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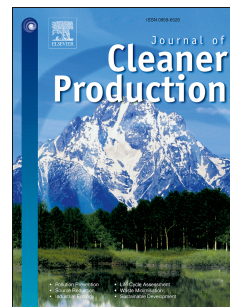
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Minimizing the environmental impact of cleaning in winemaking industry by using ozone for cleaning-in-place (CIP) of wine bottling machine

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## Artificially contaminated wine



## Wine circulation



## CIP treatments

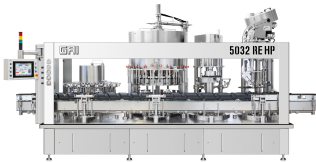
H<sub>2</sub>O (control)  
Peracetic acid  
Aqueous ozone  
Gaseous ozone



## Microbiological control



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



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2

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

5

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18

19

20 **ABSTRACT**

21

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

42

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

45

46

## 47 1. Introduction

48

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

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

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

86 Frequent and automatic cleaning, namely CIP (Cleaning-In-Place), is often applied.  
87 The definition of CIP is given in the 1990 edition of the Society of Dairy Technology  
88 manual, as “cleaning of plant or pipelines circuits without dismantling or opening the  
89 equipment and with little or no manual involvement on the part of the operator” and “The  
90 process involves the jetting or spaying of surfaces or circulation of cleaning solutions  
91 throughout the plant under conditions of increased turbulence and flow velocity” (Romney,  
92 1990). The use of CIP in food processing industry, like wine industry, usually consists of  
93 flushing cold or hot water, alkaline cleaning with detergents, acidic cleaning with detergents  
94 and disinfection by chemical disinfecting agents (Wirtaren and Salo, 2003). In the last  
95 decade, increasing environmental awareness has brought issues such as water scarcity and  
96 depletion of physical energy to the attention of the food and beverage industry (Pettigrew et  
97 al., 2015). Additionally, the chemical cleaning solutions used are not always biologically  
98 degradable (Tanmnay et al., 2014), while the cleaning processes contribute significantly to  
99 the overall wastewater in food processing. Hence, there is an increasing interest in the  
100 research of innovative technologies able to minimize the use of water and biologically non-  
101 degradable chemicals for CIP operation, since this problem represents one of the components  
102 of sustainable development from economic, environmental, safety and social aspects  
103 (Christaki and Tzia, 2002).

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

115 subsequently, gained approval as a direct additive for the treatment, storage, and processing  
116 of foods in the aqueous and gaseous phases (Morata et al., 2017). Ozone has also been used  
117 in the food industry in order to enhance food surface hygiene, sanitize food plant equipment,  
118 reuse wastewater, and reduce energy usage over time and plant waste (Guzel-Seydim et al.,  
119 2004).

120 In wine industry, applications of ozone have been proposed at different stages in  
121 winemaking, including sanitization of Petit Verdot (Bellincontro et al., 2017) and Barbera  
122 grape berries (Cravero et al., 2016), barrels (Guzzon et al., 2017) and tanks (Guillen et al.,  
123 2010). The antimicrobial potential of ozone (either in gaseous and aqueous form) was also  
124 evaluated against *B. bruxellensis* inoculated on post-harvest Barbera grapes (Cravero et al.,  
125 2016). Despite such uses of ozone in wine industry, little is known about the efficacy of this  
126 sanitizing agent in a CIP system. Therefore, this study aimed at investigating the  
127 effectiveness of gaseous and aqueous ozone in reducing the microbial load (including both  
128 yeasts and bacteria) present in flexible and rigid pipes (as components of the filling line) and  
129 in a bottling machine.

