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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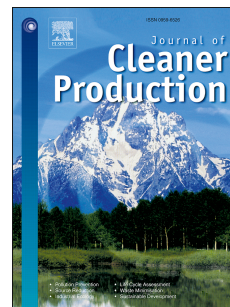
Artificially contaminated wine

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

Wine circulation
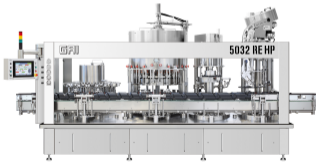
CIP treatments

H_2O (control)
Peracetic acid
Aqueous ozone
Gaseous ozone

Microbiological control

1 **Word Count: 6025**

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3 <u>Minimizing the environmental impact of cleaning in winemaking industry by using ozone for</u>

4 <u>Cleaning-in-Place (CIP) of wine bottling machine</u>

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*.

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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

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,

4

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[®] 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 $^{\circ}$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 ^{o}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_3/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 ^{o}C and 30 ^{o}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 ^{o}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   1×10^6 cells/mL and incubated at 25 ^{o}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   1×10^6 cells/mL and incubated for 4 days and 8 days at 25 ^{o}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 ^{o}C) containing

205   3.50 ± 0.25 mg/L of ozone for 15 and 30 mins; d) circulation of enriched air with 30 ± 1 μL/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 $^{\circ}$C) containing 3.50 ± 0.25 mg/L of ozone for 30 mins at 200

218 mL/min; and d) circulation of enriched air with 30 ± 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 $^{\circ}$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), delvocid (25 mg/L; DSM

241 Specialties, Heerlen, The Netherlands) and incubated at 30 $^{\circ}$C for 7 days; (4) AAB on ethanol

242 agar [10 g/L yeast extract, 20 g/L CaCO$_3$ (Sigma), 20 g/L and 20 mL ethanol (Sigma)],

243 supplemented with delvocid and incubated at 30 $^{\circ}$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 ± 0.21 Log CFU/mL for *S.*
260   *cerevisiae*, 5.24 ± 0.34 Log CFU/mL for *Starm. bacillaris,* 5.15 ± 0.21 Log CFU/mL for *B.*
261   *bruxellensis,* 5.45 ± 0.24 Log CFU/mL for *Z. bailii*, 4.30 ± 0.43 Log CFU/mL for *A. aceti,*
262   4.50 ± 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 ± 0.23 Log

293   CFU/mL to 1.10 ± 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 ± 0.24 Log CFU/mL to 1.20 ± 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 ± 0.28 Log CFU/mL for *S. cerevisiae*, 5.22

309   ± 0.37 Log CFU/mL for *Starm. bacillaris,* 5.45 ± 0.21 Log CFU/mL for *B. bruxellensis,* 5.15

310   ± 0.22 Log CFU/mL for *Z. bailii,* 4.54 ± 0.09 Log CFU/mL for *A. aceti,* and 4.77 ± 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

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

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-Fereira, 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 (Donelly, 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**

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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.

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423 **Acknowledgments**

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428 **References**

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430 Arriagada-Carrazana, J.P., Sáez-Navarrete, C., Bordeu, E., 2005. Membrane filtration effects on aromatic and

431   phenolic quality of Cabernet Sauvignon wines. J. Food Eng. 68, 363-368.

432   https://doi.org/10.1016/j.jfoodeng.2004.06.011.

433 Bartowsky, E.J., 2009. Bacterial spoilage of wine and approaches to minimize it. Lett. Appl. Microbiol. 48, 149-

434   156. https://doi: 10.1111/j.1472-765X. 2008.02505.x.

435 Bellincontro, A., Catelli, C., Cotarella, R. and Mencarelli, F., 2017. Postharvest ozone fumigation of Petit

436   Verdot grapes to prevent the use of sulfites and to increase anthocyanin in wine. Aust. J. Grape Wine

437   Res, 23, 200-206. https://doi.org/10.1111/ajgw.12257

438 Christaki, T., Tzia, C., 2002. Quality and safety assurance in winemaking. Food Control. 13, 503-517.

439   https://doi.org/10.1016/S0956-7135(02)00030-0.

