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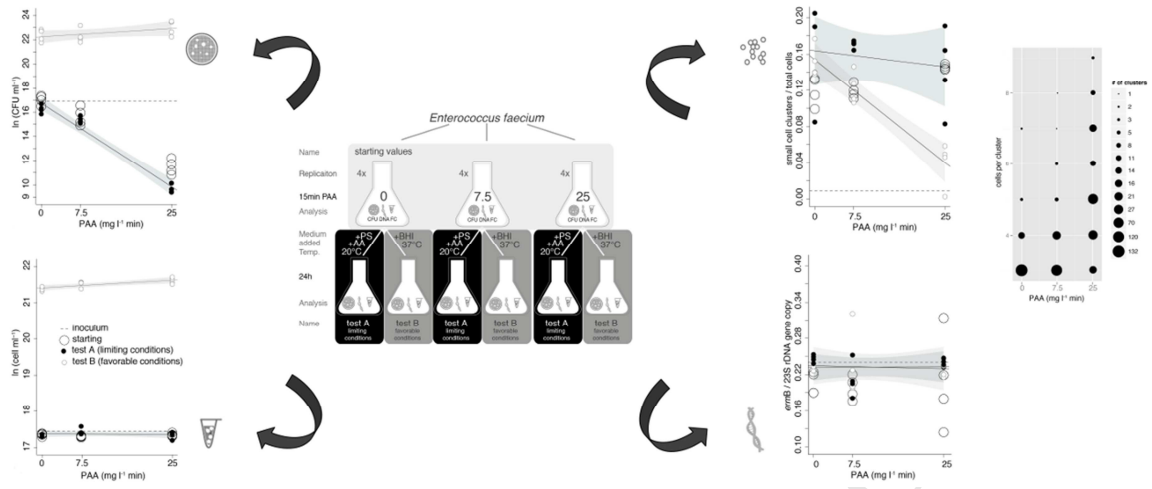
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ACCEPTED MANUSCRIPT

## Defence strategies and antibiotic resistance gene abundance in enterococci under stress by exposure to low doses of peracetic acid

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1 **Abstract**

2 Peracetic acid (PAA) is an organic compound used efficiently as disinfectant in wastewater  
3 treatments. Yet, at low doses it may cause selection; thus, the effect of low doses of PAA on  
4 *Enterococcus faecium* as a proxy of human-related microbial waste was evaluated. Bacteria were  
5 treated with increasing doses of PAA (from 0 to 25 mg L<sup>-1</sup> min) and incubated in regrowth  
6 experiments under non-growing, limiting conditions and under growing, favorable conditions. The  
7 changes in bacterial abundance, in bacterial phenotype (number and composition of small cell  
8 clusters), and in the abundance of an antibiotic resistance gene (ARG) was evaluated. The  
9 experiment demonstrated that the selected doses of PAA efficiently removed enterococci, and  
10 induced a long-lasting effect after PAA inactivation. The relative abundance of small clusters  
11 increased during the experiment when compared with that of the inoculum. Moreover, under  
12 growing favorable conditions the relative abundance of small clusters decreased and the number of  
13 cells per cluster increased with increasing PAA doses. A strong stability of the measured ARG was  
14 found, not showing any effect during the whole experiment. The results demonstrated the feasibility  
15 of low doses of PAA to inactivate bacteria. However, the stress induced by PAA disinfection  
16 promoted a bacterial adaptation, even if potentially without affecting the abundance of the ARG.

## 17 **Introduction**

18 Enterococci are commensal bacteria from guts of warm blooded animal (Byappanahalli et al.,  
19 2012). Generally, they are harmless for healthy individuals (Sava et al., 2010). However, they can  
20 become important infectious agents in patients with an impaired immune system and nowadays they  
21 are considered among the main opportunistic pathogens directly causing nosocomial infections  
22 (Arias et al., 2010).

23 Enterococci are present not only in animal intestines but they have also been found in beach sands,  
24 soils, sediments, and open waters (Byappanahalli et al., 2012). Moreover, they are used as faecal  
25 indicator bacteria (FIB), to evaluate the microbiological quality of waters (ECC, 2006; US EPA,  
26 2012).