130

## 131 2. Materials and method

132

### 133 2.1. Bacteria and yeast strains

134

135 Four yeasts and two bacteria species were used in the present study (Table 1). In  
136 particular, two commercial strains *Saccharomyces cerevisiae* Uvaferm BC<sup>®</sup> and *Oenococcus*  
137 *oeni* VP41 (Lallemand Inc., Montreal, Canada) and four strains belonging to the culture  
138 collection of DISAFA, namely *Zygosaccharomyces bailii* Zb23, *Brettanomyces bruxellensis*  
139 B23F, *Acetobacter aceti* Sc10 and *Starmerella bacillaris* FC54 (Department of Agricultural,  
140 Forest and Food Sciences, University of Turin, Italy) were selected to artificially contaminate  
141 cv. Barbera red wine. For each yeast and bacteria species, an aliquot of a cryopreserved  
142 culture, conserved at  $-80^{\circ}\text{C}$ , was transferred to YPD broth (1% yeast extract, 2% peptone,  
143 2% dextrose, all from Biogenetics, Italy) and MRS broth (Biogenetics) and then streaked to  
144 YPD and MRS agar plates, respectively.

145

### 146 2.2. Wine preparation

147



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

157

### 158 2.3. Pipes and bottling machine characteristics

159

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

166

### 167 2.4. CIP agents preparation

168

169 The cleaning agents used in the experiment are reported in Table 2. Peracetic acid  
170 (AEB Group, Brescia, Italy) was diluted with tap water to achieve a concentration of 1%.  
171 Ozone was produced either in aqueous or gaseous form using a C32-AG ozone generator  
172 (Industrie De Nora SpA, Milan, Italy), with a nominal production of 32 g O<sub>3</sub>/h, equipped with  
173 a UV-photometric analyzer BMT 964 (BMT Messtechnik GmbH, GE) to control  
174 continuously the ozone concentration provided. For each experiment, artificially  
175 contaminated wine, water, 1% peracetic acid and 3.50 mg/L ozone solution were separately  
176 circulated in the rigid and flexible pipes with a peristaltic pump (SP311, Velp Scientifica,  
177 Usmate, Monza and Brianza, Italy) to maintain a constant flux. The treatment conditions  
178 were: flow of 200 mL/min and liquid temperature of 25 °C. The gaseous ozone treatments  
179 were carried out by fluxing of 32±1 µL/L of gaseous ozone in the pipes. The concentration of  
180 ozone was stable during the experiment and the ozone was continuously monitored using the  
181 abovementioned analyzer that controls the generator output. Finally, artificially contaminated

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

185

## 186 2.5 Wine inoculation procedure and circulation

187

188 Pre-cultures of each yeast and bacterial species were prepared by inoculating a single  
189 colony into 5 mL of YPD and MRS broth, and then incubated at 25 °C and 30 °C,  
190 respectively, for 48 hours (yeasts) and 96 hours (bacteria). The pre-inocula of each yeast and  
191 bacterium were then sub-cultured in 50 mL of sterile Barbera must with 202.2 g/L of sugars  
192 in 100 mL Erlenmeyer flasks for 48 h and 96 h at 25 °C, for yeasts and bacteria respectively.  
193 The cells of each yeast and bacteria were then inoculated in 2 L of the same must at  
194  $1 \times 10^6$  cells/mL and incubated at 25 °C for the same period of time. The pre-inocula were  
195 then inoculated into an adaptation medium (80.2 g/L of sugars and 7.1 % (v/v) of ethanol) at  
196  $1 \times 10^6$  cells/mL and incubated for 4 days and 8 days at 25 °C, for yeasts and bacteria  
197 respectively. Finally, the preadapted inoculum was used to inoculate 180 L of sterile wine  
198 (14.0 g/L of sugars and 10.3 % (v/v) of ethanol). *S. cerevisiae* and *O. oeni* were inoculated as  
199 active dry preparations and rehydrated according to manufacturer's instructions.

