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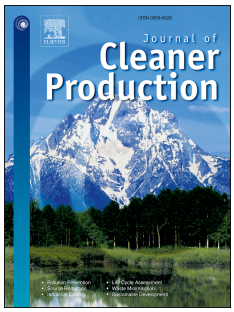

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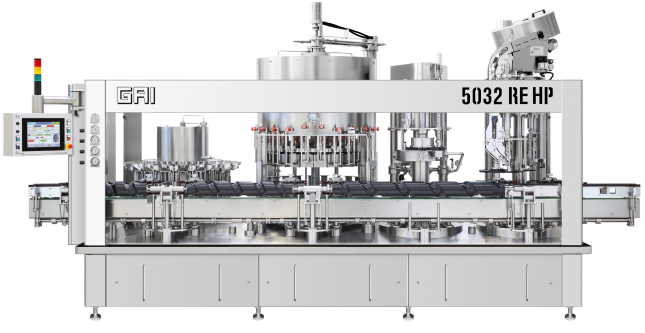
Artificially contaminated wine

Saccharomyces cerevisiae
Starmerella bacillaris
Brettanomyces bruxellensis
Zygosaccharomyces bailii
Acetobacter aceti
Oenococcus oeni

Wine circulation



CIP treatments

H_2O (control)
Peracetic acid
Aqueous ozone
Gaseous ozone

Microbiological control

Word Count: 6025

Minimizing the environmental impact of cleaning in winemaking industry by using ozone for Cleaning-in-Place (CIP) of wine bottling machine

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ABSTRACT

In winemaking industry, good cleaning and sanitization practices are essential in bottle filling process to preserve quality and avoid subsequent alterations after bottling, when microbes find environment favourable for their development. Devices connected by pipelines, like wine bottling machines, are usually cleaned using Cleaning-in-Place (CIP) method, generally requiring a high consumption of water and the use of chemical cleaning detergents with a negative impact on the environment. Ozone has recently attracted attention due to its efficacy against a broad spectrum of microorganisms and its ability to clean leaving no residues on treated surfaces, protecting the environment and human health. This study aimed to investigate the impact of aqueous (3.5 mg/L for 15 and 30 mins of contact time) and gaseous ozone (30 mg/L for 30 and 60 mins of contact time) treatments in comparison with usual sanitizing treatment with peracetic acid (1% for 15 mins of contact time) on six wine related microorganisms of oenological significance for their potential proliferation in the bottled wine. To this end, an artificially contaminated wine was used to fill rigid and flexible stainless-steel pipes and a bottling machine. The effectiveness of each treatment was evaluated using culture-dependent approach. The microorganisms showed different sensibilities to the treatments, dependent on the sanitization method used. The exposure to aqueous ozone for 30 mins was the most effective treatment for pipes cleaning, followed by peracetic acid. On the other hand, when considering the bottling machine, the use of peracetic acid as sanitizing agent led to a complete removal of the cells, while aqueous ozone for a contact of 30 mins was able to eliminate all microorganisms except *S. cerevisiae*.

Keywords: Cleaning-in-place; Peracetic acid; Ozone; Innovative sanitizing; Wine microorganisms

1. Introduction

Yeasts and bacteria are well known for their beneficial contribution in the fermentation of wine (Fleet, 2008). However, their presence in bottled wine during its shelf-life is undesirable for two reasons: (a) they depreciate the sensory appeal of the wine, and (b) some species can modify desired characteristics of the wine (Fleet, 1992). Wines are considered spoiled when they no longer appeal to the consumer. Generally, they have an unpleasant odor, appearance, taste, texture, or a combination of these defects. Microorganisms like yeasts and bacteria are well known as agents able to cause spoilage when their growth is not desirable (Du Toit and Pretorius, 2000). This alteration can occur at any phase throughout the production chain, from the grapes prior to harvest, during harvest and processing (Pinto et al., 2015), but also in the bottled wine (Loureiro and Malfeito-Ferreira, 2003).

In wine production, the bottling process is the point after which any microorganism present is undesirable and generally deleterious for wine quality (Jacobson, 2005). In particular, many bottled wines may contain small amounts of residual glucose, fructose, or malic acid that are good growth substrates for microorganisms (Loureiro and Malfeito-Ferreira, 2003). In the event of microbial alteration, species of *Acetobacter*, *Zygosaccharomyces bailii* (Zuehlke et al., 2013) and *Brettanomyces bruxellensis* (Oelofse et al., 2008) are often responsible for this process, but other species of yeasts and bacteria able to grow in bottled wine conditions may occur (Cimaglia et al., 2018). In addition, wines could undergo undesired malolactic fermentation by lactic acid bacteria (LAB) generally *Oenococcus oeni* (Valdes La Hens et al., 2014), if the concentration of malic acid in bottled wine is higher than 0.1 g/L (Ribéreau-Gayon et al., 2006). Since wines are more likely to be contaminated at the time of bottling, winemakers have to prevent these problems before and during the wine bottling process itself as a *point of no return* in wine production. Effective management of hygiene conditions, sterile (membrane) filtration and correct dosage of antimicrobial agents at this stage are essential, in order to prevent the growth of spoilage yeasts (Du Toit and Pretorius, 2000) and bacteria (Bartowsky, 2009), and to reduce organoleptic alterations during wine storage. However, some winemakers believe that wine filtration compromises red wine quality. Consequently, there is a trend to bypass this process (Arriagada-Carrazana et al., 2005). To allow a clean bottling process, pipes and bottling machines that come into direct contact with unfiltered wine must be thoroughly cleaned and sanitized to reduce possible cross-contamination. Furthermore, in many wineries, the same

production line is used to bottle multiple wines with different vintages and styles (such as red and white wines, sweet and aromatic wines). In such cases, usually only hot water is used for the cleaning of the production line and bottling machine, before changing to a different wine, and therefore the lack of sanitization could cause cross-contamination during bottling (Jacobson, 2005).