440 Cimaglia, F., Tristezza, M., Saccomanno, A., Rampino, P., Perrotta, C., Capozzi, V., Spano, G., Chiesa, M.,

441   Mita, G., Grieco, F., 2018. An innovative oligonucleotide microarray to detect spoilage

442   microorganisms in wine. Food Control 87, 169-179. https://doi.org/10.1016/j.foodcont.2017.12.023

443 Cravero, F., Englezos, V., Rantsiou, K., Torchio, F., Giacosa, S., Río Segade, S., Gerbi, V., Rolle, L., Cocolin,

444   L., 2016. Ozone treatments of post harvested wine grapes: Impact on fermentative yeasts and wine

445   chemical properties. Food Res. Int. 87, 134-141. https://doi.org/10.1016/j.foodres.2016.06.031.

446 Cravero, F., Englezos, V., Rantsiou, K., Torchio, F., Giacosa, S., Río Segade, S., Gerbi, V., Rolle, L., Cocolin,

447   L., 2018. Control of *Brettanomyces bruxellensis* on wine grapes by post-harvest treatments with

448   electrolyzed water, ozonated water and gaseous ozone. Innov. Food Sci. Emerg. Technol. 47, 309-316.

449   https://doi.org/10.1016/j.ifset.2018.03.017.

450 Donnelly, D.M., 1977. Elimination from table wines of yeast contamination by filling machines. Am. J. Enol.

451   Vitic. 28, 182-184.

452 Du Toit, M., Pretorius, I.S., 2000. Microbial spoilage and preservation of wine: using weapons from nature's

453   own arsenal–a review. S. Afr. J. Enol. Vitic. 21, 74-96.

454 FDA, Food and Drug Administration. 2001. FR21 CFR Part 173", 66(123): June 26, 2001, Silver Spring, MD:

455   US Department of Health and Human Services.

456 Fleet, G.H., 1992. Spoilage yeasts. Crit. Rev. Biotechnol. 12, 1-44.

457   https://doi.org/10.3109/07388559209069186.

458   Fleet, G.H., 2008. Wine yeasts for the future. FEMS Yeast Res. 8, 979-995.

459   https://doi.org/10.1111/j.1567-1364.2008.00427.x.

460   Garrett, T.R., Bhakoo, M. and Zhang, Z., 2008. Bacterial adhesion and biofilms on surfaces. Prog. Nat. Sci, 18,

461   1049-1056. https://doi.org/10.1016/j.pnsc.2008.04.001.

462   Gibson, H., Taylor, J.H., Hall, K.E. Holah, J.T., 1999. Effectiveness of cleaning techniques used in the food

463   industry in terms of the removal of bacterial biofilms. J Appl Microbiol, 87, 41-48.

464   https://doi.org/10.1046/j.1365-2672.1999.00790.x

465   Guillen, A.C., Kechinski, C.P., Manfroi, V., 2010. The use of ozone in a CIP system in the wine industry.

466   Ozone: Sci. Eng. 32, 355-360. https://doi.org/10.1080/01919512.2010.508482.

467   Guzel-Seydim, Z.B., Greene, A.K., Seydim, A.C., 2004. Use of Ozone in the Food Industry. Lebensm.

468   Wiss.Technol. 37, 453-460. https://doi.org/10.1016/j.lwt.2003.10.014

469   Guzzon, R., Widmann, G., Malacarne, M., Nardin, T., Nicolini, G., Larcher, R., 2011. Survey of the yeast

470   population inside wine barrels and the effects of certain techniques in preventing microbiological

471   spoilage. Eur. Food Res. Technol. 233, 285-291. https://doi.org/10.1007/s00217-011-1523-8.