200 The artificially contaminated wine was circulated for 30 mins using the peristaltic  
201 pump through the pipes to allow the possible attachment of the abovementioned  
202 microorganisms to their surfaces and then the following treatments were applied: a)  
203 circulation of sterile tap water for 15 mins and 30 mins, designating as "no CIP" control  
204 treatments; b) of 1% peracetic acid for 15 mins; c) circulation of water (25 °C) containing  
205  $3.50 \pm 0.25$  mg/L of ozone for 15 and 30 mins; d) circulation of enriched air with  $30 \pm 1$   $\mu$ L/L  
206 of ozone for 30 mins. Before and after each treatment, the determination of yeast and bacteria  
207 population was performed as follows: 400 mL of sterile physiological solution (9.0 g/L NaCl)  
208 was circulated under orbital shaking for 10 mins. From this volume, 10 mL were collected in  
209 50 mL Falcon tubes and subjected to microbiological analysis, in order to quantify the  
210 microbial load of each species that was present in the pipes surfaces before and after  
211 sanitization. Each treatment was performed in triplicate.

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

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

231

## 232 2.6. Microbiological analyses

233

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

245

## 246 2.7. Statistical analyses

247

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

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

251

### 252 3. Results

253

#### 254 3.1. Flexible and rigid pipes sanitization treatments and effect on yeasts and bacteria

255

256 The viable count of each of the six microorganisms (4 yeasts and 2 bacteria),  
257 recovered from rigid and flexible pipes before and after each treatment, is reported in Fig. 3.  
258 The initial load of yeasts and bacteria, after circulation of the artificially contaminated wine,  
259 in rigid and flexible pipes, and before the treatments, were:  $5.45 \pm 0.21$  Log CFU/mL for *S.*  
260 *cerevisiae*,  $5.24 \pm 0.34$  Log CFU/mL for *Starm. bacillaris*,  $5.15 \pm 0.21$  Log CFU/mL for *B.*  
261 *bruxellensis*,  $5.45 \pm 0.24$  Log CFU/mL for *Z. bailii*,  $4.30 \pm 0.43$  Log CFU/mL for *A. aceti*,  
262  $4.50 \pm 0.28$  Log CFU/mL for *O. oeni*. As seen in Fig. 3, plate counts highlighted significant  
263 differences between the treated and untreated rigid and flexible pipes. Almost all treatments  
264 with peracetic acid and ozone had a significantly stronger effect on yeast vitality with respect  
265 to the control treatments (sterile tap water for 15 and 30 mins) even though controls reduced  
266 the population of yeasts and bacteria, independently in both types of pipe tested. Indeed,  
267 washing the pipes with sterile tap water significantly reduced the yeast/bacterial populations  
268 by 0.7 to 3.8 Log CFU/mL. Greater reduction was mostly registered after cleaning with  
269 sterile tap water for 30 mins than for 15 mins. However, no significant differences were  
270 registered between the two control treatments (15 mins and 30 mins) for some  
271 microorganisms, like *O. oeni* (rigid and flexible pipes), *S. cerevisiae*, *Starm. bacillaris*, *B.*  
272 *bruxellensis* and *Z. bailii* (flexible pipes). Aqueous ozone treatment for 30 mins was the most  
273 effective in reducing the yeasts and bacteria population to undetectable levels ( $< 10$   
274 CFU/mL), independently of the species and type of pipes used. In most cases, peracetic acid  
275 (1 %), aqueous ozone (15 mins) and gaseous ozone (30 mins) were less effective than  
276 aqueous ozone for 30 mins, but they had higher populations reductions compared to the  
277 sterile tap water control.

