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

1. Introduction

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, Ferreira, & Loureiro, 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, Fleet, & Heard, 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, Pimentel, Teixeira, & Fonseca, 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, Coulon, De Revel, Barbe, & Bely, 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, Varela, Isak, & Pretorius, 2013). In addition, on the grape surface, several studies have found *Brettanomyces* spp. (Campolongo, Rantsiou, Giordano, Gerbi, & Cocolin, 2010), which in concentrations higher than 10^4 cells/mL could produce high levels of acetic acid and undesirable volatile phenols (Kheir, Salameh, Strehaiano, Brandam, & Lteif, 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 & Thompson, 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, Nunes, Saraiva, & Coimbra, 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, Koutchma, Margas, Leadley, & Mapping, 2015; Oztekin, Zorlugenc, & Zorlugenc, 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, Yousef, & Kim, 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, & Cantalejo, 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, Margosan, & Mlikota Gabler, 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.

2. Materials and methods

2.1. 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).

2.2. 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 %.

2.3. 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.

2.4. 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, Grando, & Zini, 1992; Urso 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 and2% (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.

2.5. Molecular analysis

2.5.1. 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, Bisson, & Mills (2000). Molecular identification of the isolates was carried out by Restriction Fragment Length Polymorphism (RFLP) analysis of theITS1-5.8S ribosomal RNA (rRNA)-ITS2 region using the protocols described by Alessandria et al. (2015).

2.5.2 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, Colin, Alais, & Legras, 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.

2.5.3. 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.

2.5.4. 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).

2.5.5. 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%.

2.5.6. 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).

2.6. Chemical analysis

2.6.1. 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, Rolle, Zeppa, & Gerbi 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).

2.6.2. 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, Torchio, Giacosa, & Río Segade (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).

2.7. 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).

3. Results and discussion

3.1. 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, Comitini, & Ciani, 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. 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, Nardini, Micheletti, Nicolini, & Larcher 2013; Khadre et al., 2001).

3.2. 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 LogCFU/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, reaching undetectable levels on Lysine medium at the end of the monitored period. This sharp decrease 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, Boido, Dellacassa, & Carrau, 2012; Nissen, Nielsen, & Arneborg, 2003).

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 day2, 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*.

3.3. 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). The DGGE profile of RNAs extracted from grapes is show in Fig. S1. 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 day14. 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-culturable 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.

3.4. 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, Romano, Cocolin, & Fiore, 2001;Jolly, Augustyn, & Pretorius, 2006; Romano, Suzzi, Comi, & Zironi, 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.

3.5. 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 (Tab. S1). 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, Fiore, Paraggio, Caruso, & Capece, 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).

4. 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.

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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.

Treatments	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)	Residual sugars (g/L)
Grape 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.

Figure captions

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

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.

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.

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.

Fig.5. Score plot (A) and loading plot (B) 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.

Fig.1.