472   Guzzon, R., Bernard, M., Barnaba, C., Bertoldi, D., Pixner, K., Larcher, R., 2017. The impact of different barrel

473   sanitation approaches on the spoilage microflora and phenols composition of wine. J. Food Sci.

474   Technol. 54, 810-821. https://doi: 10.1007/s13197-017-2527-6.

475   Guzzon, R., Franciosi, E., Moser, S., Carafa, I., Larcher, R. 2018. Application of ozone during grape drying for

476   the production of straw wine. Effects on the microbiota and compositive profile of grapes. J. Appl.

477   Microbiol. 125, 513-527. https://doi: 10.1111/jam.13774.

478   Horvitz, S., Cantalejo, M.J., 2014. Application of ozone for the postharvest treatment o fruits and vegetables.

479   Crit. Rev. Food Sci. Nutr. 54, 312-339. https://doi: 10.1080/10408398.2011.584353.

480   Jacobson, J.L., 2005. Introduction to wine laboratory practices and procedures. Springer book

481   Khadre, M.A., Yousef, A.E., Kim, J.G., 2001. Microbiological aspects of ozone applications in food: a review.

482   J. Food Sci. 66,1242-1252. https://doi.org/10.1111/j.1365-2621.2001.tb15196.x.

483   Kim, J.G., Yousef, A.E., Khadre, M.A., 2003. Ozone and its current and future application in the food industry.

484   Adv. Food Nutr. Res. 45, 167–218. https://doi.org/10.1016/S1043-4526(03)45005-5.

485   Loureiro, V., Malfeito-Ferreira, M., 2003. Spoilage yeasts in the wine industry. Int. J. Food Microbiol. 86, 23-

486   50. https://doi.org/10.1016/S0168-1605(03)00246-0.

487   Oelofse, A., Pretorius, I.S., du Toit, M., 2008. Significance of *Brettanomyces* and *Dekkera* during winemaking:

488   a synoptic review. S. Afr. J. Enol. Vitic. 29, 128-144. https://doi.10.21548/29-2-1445.

489   Mahapatra, A.K., Muthukumarappan, K., Julson, J.L., 2005. Applications of Ozone, Bacteriocins and Irradiation

490   in Food Processing: A Review, Crit. Rev. Food Sci. Nutr. 45, 447-461.

491   https://doi.org/10.1080/10408390591034454.

492   Morata, A., Loira, I., Vejarano, R., González, C., Callejo, M.J. and Suárez-Lepe, J.A., 2017. Emerging

493   preservation technologies in grapes for winemaking. Trends Food Sci Technol, 67, 36-43.

494   https://doi.org/10.1016/j.tifs.2017.06.014.

495   Pettigrew, L., Blomenhofer, V., Hubert, S., Groß, F., Delgado, A., 2015. Optimisation of water usage in a

496   brewery clean-in-place system using reference nets. J. Clean. Prod. 87, 583-593.

497   https://doi.org/10.1016/j.jclepro.2014.10.072.

498   Pinto, C., Pinho, D., Cardoso, R., Custódio, V., Fernandes, J., Sousa, S., Pinheiro, M., Egas, C., Gomes, A.C.,

499   2015. Wine fermentation microbiome: a landscape from different Portuguese wine appellations. Front.

500   Microbiol. 1, 6:905. doi: 10.3389/fmicb.2015.00905.

501   Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B., Lonvaud, A., 2006. The microbiology of wine and

502   vinifications. Handbook of enology, second ed. Chichester, England: Wiley.

503   Rodrigues, N., Goncalves, G., Pereira-da-Silva, S., Malfeito-Ferreira, M., Loureiro, V., 2001. Development and

504   use of a new medium to detect yeasts of the genera *Dekkera/Brettanomyces*. J. Appl. Microbiol. 90,

505   588–599. https://doi.org/10.1046/j.1365-2672.2001.01275.x.