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

283 and 30 mins led to a reduction of 2.20 to 3.30 Log CFU/mL; treatment with 1% peracetic  
284 acid led to a reduction of 3.0 to 4.35 Log CFU/mL; the aqueous ozone for 15 and 30 mins led  
285 to a reduction of 3.05 to 5.45 Log CFU/mL; and gaseous ozone for 30 mins to a reduction of  
286 2.54 to 3.97 Log CFU/mL. Therefore, the reduction level of *S. cerevisiae* population was  
287 affected by the type of treatment, and also by the type of pipe used, as the reduction level was  
288 found higher in flexible tubes with the exception of gaseous ozone treatment. Similar results  
289 were obtained for *Starm. bacillaris* cells present in rigid pipes, since the peracetic acid for 15  
290 mins and aqueous ozone treatments (either for 15 and 30 mins) removed it completely from  
291 the flexible pipes. Concerning the two spoilage yeasts, *B. bruxellensis* and *Z. bailii*, the  
292 aqueous ozone (15 mins) treatment decreased their populations from  $5.40 \pm 0.23$  Log  
293 CFU/mL to  $1.10 \pm 0.10$  Log CFU/mL in rigid pipes, which corresponds to an average  
294 reduction of 4.3 Log CFU/mL, while the other sanitizing treatments removed completely  
295 these yeasts from the pipes surface, independently by the type of pipe used. The populations  
296 of the artificially inoculated bacteria, *A. aceti* and *O. oeni* on rigid pipes, significantly  
297 decreased from  $4.40 \pm 0.24$  Log CFU/mL to  $1.20 \pm 1.20$  Log CFU/mL after treatments with  
298 peracetic acid and ozone (15 and 30 mins), which corresponds to a reduction of 2.2–3.4 Log  
299 CFU/mL. It appeared that 30-mins aqueous ozone treatment was the most effective in  
300 eliminating these bacteria from rigid and flexible pipes.

301

### 302 3.2. Effect of bottling equipment sanitization treatments on yeasts and bacteria

303

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

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

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

331

#### 332 4. Discussion

333

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



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

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

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

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

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

405

## 406 **5. Conclusion**

407

408 This is the first time that peracetic acid (common antimicrobial agent) and ozone  
409 (alternative innovative agent) were compared to reduce the population of six wine related  
410 microorganisms present in stainless steel pipes and bottling machine, after circulation of  
411 artificially contaminated red wine. Among treatments, aqueous ozone for 30 mins contact  
412 time displayed enhanced antimicrobial activity, since it was the only treatment able to  
413 guarantee sanitization in rigid and flexible pipes. In the case of ozone-treated bottling  
414 machine, the same situation was observed, except for *S. cerevisiae*, which was found in the  
415 bottled wines although in significantly lower populations. Ozone technology can fulfil the  
416 growing demand of winemakers for increasing the shelf-life of bottled wines and for reducing  
417 the use of biologically non-degradable chemicals for CIP operation. However, the choice of  
418 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

## 423 **Acknowledgments**

424

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426 Nora Next for providing the ozone generator apparatus.

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 <sup>®</sup>	<i>Saccharomyces cerevisiae</i>	Lallemand <sup>a</sup>
FC54	<i>Starmerella bacillaris</i>	DISAFA <sup>b</sup>
B23F	<i>Brettanomyces bruxellensis</i>	DISAFA <sup>b</sup>
MT1	<i>Zygosaccharomyces bailii</i>	DISAFA <sup>b</sup>
BA23	<i>Acetobacter aceti</i>	DISAFA <sup>b</sup>
VP41	<i>Oenococcus oeni</i>	Lallemand <sup>a</sup>

<sup>a</sup> Lallemand Inc. (Montreal, Canada)<sup>b</sup> Yeast culture collection of DISAFA, Department of Agricultural, Forest and Food Sciences, University of Turin, Italy

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**Table 2**

Cleaning agents used in this study; PAA: peracetic acid

Treatment	Contact time	Pipes	Bottling machine
H <sub>2</sub> O	15 mins	x	
H <sub>2</sub> O	30 mins	x	x
PAA	15 mins	x	x
O <sub>3</sub> aqueous	15 mins	x	
O <sub>3</sub> aqueous	30 mins	x	x
O <sub>3</sub> gaseous	30 mins	x	x
O <sub>3</sub> gaseous	60 mins		x