27 The presence in waters of enterococci carrying antibiotic resistance and virulence traits has been  
28 reported by several authors (Di Cesare et al., 2013, 2012; Vignaroli et al., 2013). These features,  
29 coupled with their ability to survive in human macrophages (Sabatino et al., 2015), highlight that  
30 the occurrence of enterococci in the environment may pose a threat to human health both directly  
31 and indirectly through the spread, by horizontal gene transfer (HGT), of their antibiotic resistance  
32 genes (ARGs) to human strains (Morrone et al., 2016).

33 As a consequence of their role as FIB and of their potential pathogenicity, it becomes crucial to  
34 understand their response to the most widely used disinfectants, in order to allow the design of new  
35 and more efficient disinfection processes in wastewater treatment plants (WWTPs). A growing  
36 concern for the sanitary implications of disinfection by products (DBPs) generated by chlorine-  
37 based compounds is promoting the use of alternative treatments (Richardson et al., 2007), including  
38 UV radiation, membranes, and several new disinfectants, such as peracetic acid (PAA) (Metcalf and  
39 Eddy, 2014).

40 PAA is an organic peroxide that has been used for many years as disinfectant in various human  
41 activities, including food and healthcare industries (MarketsandMarkets, 2015). It is a broad-  
42 spectrum disinfectant, not known for the generation of known DBPs (Dell'Erba et al., 2007;

43 Nurizzo et al., 2005). PAA is stored in a liquid concentrated solution, where it is in equilibrium with  
44 hydrogen peroxide ( $H_2O_2$ ) and acetic acid (AA) (Kitis, 2004). PAA can be dosed in WWTPs using  
45 the same equipment used for sodium hypochlorite ( $NaOCl$ ), without the need for expensive  
46 modifications (Antonelli et al., 2013).

47 Commonly investigated PAA doses for disinfection treatment range between 10 and 400  $mg L^{-1}$   
48 min (Santoro et al., 2015), suggesting values below 50  $mg L^{-1}$  min as low doses. However, most  
49 previous works do not estimate the actual PAA dose but only report initial PAA concentration and  
50 contact time, although these operating conditions are often insufficient to exhaustively describe the  
51 disinfection process because of PAA decay. Initial PAA concentration between 1 and 15  $mg L^{-1}$  and  
52 contact time between 10 and 60 minutes are usually adopted for secondary and tertiary effluents  
53 (Luukkonen and Pehkonen, 2016). Coliform bacteria and enterococci are by far the most studied  
54 target microorganisms in wastewater disinfection (Luukkonen et al., 2015), and the effectiveness of  
55 PAA on their inactivation has been widely documented (Stampi et al., 2002). The inactivation is  
56 strongly dependent on effluent composition, since it can determine rapid PAA decay (Liu et al.,  
57 2014; Pedersen et al., 2013). Low PAA concentrations (about 2  $mg L^{-1}$ ) with short contact times  
58 (minimum value of 12 minutes) were demonstrated to be sufficient for complying with stringent  
59 regulations on agricultural reuse, also resulting in long term disinfection action and, thus, in the  
60 preservation of the quality of reclaimed wastewater at point-of-use (Antonelli et al., 2006).

61 While most of the studies on PAA disinfection addressed engineering aspects, a recent study  
62 highlighted the occurrence of peculiar ecological responses and change in the specific ARGs  
63 abundance of the microbial community when exposed to PAA (Di Cesare et al., 2016). Although  
64 bacterial aggregations, or similar phenotypic adaptations of the community, are not detected while  
65 assessing the microbiological quality of the discharged effluents (being this evaluation based on  
66 FIB count only), such phenotypic variability can heavily influence the overall response of a  
67 bacterial community to disinfection (Rizzo et al., 2013). Moreover, it is known that disinfection  
68 treatments could be inefficient in removing ARGs within specific bacterial populations (Ferro et al.,

69 2017), or can even drive the selection of ARGs in microbial communities from WWTPs (Di Cesare  
70 et al., 2016). Such evidence highlights the role of WWTPs, and in particular of the contribution of  
71 chemical disinfection treatments, in the spread of ARGs in the environment, hinting to the need for  
72 further investigations on the phenotypic and genotypic responses by bacteria subjected to best  
73 practices for wastewater treatment, such as disinfection by PAA.