506   Rolle, L., Englezos, V., Torchio, F., Cravero, F., Río Segade, S., Rantsiou, K., Giacosa, S., Gambuti, A., Gerbi,

507   V., Cocolin, L., 2018. Ethanol reduction in red wines by technological and microbiological approaches:

508   a comparative study. Aust. J. Grape Wine Res. 24, 62-74. https://doi.org/10.1111/ajgw.12301.

509   Romney, A.J.D., 1990. Cip: Cleaning in Place. The Society for Dairy Technology, Cambridgeshire, UK.

510   Santos, M. C., Nunes, C., Saraiva, J. A., Coimbra, M. A. 2012. Chemical and physical methodologies for the

511   replacement/reduction of sulfur dioxide use during winemaking: Review of their potentialities and

512   limitations. Eur. Food Res. Technol. 234, 1–12. https://doi.org/10.1007/s00217-011-1614-6.

513   Schuller, D., Corte-Real, M., Leao, C., 2000. A differential medium for the enumeration of the spoilage yeast

514   *Zygosaccharomyces bailii* in wine. J. Food Prot. 63, 1570-1575. https://doi.org/10.4315/0362-028X-

515   63.11.1570.

516   Tanmay, P., Arijit, J., Arpan, D., Arpita, M., Suman, K.H., Pradeep, K.D.M., Bikas, R.P., Keshab, C.M., 2014.

517   Smart cleaning-in-place process through crude keratinase: an eco-friendly cleaning techniques towards

518   dairy industries. J. Clean. Prod. 76, 140-153. https://doi.org/10.1016/j.jclepro.2014.04.028.

519   Valdes la Hens, D., Bravo☐Ferrada, B. M., Delfederico, L., Caballero, A. C., Semorile, L.C., 2015. Prevalence

520   of *Lactobacillus plantarum* and *Oenococcus oeni* during spontaneous malolactic fermentation in

521   Patagonian red wines revealed by polymerase chain reaction☐denaturing gradient gel electrophoresis

522   with two targeted genes. Aust. J Grape Wine R, 21, 4-56. https://doi.org/10.1111/ajgw.12110

523   White, F.M.,1999. Fluid mechanics, fourth ed. WCB/McGraw-Hill, Boston,

524   Wirtanen, G., Salo, S., 2003. Disinfection in food processing–efficacy testing of disinfectants. Rev. Environ.

525   Sci. Biotechnol. 2, 293-306. https://doi.org/10.1023/B:RESB.0000040471.15700.03.

526   Zuehlke, J. M., Petrova, B., Edwards, C.G., 2013. Advances in the control of wine spoilage by

527   *Zygosaccharomyces* and *Dekkera/Brettanomyces*. Annu. Rev. Food Sci. Technol. 4, 57-78. https://doi:

528   10.1146/annurev-food-030212-182533.

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549 **Table 1**

550 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]

551 [a] Lallemand Inc. (Montreal, Canada)

552 [b] Yeast culture collection of DISAFA, Department of Agricultural, Forest and Food Sciences,

553 University of Turin, Italy

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577 **Table 2**

578 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

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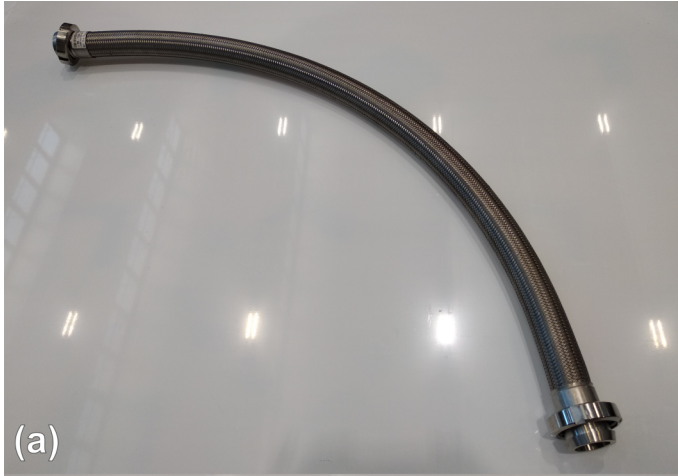
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604 **Figure captions**