Frequent and automatic cleaning, namely CIP (Cleaning-In-Place), is often applied. The definition of CIP is given in the 1990 edition of the Society of Dairy Technology manual, as "cleaning of plant or pipelines circuits without dismantling or opening the equipment and with little or no manual involvement on the part of the operator" and "The process involves the jetting or spaying of surfaces or circulation of cleaning solutions throughout the plant under conditions of increased turbulence and flow velocity" (Romney, 1990). The use of CIP in food processing industry, like wine industry, usually consists of flushing cold or hot water, alkaline cleaning with detergents, acidic cleaning with detergents and disinfection by chemical disinfecting agents (Wirtaren and Salo, 2003). In the last decade, increasing environmental awareness has brought issues such as water scarcity and depletion of physical energy to the attention of the food and beverage industry (Pettigrew et al., 2015). Additionally, the chemical cleaning solutions used are not always biologically degradable (Tanmnay et al., 2014), while the cleaning processes contribute significantly to the overall wastewater in food processing. Hence, there is an increasing interest in the research of innovative technologies able to minimize the use of water and biologically non-degradable chemicals for CIP operation, since this problem represents one of the components of sustainable development from economic, environmental, safety and social aspects (Christaki and Tzia, 2002).

To this regard, the use of ozone (O_3) as sanitizing agent is gaining attention in the last decades, mainly due to its simple use and the high antimicrobial activity against a wide spectrum of microorganisms (Khadre et al., 2001). Ozone can be an alternative to traditional chemical solutions for microbial control (Morata et al., 2017). This molecule, generated from atoms rearrangement when oxygen molecules are subjected to intense electric discharge, has some attractive features with potential applications in food and beverage industry (Horvitz and Cantalejo, 2014). Ozone auto-decomposes into oxygen without leaving residues in food, therefore its use does not require a final rinse of the treated material to remove any residual disinfectant. Such advantages make ozone attractive to the food and beverage industry, and consequently it has been declared as GRAS (Generally Recognised As Safe) for use in food processing by the United States Food and Drug Administration (FDA, 2001). Ozone,

subsequently, gained approval as a direct additive for the treatment, storage, and processing of foods in the aqueous and gaseous phases (Morata et al., 2017). Ozone has also been used in the food industry in order to enhance food surface hygiene, sanitize food plant equipment, reuse wastewater, and reduce energy usage over time and plant waste (Guzel-Seydim et al., 2004).

In wine industry, applications of ozone have been proposed at different stages in winemaking, including sanitization of Petit Verdot (Bellincontro et al., 2017) and Barbera grape berries (Cravero et al., 2016), barrels (Guzzon et al., 2017) and tanks (Guillen et al., 2010). The antimicrobial potential of ozone (either in gaseous and aqueous form) was also evaluated against *B. bruxellensis* inoculated on post-harvest Barbera grapes (Cravero et al., 2016). Despite such uses of ozone in wine industry, little is known about the efficacy of this sanitizing agent in a CIP system. Therefore, this study aimed at investigating the effectiveness of gaseous and aqueous ozone in reducing the microbial load (including both yeasts and bacteria) present in flexible and rigid pipes (as components of the filling line) and in a bottling machine.

2. Materials and method

2.1. *Bacteria and yeast strains*

Four yeasts and two bacteria species were used in the present study (Table 1). In particular, two commercial strains *Saccharomyces cerevisiae* Uvaferm BC® and *Oenococcus oeni* VP41 (Lallemand Inc., Montreal, Canada) and four strains belonging to the culture collection of DISAFA, namely *Zygosaccharomyces bailii* Zb23, *Brettanomyces bruxellensis* B23F, *Acetobacter aceti* Sc10 and *Starmerella bacillaris* FC54 (Department of Agricultural, Forest and Food Sciences, University of Turin, Italy) were selected to artificially contaminate cv. Barbera red wine. For each yeast and bacteria species, an aliquot of a cryopreserved culture, conserved at –80 ^{o}C, was transferred to YPD broth (1% yeast extract, 2% peptone, 2% dextrose, all from Biogenetics, Italy) and MRS broth (Biogenetics) and then streaked to YPD and MRS agar plates, respectively.

2.2. Wine preparation

Vitis vinifera L. cultivar Barbera red wine containing about 14.0 g/L of residual sugars, 0.8 g/L of malic acid, 8.4 g/L of glycerol, 10.3% (v/v) ethanol, 8.90 g/L titratable acidity (expressed as g/L of tartaric acid) and with a pH of 3.44 was used in this study. Wine chemical analysis was performed using the protocols described by Rolle et al. (2018). This type of wine is susceptible to contamination because it contains residual amounts of sugars and malic acid that could be potentially consumed by the microorganisms that cause microbial degradation of wine. Prior to treatments the wine was heated to 60 ^{o}C and the absence of microorganisms was checked by plate counting using specific mediums, according to the needs of the different species examined in this study (see section 2.5).