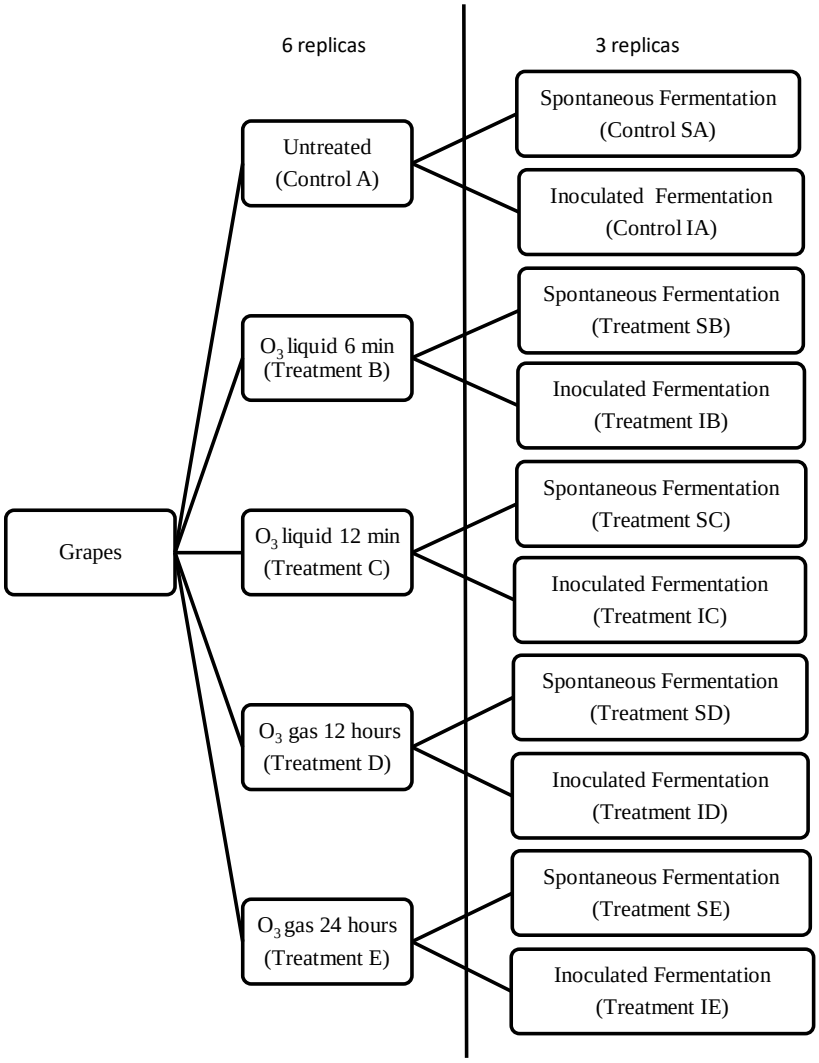

6 replicas
3 replicas
Grapes
Untreated (Control A)
O_3 liquid 6 min (Treatment B)
O_3 liquid 12 min (Treatment C)
O_3 gas 12 hours (Treatment D)
O_3 gas 24 hours (Treatment E)
Spontaneous Fermentation (Control SA)
Inoculated Fermentation (Control IA)
Spontaneous Fermentation (Treatment SB)
Inoculated Fermentation (Treatment IB)
Spontaneous Fermentation (Treatment SC)
Inoculated Fermentation (Treatment IC)
Spontaneous Fermentation (Treatment SD)
Inoculated Fermentation (Treatment ID)
Spontaneous Fermentation (Treatment SE)
Inoculated Fermentation (Treatment IE)

Fig.2.

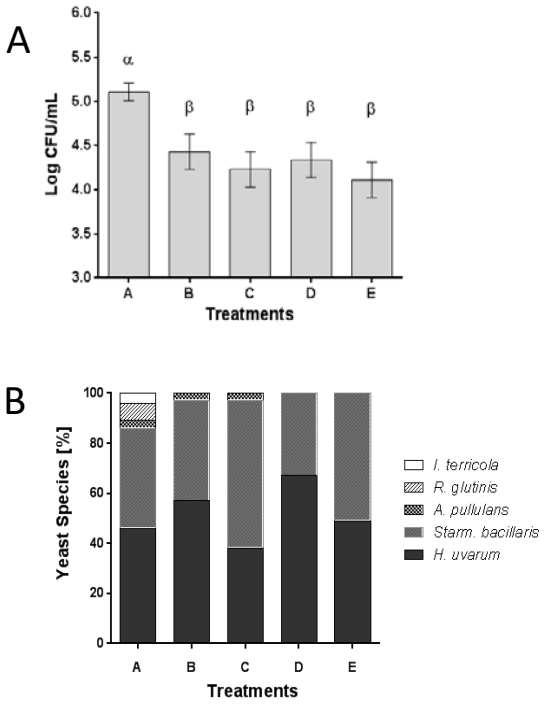

A
6.0
5.5
5.0
4.5
4.0
3.5
3.0
Log CFU/mL
α
β
β
β
β
A
B
C
D
E
Treatments
B
100
80
60
40
20
0
Yeast Species [%]
A
B
C
D
E
Treatments
I. terricola
R. glutinis
A. pullulans
Starm. bacillaris
H. uvarum

Fig.3.