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606 **Fig. 1** Flexible (Panel A) and rigid pipes (Panel B) used in this study.
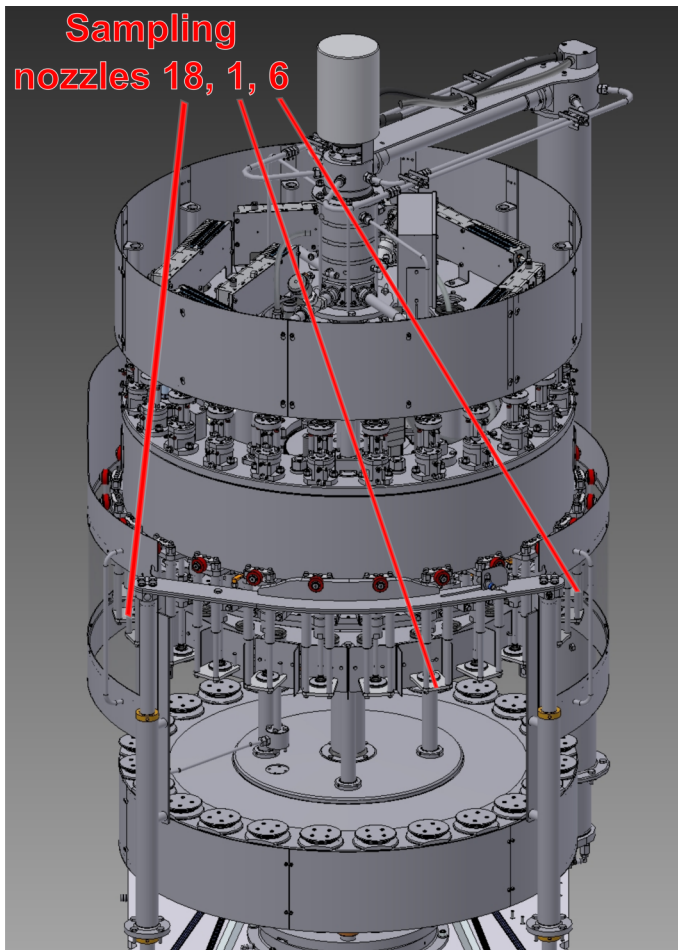