2.3. Pipes and bottling machine characteristics

The rigid and flexible pipes used in this study are shown in Fig. 1 (Panel A and B). They are made of stainless steel and have a length of 250 cm and inner diameter of 5 cm. These pipes were used inside the bottling machine. The bottling machine used to fill the bottles with artificially contaminated wine was the model 5032RE-HO from GAI (Ceresole d'Alba, Italy). A detailed illustration of a part of the bottling filling machine used in this study is given in Fig. 2.

2.4. CIP agents preparation

The cleaning agents used in the experiment are reported in Table 2. Peracetic acid (AEB Group, Brescia, Italy) was diluted with tap water to achieve a concentration of 1%. Ozone was produced either in aqueous or gaseous form using a C32-AG ozone generator (Industrie De Nora SpA, Milan, Italy), with a nominal production of 32 g O_3/h, equipped with a UV-photometric analyzer BMT 964 (BMT Messtechnik Gmbh, GE) to control continuously the ozone concentration provided. For each experiment, artificially contaminated wine, water, 1% peracetic acid and 3.50 mg/L ozone solution were separately circulated in the rigid and flexible pipes with a peristaltic pump (SP311, Velp Scientifica, Usmate, Monza and Brianza, Italy) to maintain a constant flux. The treatment conditions were: flow of 200 mL/min and liquid temperature of 25 °C. The gaseous ozone treatments were carried out by fluxing of 32±1 μL/L of gaseous ozone in the pipes. The concentration of ozone was stable during the experiment and the ozone was continuously monitored using the abovementioned analyzer that controls the generator output. Finally, artificially contaminated

wine, peracetic acid, ozone solution, water and physiological solution were separately circulated in the bottling machine, using a pump to guarantee a constant flux during filling, using the abovementioned protocols.

2.5 Wine inoculation procedure and circulation

Pre-cultures of each yeast and bacterial species were prepared by inoculating a single colony into 5 mL of YPD and MRS broth, and then incubated at 25 ^{o}C and 30 ^{o}C, respectively, for 48 hours (yeasts) and 96 hours (bacteria). The pre-inocula of each yeast and bacterium were then sub-cultured in 50 mL of sterile Barbera must with 202.2 g/L of sugars in 100 mL Erlenmeyer flasks for 48 h and 96 h at 25 ^{o}C, for yeasts and bacteria respectively. The cells of each yeast and bacteria were then inoculated in 2 L of the same must at 1×10^{6} cells/mL and incubated at 25 ^{o}C for the same period of time. The pre-inocula were then inoculated into an adaptation medium (80.2 g/L of sugars and 7.1 % (v/v) of ethanol) at 1×10^{6} cells/mL and incubated for 4 days and 8 days at 25 ^{o}C, for yeasts and bacteria respectively. Finally, the preadapted inoculum was used to inoculate 180 L of sterile wine (14.0 g/L of sugars and 10.3 % (v/v) of ethanol. *S. cerevisiae* and *O. oeni* were inoculated as active dry preparations and rehydrated according to manufacturer's instructions.

The artificially contaminated wine was circulated for 30 mins using the peristaltic pump through the pipes to allow the possible attachment of the abovementioned microorganisms to their surfaces and then the following treatments were applied: a) circulation of sterile tap water for 15 mins and 30 mins, designating as "no CIP" control treatments; b) of 1% peracetic acid for 15 mins; c) circulation of water (25 ^{o}C) containing 3.50 ± 0.25 mg/L of ozone for 15 and 30 mins; d) circulation of enriched air with 30 ± 1 μL/L of ozone for 30 mins. Before and after each treatment, the determination of yeast and bacteria population was performed as follows: 400 mL of sterile physiological solution (9.0 g/L NaCl) was circulated under orbital shaking for 10 mins. From this volume, 10 mL were collected in 50 mL Falcon tubes and subjected to microbiological analysis, in order to quantify the microbial load of each species that was present in the pipes surfaces before and after sanitization. Each treatment was performed in triplicate.

In addition, artificially contaminated wine (130 L) was pumped through the bottling machine for 30 mins and used to fill three sterile glass bottles, which were located at three different sites (nozzle 1, 6 and 18) (Fig. 2). At the end of the circulation, the bottling machine was cleaned using the following treatments: a) circulation of sterile water for 30 mins,

designating as "no CIP" control treatment; b) circulation with 1% peracetic acid for 15 mins; c) circulation of water (25 ^{o}C) containing 3.50 ± 0.25 mg/L of ozone for 30 mins at 200 mL/min; and d) circulation of enriched air with 30 ± 1 μL/L of ozone for 60 mins (in this case the contact time with gaseous ozone was extended for another 30 mins, due to the longer circuit present in the bottling machine than the pipes). Before and after each treatment, the yeasts and bacteria load present in the circuit of the bottling machine were determined by circulating 130 L of the abovementioned physiological solution through the bottling machine for 10 mins. At the end of each circulation, three sterile glass bottles located at three different sites (nozzle 1, 6 and 18) along the filling line were filled with sterile physiological solution, which was subjected to microbiological analysis in order to evaluate the population of the inoculated yeasts and bacteria in the bottle filling machine before and after CIP treatments. It is worth mentioning that the absence of microorganisms from the circuit of the bottling machine prior to bottling initiation is based on measuring the microbial load present in sterile glass bottles, containing sterile physiological solution that is previously circulated through the bottling machine for 10 mins. Each treatment was performed in triplicate.