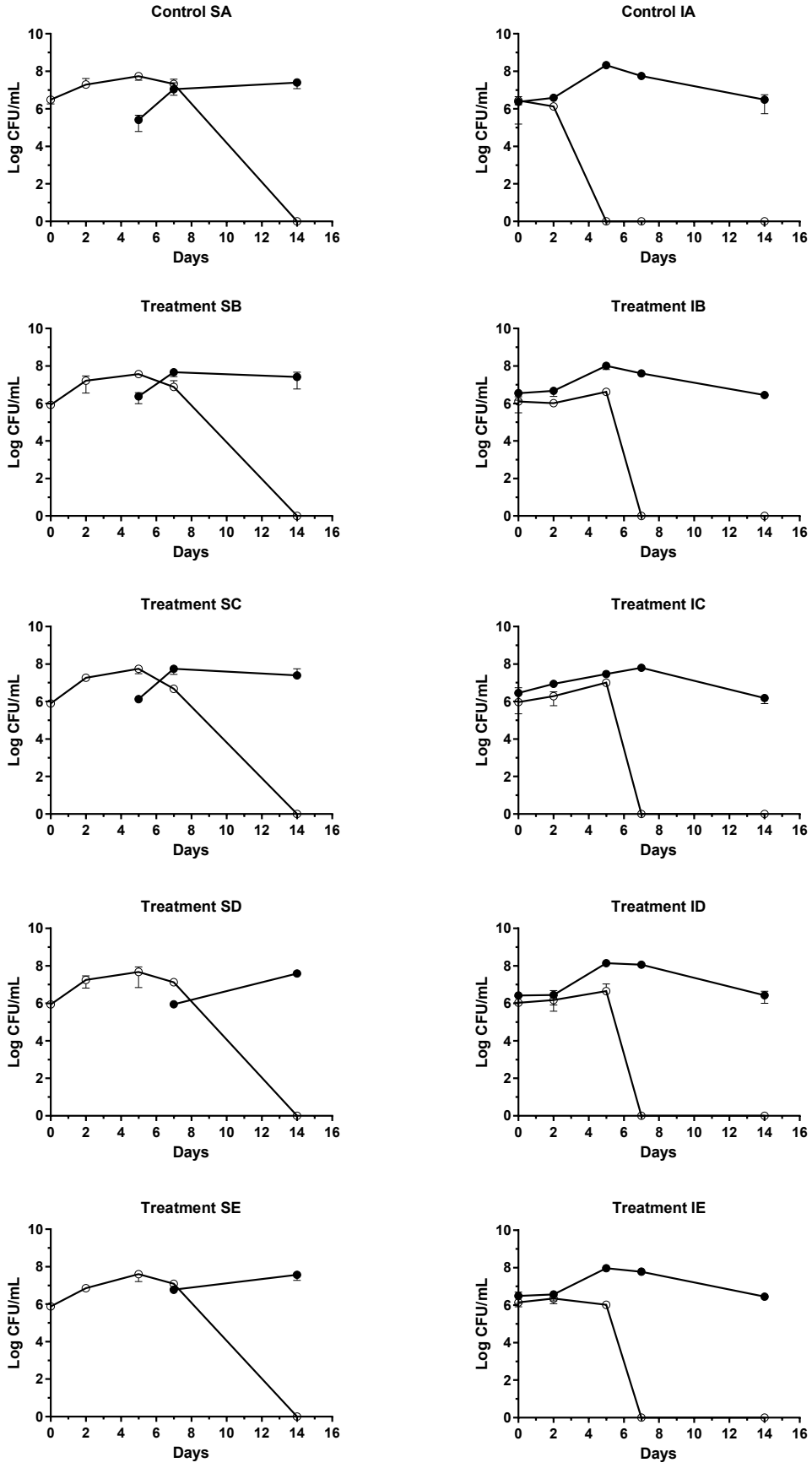

Control SA
Control IA
Treatment SB
Treatment IB
Treatment SC
Treatment IC
Treatment SD
Treatment ID
Treatment SE
Treatment IE
Log CFU/mL
Days
0 2 4 6 8 10 12 14 16
0 2 4 6 8 10