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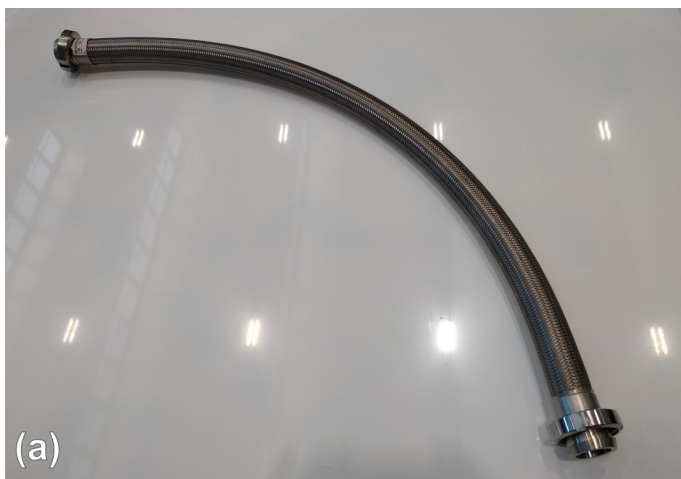
**Figure captions**

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

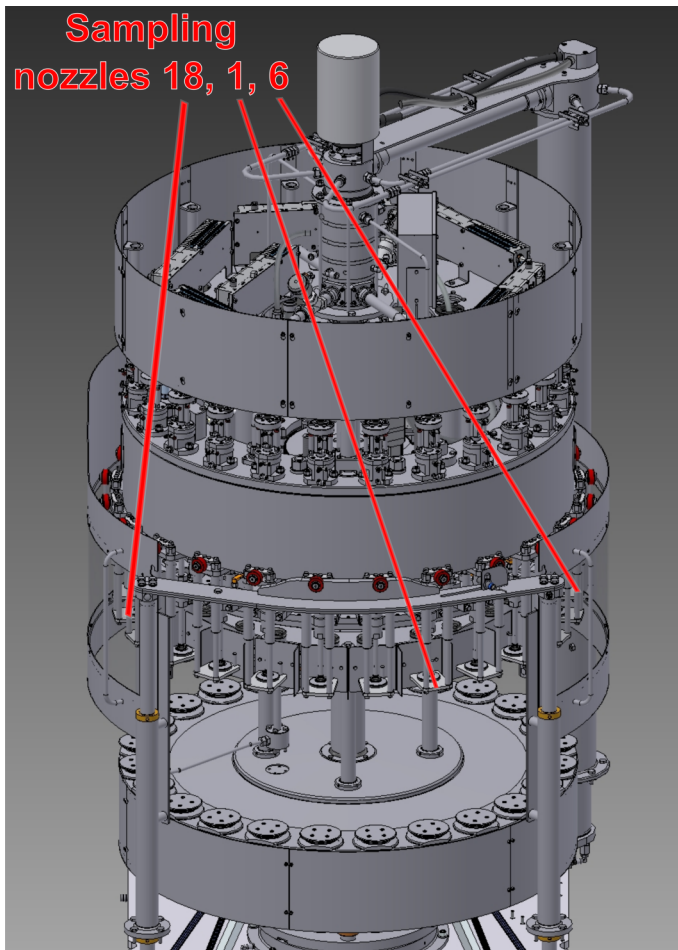
**Fig. 2** Detailed illustration of the bottling filling machine used in this study.

**Fig. 3** Viable counts ( $\text{Log}_{10}\text{CFU/mL}$ ) of yeast and bacterial populations recovered from rigid and flexible pipes, before and after treatments with  $\text{H}_2\text{O}$  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 ( $\pm\text{SD}$ ) of three biological replicates. The different letters in each column indicated significant differences according to ANOVA and Tukey-HSD test ( $p < 0.05$ ).