2.6. Microbiological analyses

For all samples, decimal dilutions in sterile physiological solution were made. The enumeration of yeasts and bacteria was carried out by plating adequate dilutions onto plates (duplicate) of several culture mediums: (1) *S. cerevisiae* and *Starm. bacillaris* on Wallerstein laboratory Nutrient agar medium (Biogenetics) and incubated at 28 ^{o}C for 5 days, (2) *Z. bailii* and *B. bruxellensis* on selective/differential medium ZDM (Sculler et al., 2000) and DBDM (Rodrigues et al., 2001), respectively, (3) *O. oeni* on double-layer MRS agar (pH 5.2), supplemented with malic acid (10 g/L, Sigma, Milan, Italy), delvocid (25 mg/L; DSM Specialties, Heerlen, The Netherlands) and incubated at 30 ^{o}C for 7 days; (4) AAB on ethanol agar [10 g/L yeast extract, 20 g/L $CaCO_3$ (Sigma), 20 g/L and 20 mL ethanol (Sigma)], supplemented with delvocid and incubated at 30 ^{o}C for 7 days. After counting, means and deviation standards were calculated.

2.7. Statistical analyses

All data were statistically analyzed using the software IBM SPSS Statistics (IBM Corp., Armonk, NY, USA). Tukey-HSD post-hoc test was used to establish significant

differences by one-way ANOVA ($p < 0.05$).

3. Results

3.1. Flexible and rigid pipes sanitization treatments and effect on yeasts and bacteria

The viable count of each of the six microorganisms (4 yeasts and 2 bacteria), recovered from rigid and flexible pipes before and after each treatment, is reported in Fig. 3. The initial load of yeasts and bacteria, after circulation of the artificially contaminated wine, in rigid and flexible pipes, and before the treatments, were: 5.45 ± 0.21 Log CFU/mL for *S. cerevisiae*, 5.24 ± 0.34 Log CFU/mL for *Starm. bacillaris*, 5.15 ± 0.21 Log CFU/mL for *B. bruxellensis*, 5.45 ± 0.24 Log CFU/mL for *Z. bailii*, 4.30 ± 0.43 Log CFU/mL for *A. aceti*, 4.50 ± 0.28 Log CFU/mL for *O. oeni*. As seen in Fig. 3, plate counts highlighted significant differences between the treated and untreated rigid and flexible pipes. Almost all treatments with peracetic acid and ozone had a significantly stronger effect on yeast vitality with respect to the control treatments (sterile tap water for 15 and 30 mins) even though controls reduced the population of yeasts and bacteria, independently in both types of pipe tested. Indeed, washing the pipes with sterile tap water significantly reduced the yeast/bacterial populations by 0.7 to 3.8 Log CFU/mL. Greater reduction was mostly registered after cleaning with sterile tap water for 30 mins than for 15 mins. However, no significant differences were registered between the two control treatments (15 mins and 30 mins) for some microorganisms, like *O. oeni* (rigid and flexible pipes), *S. cerevisiae*, *Starm. bacillaris*, *B. bruxellensis* and *Z. bailii* (flexible pipes). Aqueous ozone treatment for 30 mins was the most effective in reducing the yeasts and bacteria population to undetectable levels (< 10 CFU/mL), independently of the species and type of pipes used. In most cases, peracetic acid (1 %), aqueous ozone (15 mins) and gaseous ozone (30 mins) were less effective than aqueous ozone for 30 mins, but they had higher populations reductions compared to the sterile tap water control.

All treatments of flexible pipes with aqueous ozone (15 mins and 30 mins) and peracetic acid reduced the population of *Starm. bacillaris* to undetectable levels (<10 CFU/mL). The *S. cerevisiae* population was significantly reduced after treatments in rigid and flexible pipes by approximately 2.2–5.4 Log CFU/mL (initial population 5.4 Log CFU/mL). More specifically, the performance of each treatment was as follows: water for 15

and 30 mins led to a reduction of 2.20 to 3.30 Log CFU/mL; treatment with 1% peracetic acid led to a reduction of 3.0 to 4.35 Log CFU/mL; the aqueous ozone for 15 and 30 mins led to a reduction of 3.05 to 5.45 Log CFU/mL; and gaseous ozone for 30 mins to a reduction of 2.54 to 3.97 Log CFU/mL. Therefore, the reduction level of *S. cerevisiae* population was affected by the type of treatment, and also by the type of pipe used, as the reduction level was found higher in flexible tubes with the exception of gaseous ozone treatment. Similar results were obtained for *Starm. bacillaris* cells present in rigid pipes, since the peracetic acid for 15 mins and aqueous ozone treatments (either for 15 and 30 mins) removed it completely from the flexible pipes. Concerning the two spoilage yeasts, *B. bruxellensis* and *Z. bailii*, the aqueous ozone (15 mins) treatment decreased their populations from 5.40 ± 0.23 Log CFU/mL to 1.10 ± 0.10 Log CFU/mL in rigid pipes, which corresponds to an average reduction of 4.3 Log CFU/mL, while the other sanitizing treatments removed completely these yeasts from the pipes surface, independently by the type of pipe used. The populations of the artificially inoculated bacteria, *A. aceti* and *O. oeni* on rigid pipes, significantly decreased from 4.40 ± 0.24 Log CFU/mL to 1.20 ± 1.20 Log CFU/mL after treatments with peracetic acid and ozone (15 and 30 mins), which corresponds to a reduction of 2.2–3.4 Log CFU/mL. It appeared that 30-mins aqueous ozone treatment was the most effective in eliminating these bacteria from rigid and flexible pipes.