Fig.4

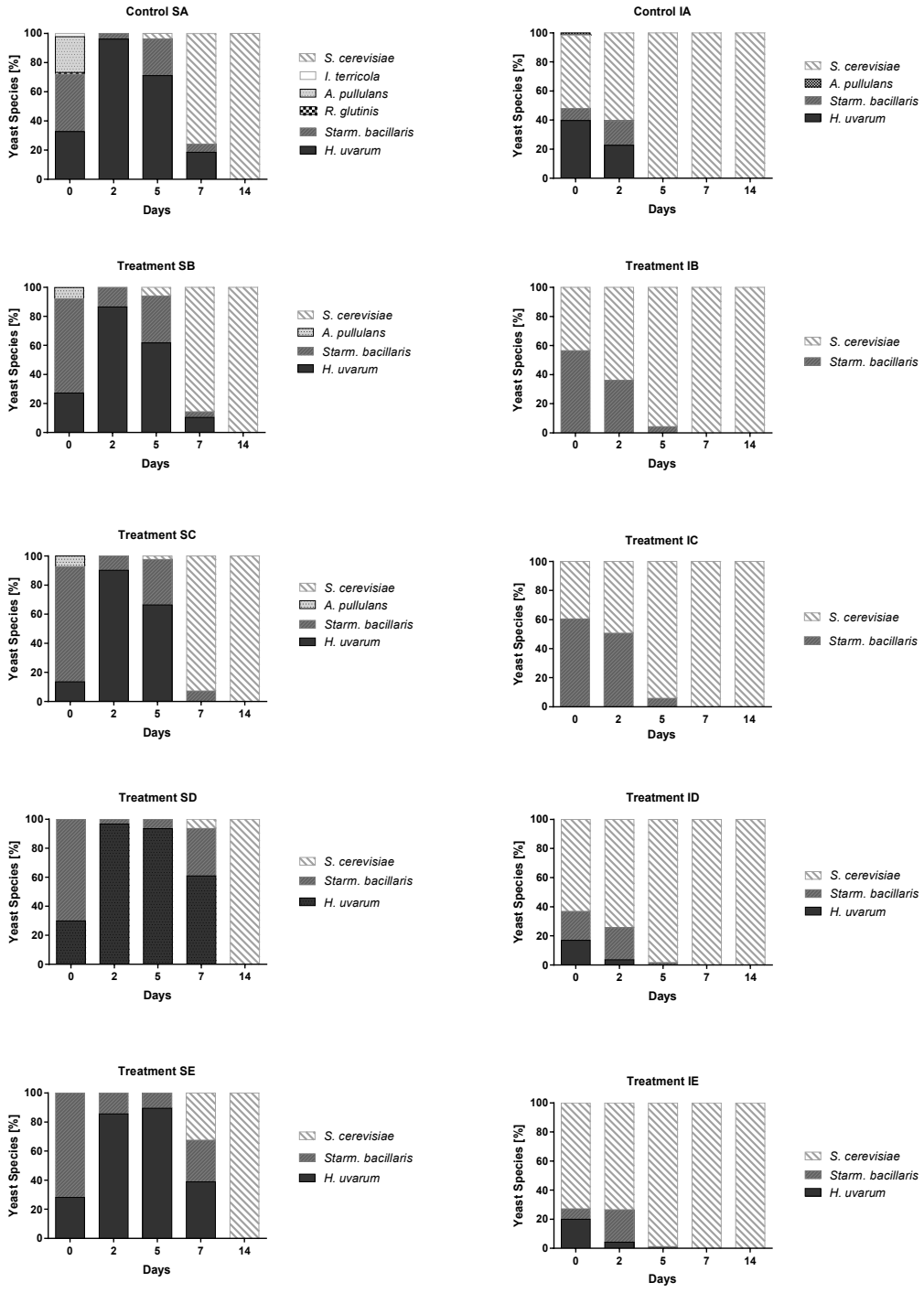
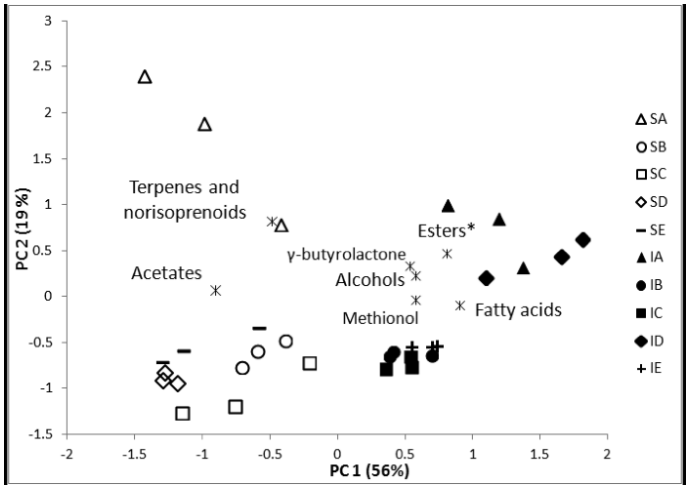

Control SA
Yeast Species [%]
Days
S. cerevisiae
I. terricola
A. pullulans
R. glutinis
Starm. bacillaris
H. uvarum
Control IA
S. cerevisiae
A. pullulans
Starm. bacillaris
H. uvarum
Treatment SB
S. cerevisiae
A. pullulans
Starm. bacillaris
H. uvarum
Treatment IB
S. cerevisiae
Starm. bacillaris
Treatment SC
S. cerevisiae
A. pullulans
Starm. bacillaris
H. uvarum
Treatment IC
S. cerevisiae
Starm. bacillaris
Treatment SD
S. cerevisiae
Starm. bacillaris
H. uvarum
Treatment ID
S. cerevisiae
Starm. bacillaris
H. uvarum
Treatment SE
S. cerevisiae
Starm. bacillaris
H. uvarum
Treatment IE
S. cerevisiae
Starm. bacillaris
H. uvarum

Fig.5.


PC2 (19%)
PC1 (56%)
Terpenes and norisoprenoids
Acetates
γ-butyrolactone
Esters*
Alcohols
Methionol
Fatty acids
SA
SB
SC
SD
SE
IA
IB
IC
ID
IE