74 This study investigated the response of enterococci to the stress exerted by two different low doses  
75 of the disinfectant; such doses were chosen within a range of potentially optimal but low values,  
76 within the rationale of a future reduction in PAA doses in wastewater treatment plants.  
77 *Enterococcus faecium* was chosen as a relevant reference microorganism because of its tendency  
78 towards the acquisition of antibiotic resistance genes (van Schaik and Willems, 2010). The efficacy  
79 of two low doses of PAA on *E. faecium* inactivation was evaluated by analysing the bacterial  
80 response in terms of abundance and phenotype after the disinfection and during regrowth tests in  
81 low and rich medium. Such low doses would allow a better understanding of the fate of *E. faecium*  
82 when growing under different environmental conditions. Moreover, we assessed the impact of low  
83 doses of PAA on the relative abundance of a specific ARG (*ermB*) acquired by the selected *E.*  
84 *faecium* by conjugation and thus located on a mobile element, implementing its variability in copy  
85 numbers when exposed to different experimental conditions.

86

## 87 **Material and methods**

### 88 *Bacterial strain*

89 The strain *E. faecium* 64/3-67/7E from the collection of the Department of Life and Environmental  
90 Sciences of the Polytechnic University of Marche, resistant to erythromycin (ERY) and tetracycline  
91 and carrying a ERY resistant gene (*ermB*), was selected for this study. This strain is a  
92 transconjugant obtained by filter mating experiment (following the protocol described by Vignaroli  
93 et al. (2011)) of the *ermB* carrying donor strain *E. faecium* 6767/7 and the recipient *E. faecium* 64/3,



94 rifampin- and fusidic acid-resistant and carrying the ERY low-level resistance gene *msrC* (Bender  
95 et al., 2015).

#### 96 *Inoculum preparation*

97 The inoculum of *E. faecium* 64/3-67/7E was obtained by growing the strain in Brain Heart Infusion  
98 broth (BHI) at 37°C for 24 h. The broth culture was centrifuged at 1000 rpm for 10 mins, and the  
99 pellet was washed twice with physiological solution (NaCl 0.9%). The pellet was then re-suspended  
100 in physiological solution in order to reach a final concentration of  $1 \cdot 10^7$  cell mL<sup>-1</sup> (as confirmed by  
101 flow cytometry).

102

#### 103 *Experimental design*

104 The experimental design consisted of two parts. In the first one, referred to as “experiment 1”, the  
105 response of the strain *E. faecium* 64/3-67/7E to PAA was investigated in order to determine the  
106 doses of a PAA commercial solution (VigorOx WWT II, Peroxychem, PAA 15%, H<sub>2</sub>O<sub>2</sub> 23%, AA  
107 16%) to be used in the following steps. In the second part, referred to as “experiment 2”, the  
108 response of a population of *E. faecium* 64/3-67/7E to the disinfection treatment and during two tests  
109 of regrowth in low (heavy limiting conditions, test A) and rich (favorable conditions, test B)  
110 medium (Figure 1). The aim of “experiment 1” was to select two doses for the subsequent  
111 experiments, within the range of what is expected as low doses of PAA, as previously discussed, in  
112 order to determine a strong (order of logs) and weak (about 50%) inactivation of FIB. The upper  
113 limit for the investigated interval of doses in experiment 1 was defined *a priori* as 25 mg L<sup>-1</sup> min  
114 and several lower doses were assessed. Decay and microbial inactivation tests were performed  
115 using 100 mL aliquots of the inoculum. As for decay tests, the decrease of residual PAA  
116 concentration over time (t) at an initial PAA concentration (PAA<sub>0</sub>) of 1.68 mg L<sup>-1</sup> was evaluated in  
117 triplicate replicates. Results were fitted with a first-order kinetic model, as reported in Equation 1,  
118 and the decay coefficient  $k_{\text{PAA}}$  was estimated.

$$PAA_t = PAA_0 \cdot e^{-k_{PAA}t} \quad (1)$$

119 Then, initial PAA concentrations required for applying PAA doses ( $CT_{PAA}$ ) ranging from 1 to 25  
120  $\text{mg L}^{-1} \text{ min}$  over a process time of 15 minutes were calculated by Equation 2, that is directly derived  
121 from the equation reported in Santoro et al. (2015) for estimating the actual PAA dose as the area  
122 under the PAA decay curve, assuming negligible oxidative demand.