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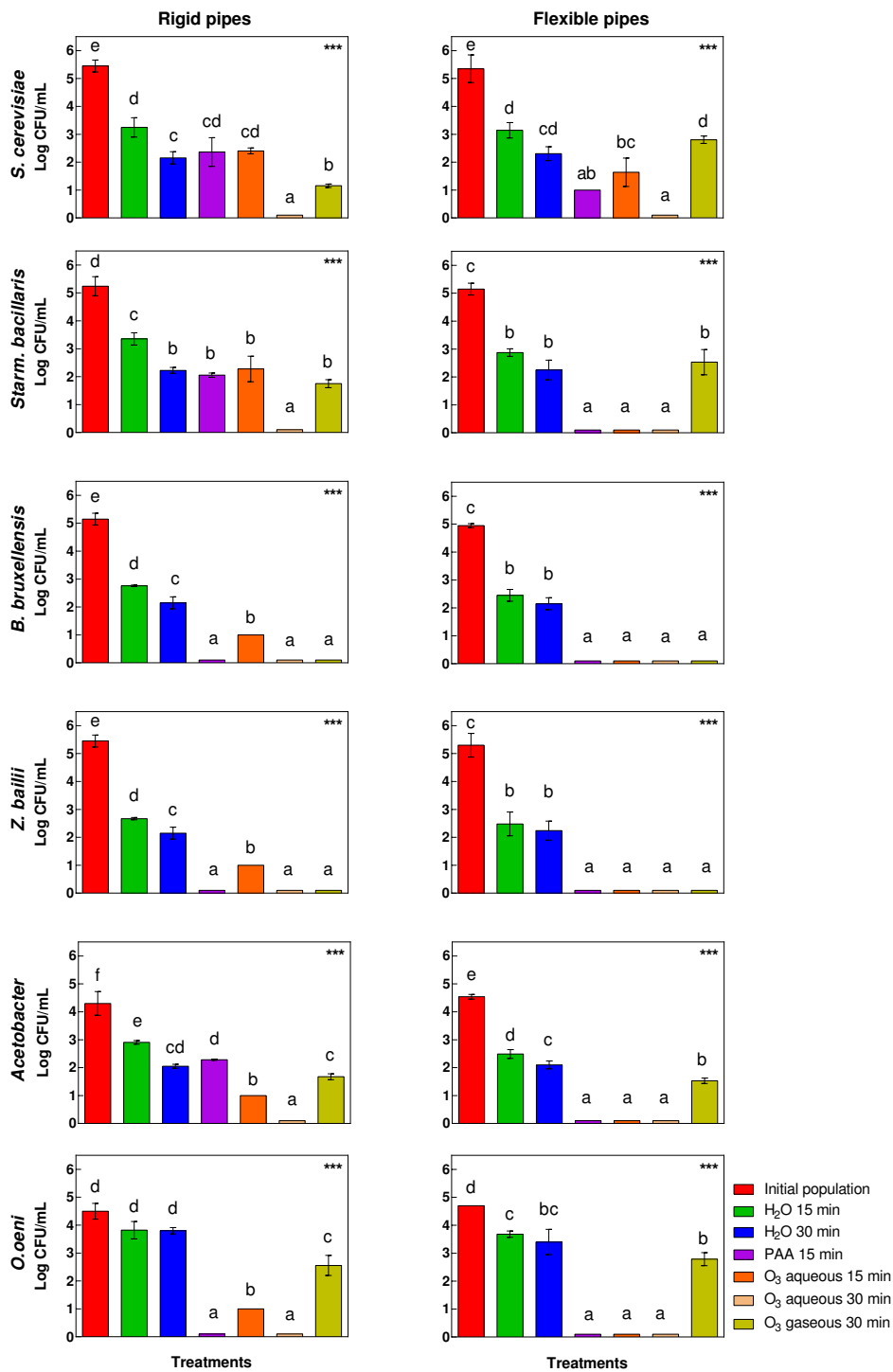608 **Fig. 2** Detailed illustration of the bottling filling machine used in this study.