3.2. Effect of bottling equipment sanitization treatments on yeasts and bacteria

The efficacy of cleaning treatments with water (30 mins), 1% peracetic acid (15 mins), aqueous ozone (30 mins) and gaseous ozone (60 mins) in reducing yeasts and bacteria populations after bottle filling of artificially contaminated wine using a wine bottling machine is presented in Fig. 4. The average population recovered from the bottling machine after bottle filling and before treatment was 4.80 ± 0.28 Log CFU/mL for *S. cerevisiae*, 5.22 ± 0.37 Log CFU/mL for *Starm. bacillaris*, 5.45 ± 0.21 Log CFU/mL for *B. bruxellensis*, 5.15 ± 0.22 Log CFU/mL for *Z. bailii*, 4.54 ± 0.09 Log CFU/mL for *A. aceti*, and 4.77 ± 0.10 Log CFU/mL for *O. oeni*. Bottling machine washed with sterile tap water for 30 mins (control) yielded average population from 3.03 to 4.10 Log CFU/mL for all inoculated species, independently on the nozzle location. Complete elimination of *Starm. bacillaris*, *A. aceti* and *O. oeni* cells from the bottling machine circuit was observed independently of the sanitizing treatment used (1% peracetic acid, aqueous ozone and gaseous ozone). It is worth noticing that the efficiency of the treatments used for bottling machine sanitization was not influenced

by the nozzles position, since non-significant differences were observed between the populations of microorganisms recovered from the different nozzles (data not shown).

In this context, washing the bottling machine with peracetic acid and ozone (either in aqueous or gaseous form) resulted in a significant reduction of the yeasts and bacteria counts compared to control treatment (sterile tap water) at the three sampling points (nozzles 1, 6 and 18) with some exceptions for gaseous ozone. Peracetic acid was the most effective in reducing to undetectable levels (< 10 CFU/mL) the population of yeasts and bacteria present on bottling machine surface, even compared to ozone treatments. The use of aqueous ozone for 30 mins decreased the populations of the inoculated yeasts and bacteria to undetectable levels, except for the *S. cerevisiae* species, whose population decreased from 4.80 Log CFU/mL to 1.00 Log CFU/mL. Moreover, gaseous ozone for 60 mins was the less effective treatment since only *Starm. bacillaris*, *A. aceti* and *O. oeni* were completely removed from the bottling machine surface, whereas about 2.0–3.5 Log CFU/mL were recovered for other microorganisms after treatment.

4. Discussion

The use of ozone as an antimicrobial agent in winemaking industry has been proposed for a number of yeasts and bacteria present on grapes (Guzzon et al., 2018) and winemaking barrels (Guzzon et al., 2017). In the present study, the possibility of using peracetic acid and ozone (either in aqueous or gaseous form) to remove yeasts and bacteria from stainless steel surfaces was investigated. The sanitizing agents used significantly improved the removal of the attached populations of each inoculated yeast and bacteria, compared to the control sterile tap water treatments, with some exceptions; particularly for *S. cerevisiae* and *Starm. bacillaris*. In addition, results demonstrated that gaseous and aqueous ozone at low dose is effective in reducing the numbers of the microorganisms used in this study, in agreement with general observations that low doses of this sanitizing agent are able to reduce the populations of bacteria, moulds, yeasts and viruses (Morata et al., 2017). However, in this study, longer than 15 mins contact time is necessary in order to ensure complete elimination of most yeasts and bacteria. Concerning the two spoilage yeasts, *B. bruxellensis* and *Z. bailii*, they were very sensitive to ozone treatments (either in aqueous or gaseous form), since they were the only microorganisms that ozone treatments (except aqueous ozone for 15 mins in rigid pipes) reduced their population to undetectable levels (<10 CFU/mL), independently of the pipe structure used. These results are in good agreement with those reported by Guzzon et

al. (2011), which have suggested greater sensitivity of ozone treatments to *Brettanomyces*/*Dekkera* than other oenological yeasts, on the basis of a survey of the effect of ozone on winemaking barrel microbiota. Additionally, the results of this study are in accordance with general observations that the efficiency of ozone as sanitizing agent depends on the strains and species of the microorganism, the age of the treated culture population, the presence of ozone demanding medium components, and the form of ozone treatment (aqueous or gaseous form) (Kim et al., 2003).

As already mentioned, in the food industry much attention is given to cleaning and sanitization operations of food-processing equipment, both in preventing product contamination and to maintain equipment functionality (Mahapatra et al., 2005). In wine industry, bottle filling is a critical operation since it is the last contamination source before wine is released to the market. In recent years, bottling line sanitization and overall plant hygiene standards in wineries have contributed to a significant improvement of the quality of the wine bottling. In addition to this, the incidence of yeast spoilage in bottled wines also decreased because of increased adoption of sterile filtration immediately before bottling (Loureiro and Malfeito-Fereira, 2003). However, these improvements have not sufficed to reduce the levels of chemical preservatives used even in sweet and dry wines sterilized by filtration before bottling. The microorganisms tested in this study were chosen carefully focusing on the risk of wine alteration in bottle, because of their resistance to high levels of ethanol and their ability to ferment residual sugars and malic acid forming turbidity, sediment and gassiness in the bottle (Du Toit and Pretorius, 2000). The results demonstrated that washing the filling machine with peracetic acid and ozone (either in aqueous and gaseous form) resulted in a significant reduction of the yeasts and bacteria counts compared to controls at the three sampling points, while no significant differences were observed between the population of microorganisms recovered from the different nozzles. This highlights the ability of all the sanitizing agents used in this study to ensure a good contact with the treated surface.