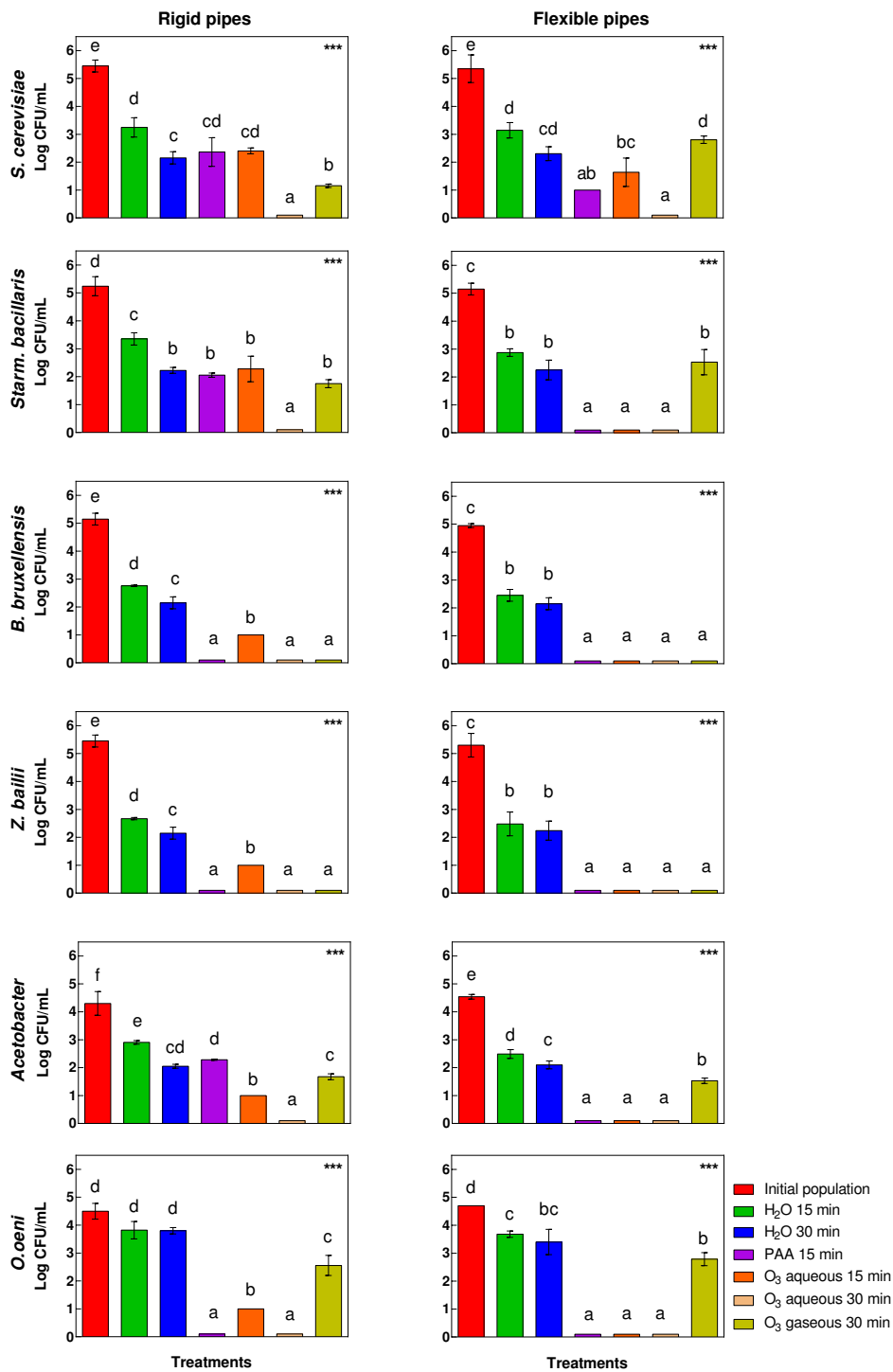
**Fig. 4** Viable counts ( $\text{Log}_{10}\text{CFU/mL}$ ) of yeasts and bacteria populations recovered from, the bottling machine, before and after cleaning with  $\text{H}_2\text{O}$  for 30 mins, 1% peracetic acid for 15 mins, aqueous (30 mins) and gaseous (60 mins) ozone. Data are the mean ( $\pm\text{SD}$ ) of three biological replicates. The different letters in each column indicated significant differences according to ANOVA and Tukey-HSD test ( $p < 0.05$ ).