609

610 **Fig. 3** Viable counts (Log_{10}CFU/mL) of yeast and bacterial populations recovered from rigid

611 and flexible pipes, before and after treatments with H_2O for 15 and 30 mins, 1% peracetic

612 acid for 15 mins, aqueous ozone for 15 and 30 mins, gaseous ozone for 30 mins. Data are the

613 mean (±SD) of three biological replicates. The different letters in each column indicated

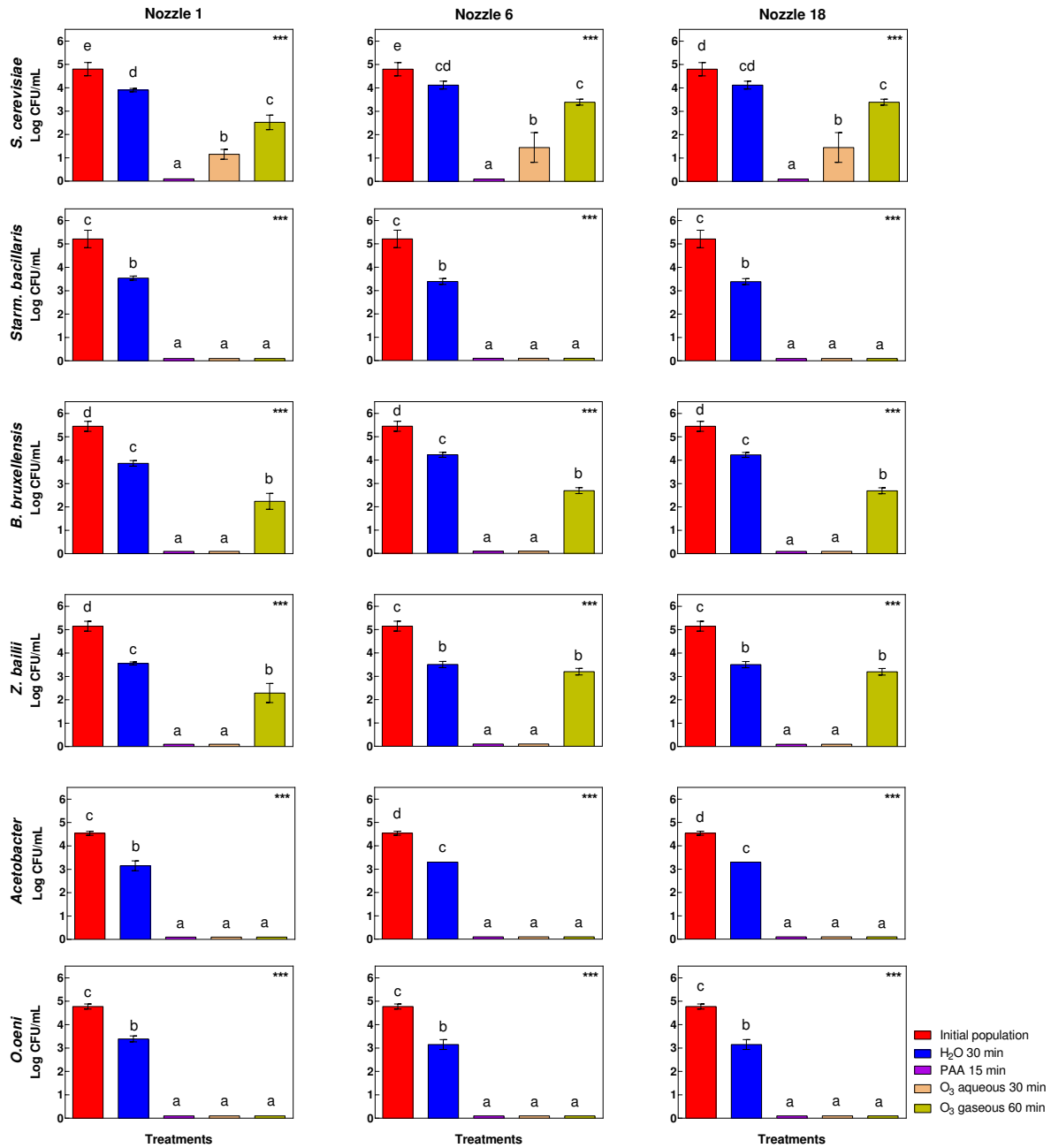614 significant differences according to ANOVA and Tukey-HSD test ($p < 0.05$).

615

616 **Fig. 4** Viable counts (Log_{10}CFU/mL) of yeasts and bacteria populations recovered from, the

617 bottling machine, before and after cleaning with H_2O for 30 mins, 1% peracetic acid for 15

618 mins, aqueous (30 mins) and gaseous (60 mins) ozone. Data are the mean (±SD) of three

619 biological replicates. The different letters in each column indicated significant differences

620 according to ANOVA and Tukey-HSD test ($p < 0.05$).

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Sampling nozzles 18, 1, 6

1 **Highlights**

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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.

9