Concerning the impact of the abovementioned sanitizing treatments on each microorganism, higher sensitivities were observed for *Starm. bacillaris*, *A. aceti* and *O. oeni*, since their populations were reduced to undetectable levels after treatments, independently on the nozzle position. Peracetic acid was the most effective treatment in killing yeasts and bacteria on filling machine surface, compared to ozone treatments. Particularly, the use of aqueous ozone for 30 mins was less effective only for *S. cerevisiae* cells (population decrease to about 1.00 Log CFU/mL) whereas higher populations of *S. cerevisiae*, *B. bruxellensis* and

Z. bailli were recovered after treatment with ozone gas (about 2.0–3.5 Log CFU/mL).

To date, there are few published studies that evaluated the efficacy of sanitizing and antimicrobial agents against yeasts and bacteria, either in suspensions or on surfaces, and the removal of biofilms (Wirtanen and Salo, 2003). Thus, effectiveness is usually determined in tests with free cells in suspension, which do not faithfully represent the conditions present on surfaces where the agents are required to inactivate microorganisms (Gibson et al., 1999). The cells adhered to surfaces are more difficult to remove (Garrett et al., 2008). These observations may explain the fact that after aqueous ozone treatments the *S. cerevisiae* cells attached to bottling machine surfaces showed a higher resistance to sanitizing agent, compared to the pipes. In addition to this, the differences observed in the efficacy of the treatments to reduce the population of the inoculated microorganisms in pipes and bottling machine could be explained by the differences in pipes shape and diameter. The latter is an important factor since pipes modulate the flow characteristics of the liquid and, consequently, cleaning efficiency (White, 1999). Some authors investigated the critical points of wine bottling machines, which were found to be the bell rubbers and rubber spacers, the outlet side of the sterilizing filter and the filler (Loureiro and Malfeito-Fereira, 2003). In particular, bell rubbers and/or spacers were observed to be continually splashed with wine and exposed to air between filling, providing an excellent environment for yeast growth (Donelly, 1977). This last aspect could explain the lower efficiency of aqueous ozone when compared to that obtained on the pipes.

5. Conclusion

This is the first time that peracetic acid (common antimicrobial agent) and ozone (alternative innovative agent) were compared to reduce the population of six wine related microorganisms present in stainless steel pipes and bottling machine, after circulation of artificially contaminated red wine. Among treatments, aqueous ozone for 30 mins contact time displayed enhanced antimicrobial activity, since it was the only treatment able to guarantee sanitization in rigid and flexible pipes. In the case of ozone-treated bottling machine, the same situation was observed, except for *S. cerevisiae*, which was found in the bottled wines although in significantly lower populations. Ozone technology can fulfil the growing demand of winemakers for increasing the shelf-life of bottled wines and for reducing the use of biologically non-degradable chemicals for CIP operation. However, the choice of this sanitizing agent is critical for keeping product quality and safety, since its efficiency

419 depends on many factors, such as type of cleaning, exposure time, and microorganisms target
420 and the characteristics of the surface treated. Future studies may focus on the industrial
421 application of the suggested protocol.
422
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427
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Table 1

Origin of the four yeasts and two bacteria strains used in this study

Strain	Species	Origin
Uvaferm BC®	*Saccharomyces cerevisiae*	Lallemand[a]
FC54	*Starmerella bacillaris*	DISAFA[b]
B23F	*Brettanomyces bruxellensis*	DISAFA[b]
MT1	*Zygosaccharomyces bailii*	DISAFA[b]
BA23	*Acetobacter aceti*	DISAFA[b]
VP41	*Oenococcus oeni*	Lallemand[a]

[a] Lallemand Inc. (Montreal, Canada)

[b] Yeast culture collection of DISAFA, Department of Agricultural, Forest and Food Sciences, University of Turin, Italy

Table 2

Cleaning agents used in this study; PAA: peracetic acid

Treatment	Contact time	Pipes	Bottling machine
H_2O	15 mins	x	
H_2O	30 mins	x	x
PAA	15 mins	x	x
O_3 aqueous	15 mins	x	
O_3 aqueous	30 mins	x	x
O_3 gaseous	30 mins	x	x
O_3 gaseous	60 mins		x

