, where the treatments have reduced of about 0.5 Log the counts of apiculate yeasts, resulting in a decrease of the acetic acid content in the wines produced by spontaneous fermentation from the treated grapes. Comparing the results obtained in this study with those obtained in the two works of Cravero et al., 2016 a, b, it is shown how the treatments are much more efficient on *B. bruxellensis* respect the other yeast species. Particularly, the reduction of *B. bruxellensis* is twice that of the apiculate yeast in treatments with EW and WO and even four times higher in GO treatments. These results suggest the potential use of EW, WO and GO as a sanitizer in post-harvest grapes to control the population of undesirable yeasts (apiculate yeasts and *B. bruxellensis*).

During the fermentation time, *S. cerevisiae* population was dominated thanks the inoculated Lalvin EC-1118®, as demonstrated by the results of interdelta-PCR and cluster analysis using the similarity coefficient of 90%

(data not shown). However, towards the end of fermentation, more *B. bruxellensis* cells were found as *S. cerevisiae* viable population started to decline. This is correlated with the higher ethanol tolerance of *B. bruxellensis* than *S. cerevisiae* in conditions of low sugar concentrations (Renouf et al., 2006). It is important to take into account that, at the end of fermentation, the 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 et al., 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.

Also 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 et al., 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 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 et al., 1995; Zuehlke et al., 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*.

Conclusion

This study demonstrated, for the first time, the efficacy of the EW, WO and GO treatments in reducing *B. bruxellensis* inoculated in post-harvest grapes. The results showed a relatively high reduction of *B. bruxellensis* in the must produced by grapes treated with GO at 24 h and 12 h, decreasing by 2.1 and 1.6 Log, respectively. EW and WO treatments have obtained lower and similar reductions ranging between 1.0 and 1.4 Log.

However, 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. Nevertheless, the treatment of post-harvest grapes with gaseous ozone permitted to reduce significantly the concentration of ethylphenols in the wine in relation to the control wine. 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.

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Conclusions and Future perspectives

These three articles describe a preliminary approach to use ozone and electrolyzed water directly on post-harvested wine grapes, particularly, the first two generally studied the impact of the two sanitizers in the early stages of winemaking, and the third specific focus on the effect on *B. bruxellensis* inoculated and on the wines produced.

To better understand the effect of EW and ozone on the yeasts ecology on the grapes wine surface and during spontaneous and inoculated fermentation, we have tested two different concentrations of EW and two different forms of ozone, aqueous and gaseous, in two different time to contact for each one. This two studies highlighted the capacity of the treatments to reduce and change the yeast population present on the grapes and in the first step of the fermentations independently to the type of treatments, concentration of the active ingredient, contact time of the treatment and to the form (aqueous and gaseous). The microbiological analysis showed a reduction at about 0.5 CFU/mL in all trials after the treatments, with an interesting greater decrease of *Hanseniaspora uvarum*. Therefore, EW and ozone reduced the risk of possible spoilage in early stages of fermentation caused by apiculate yeasts. The microbiological results linked well with the chemical analysis of the wines, where lower acetic acid values were found in all wines obtained from the treated grapes. As *H. uvarum* has been described as a large producer of acetic acid (Jolly et al., 2006), its reduction in the grapes helped containing the amount of this product in the final wines.

Thanks to the promising results obtained from the first two studies, the next step was to test the use of electrolyzed water and ozone on the wine

grapes, to eliminate *B. bruxellensis* from its surface. In this work, a large amount of *B. bruxellensis* cells were inoculated on grape berries surface, to better understand the impact of the treatments on yeast population. In this case we used two different contact time for each treatment maintaining the same concentration for each different active ingredient. The results showed a good effect of gaseous ozone, independently to the time of contact, in fact, the decrease of *B. bruxellensis* was of 1.6 and 2.1 Log for the treatment at 12 h and 24 h, respectively. A slightly lower decrease was recorded for EW and aqueous ozone treatment, although a significant population reduction was registered with respect to the control. The treatment with gaseous ozone also reduced significantly the concentration of ethylphenols in wines which grapes were treated, if compared to control wines, confirming the microbiological results. The study shows the possible use of these sanitizers as alternative to SO_2 before the fermentation process to decrease the problems related to the presence of this yeast on the grape surface.

Considering the results obtained from these three studies, future experiments have to be carried out to efficiently use these two eco-friendly approaches to sanitize the wine grapes.

For the ozone use, interesting will be to test its efficiency in containing *Botrytis cinerea* on attacked wine grapes, in order to understand its the impact on wines quality, focusing on the possibility to reduce or eliminate the laccase enzyme produce by the mycelium. Finally, ozone should be tested during the harvest period in a real cellar, to monitor parameters such as: overall cost, working times, ozone form (aqueous or gaseou) and reduction of SO_2 in the first stage of winemaking. Moreover, any practical issue related to the use of the ozone in the cellar will have to be determined.

For the electrolyze water, before using it in a real cellar, it would be better

to deepen the discourse of the residual, because some studies establish the presence of little trace of chlorine on the vegetable and fruits treated (Laureano et al., 2016). This would be a problem for the wine quality, especially for possible production of trichloroanisole, compounds that give a typical cork-taint aroma.

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Annex: Research products

ISI indexed journal articles

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