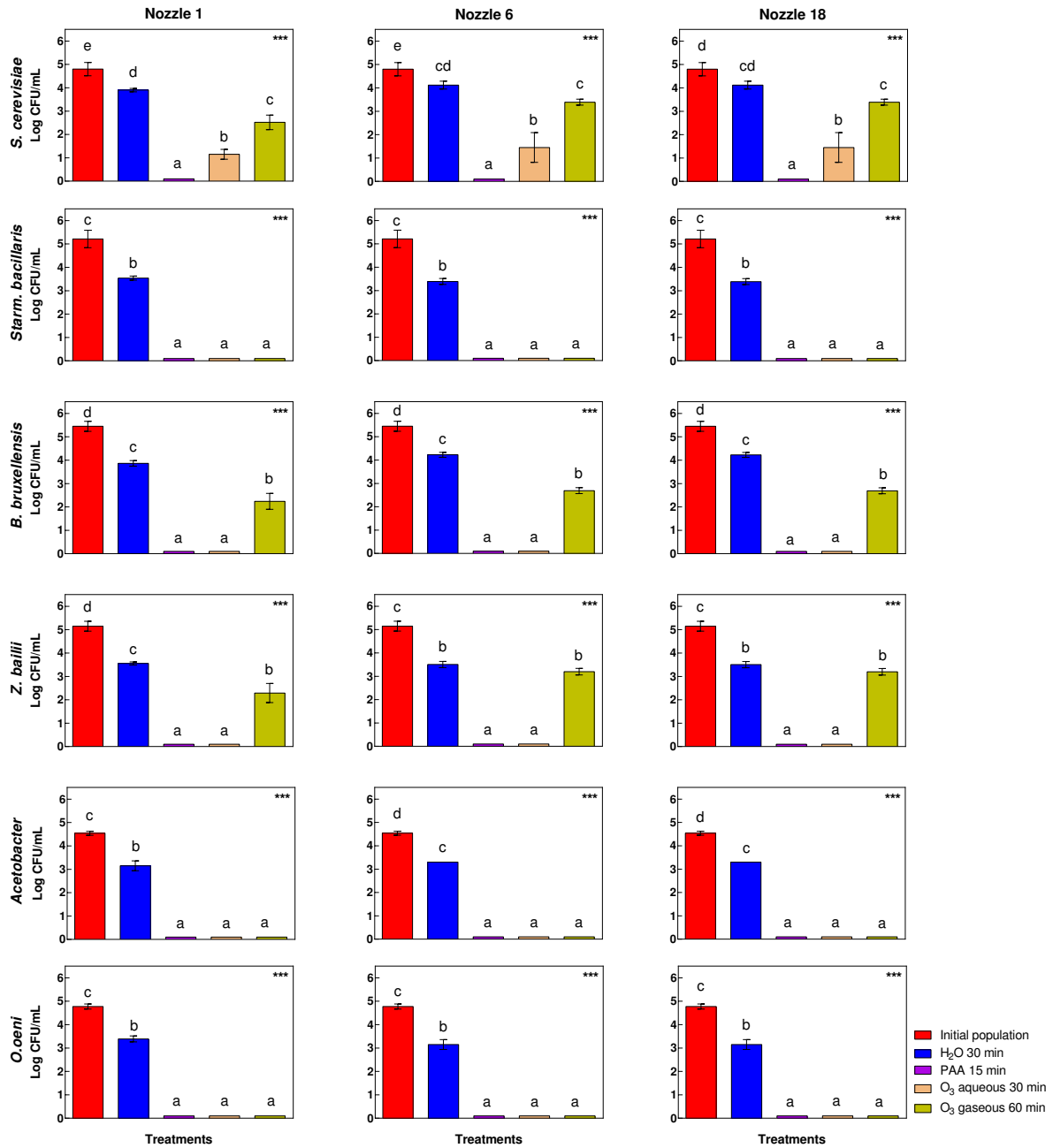


ACCEPTED MANUSCRIPT









**1 Highlights**

2

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

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