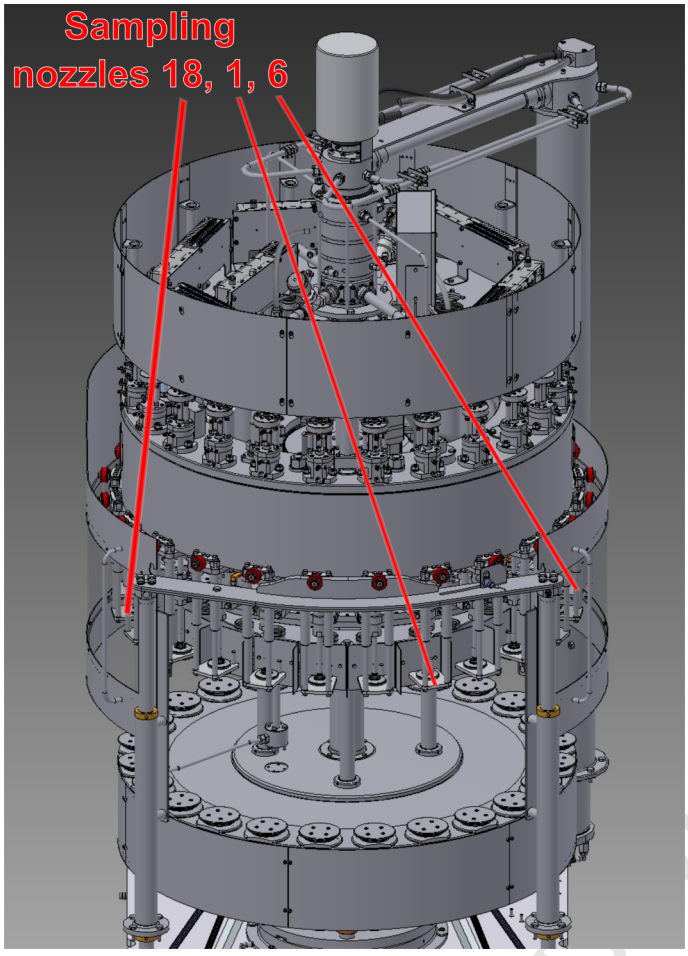
Figure captions

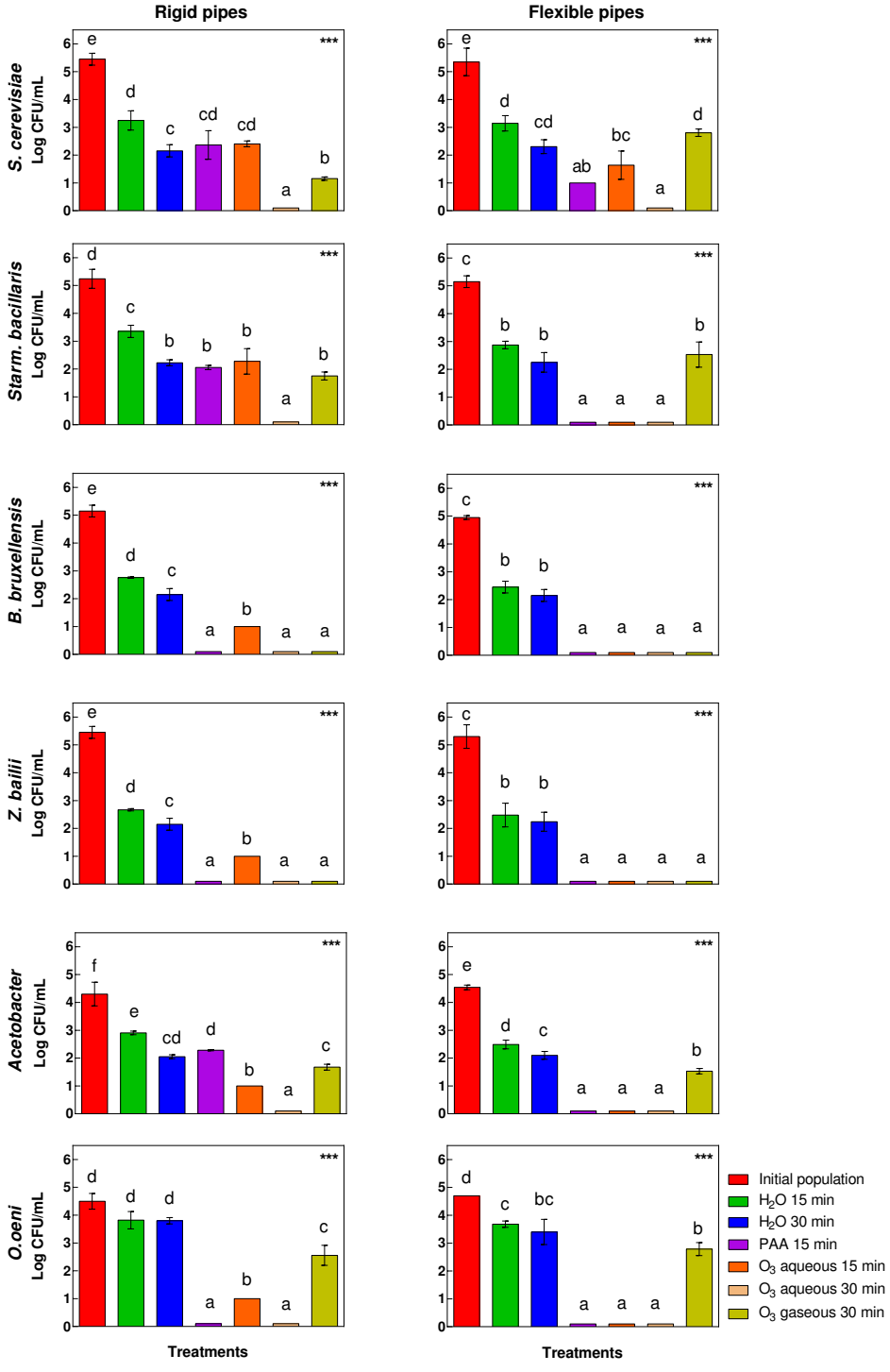
Fig. 1 Flexible (Panel A) and rigid pipes (Panel B) used in this study.

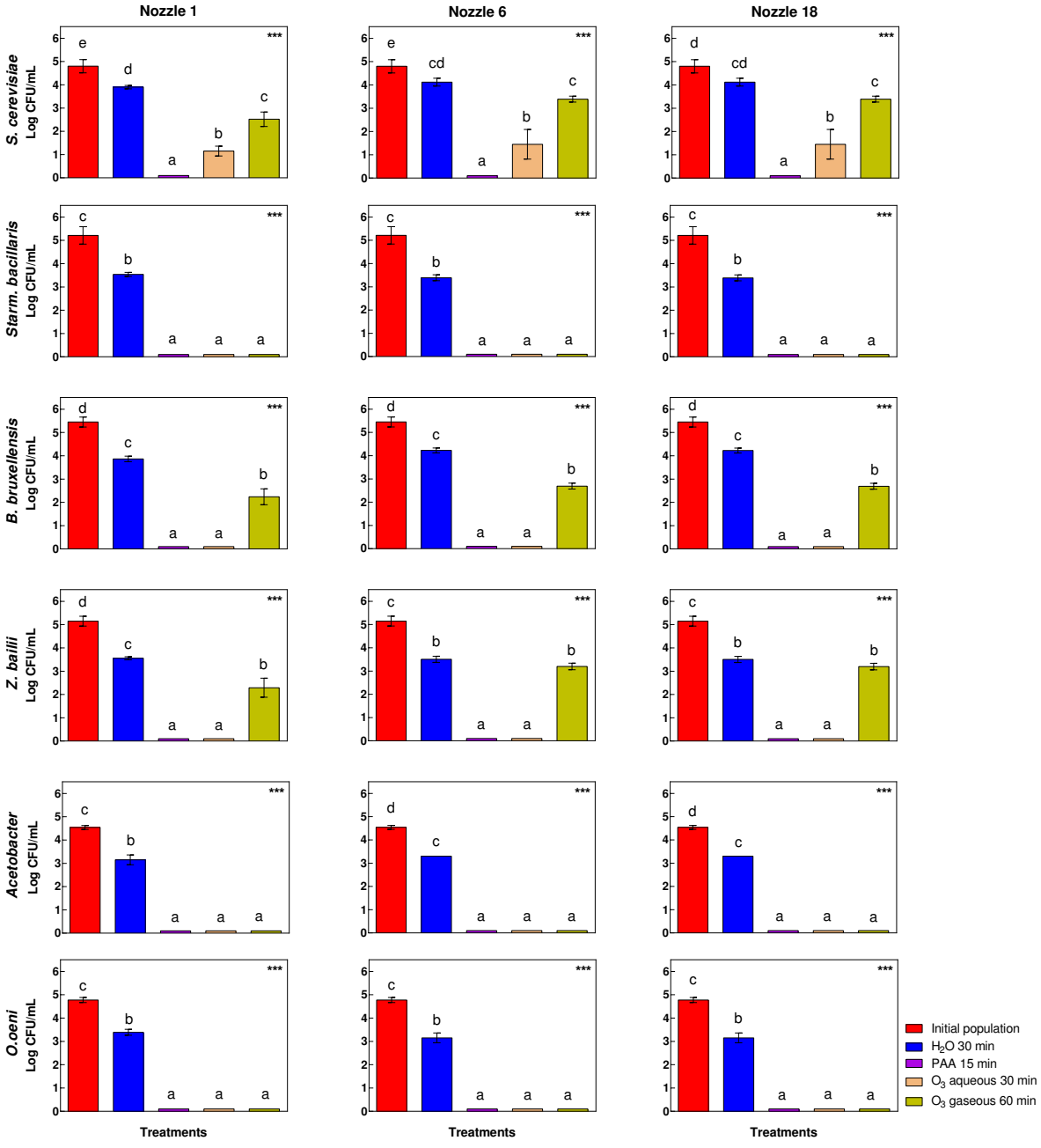
Fig. 2 Detailed illustration of the bottling filling machine used in this study.

Fig. 3 Viable counts (Log_{10}CFU/mL) of yeast and bacterial populations recovered from rigid and flexible pipes, before and after treatments with H_20 for 15 and 30 mins, 1% peracetic acid for 15 mins, aqueous ozone for 15 and 30 mins, gaseous ozone for 30 mins. Data are the mean (±SD) of three biological replicates. The different letters in each column indicated significant differences according to ANOVA and Tukey-HSD test ($p < 0.05$).

Fig. 4 Viable counts (Log_{10}CFU/mL) of yeasts and bacteria populations recovered from, the bottling machine, before and after cleaning with H_2O for 30 mins, 1% peracetic acid for 15 mins, aqueous (30 mins) and gaseous (60 mins) ozone. Data are the mean (±SD) of three biological replicates. The different letters in each column indicated significant differences according to ANOVA and Tukey-HSD test ($p < 0.05$).







Highlights

- Good cleaning and sanitization practices are essential in wine bottling process.
- Microorganisms showed different sensibilities to the sanitization treatments.
- Aqueous ozone was the most effective treatment for pipes cleaning.
- Aqueous ozone removed all microorganisms except *S. cerevisiae* from bottling machine.
- The use of ozone for CIP could reduce non-degradable biologically chemicals.