$$PAA_0 = \frac{k_{PAA}CT_{PAA}}{1 - e^{-k_{PAA}t}} \quad (2)$$

123 Disinfection tests were carried out for assessing the effect of eight PAA doses by dosing previously  
124 estimated initial PAA concentrations and by quenching the residual disinfectant after 15 minutes  
125 using sodium thiosulphate and bovine catalase. Each trial was repeated twice and two aliquots of  
126 the inoculum in which the disinfectant was not dosed were included as negative controls. After the  
127 disinfectant quenching, all samples and negative controls were analysed for the colony-forming unit  
128 (CFU) count. The log-inactivation extent, defined as the ratio between CFU grown on plates after  
129 disinfection treatment with a certain PAA dose and CFU grown on plates in the absence of  
130 disinfection treatment, was estimated to select a dose that corresponds to a microbial inactivation of  
131 50%.

132 The aim of “experiment 2” was to test the effect of PAA on the regrowth of bacteria after the  
133 disinfection treatments and kept under different culture conditions. To do this, twelve aliquots of the  
134 inoculum (210 mL) were placed in different flasks. Two series of four flasks were processed for the  
135 disinfection treatment by using the two selected PAA doses (which corresponded to 7.5 and 25  $\text{mg}$   
136  $\text{L}^{-1} \text{ min}$ ), the third series of four flasks was not disinfected, and thus used as negative control. Two  
137 series of subsamples (40 mL) for each aliquot (including the negative control) were prepared in  
138 order to evaluate the capability of *E. faecium* to regrow after the disinfection treatment in a poor  
139 medium under high nutrient limitation (test A) and in a rich medium under favorable growing  
140 conditions (test B).

141 For the experiment under limiting conditions (test A) the subsamples were incubated with shaking  
142 at 20°C for 24 h in physiological solution 0.9% (80 mL for each subsample) supplemented with  
143 different AA concentrations (as carbon source) to reach the final AA concentration of 2 mg L<sup>-1</sup>.  
144 Specifically, the amount of AA added for each subsample was calculated by considering the amount  
145 already present in the flasks after the disinfection treatment, derived from PAA decay, to avoid the  
146 unbalance of carbon content in different subsamples. For the experiment under favorable conditions  
147 (test B), the subsamples were incubated at 37°C for 24 h in BHI (80 mL for each subsample).  
148 A total of 37 subsamples were processed for bacterial count, size distribution of cell clusters, and  
149 molecular analyses. These represented: 3 subsample of the inoculum, 12 subsamples after the  
150 disinfection treatment (four at 7.5 mg L<sup>-1</sup> min, four at 25 mg L<sup>-1</sup> min, plus four controls), and 24  
151 subsamples of the four replicate for each the three groups of PAA dose after the tests under limiting  
152 and under favorable conditions.

153

#### 154 *Residual PAA concentration*

155 Residual PAA concentration was measured by adapting the DPD - colorimetric method for the  
156 determination of chlorine concentration, as reported in Standard Methods (APHA/AWWA/WEF,  
157 2012). In detail, a stoichiometric excess of potassium iodide buffer solution and DPD (N,N-diethyl-  
158 p-phenylenediaminesulphate salt) were dosed to develop a transient colour proportionally to PAA  
159 concentration. Sample absorbance was measured at 530 nm wavelength by a Dr. Lange CADAS  
160 200 spectrophotometer (optical path 40 mm) and measured values were related to PAA  
161 concentration by means of a standard curve that was previously determined.

162

#### 163 *Bacterial count and phenotype*

164 Bacterial abundance was evaluated both by plate count and by flow cytometry. Bacterial phenotype  
165 was measured as composition of single or dividing cells, small clusters (between 3 and 9 aggregated

166 cells), and large clusters (more than 10 aggregated cells) by flow cytometry (Accuri C6, BD  
167 Biosciences) and confirmed by epifluorescence microscopy (AxioPlan 10, Zeiss).  
168 For plate count, the analysis of CFU was carried out by spotting 10  $\mu\text{L}$  of the dilutions (up to  $10^{-8}$ )  
169 of the inoculum and of the samples on brain-heart infusion agar plates incubated at  $37^{\circ}\text{C}$  for 24 h.  
170 For the measurement of bacterial abundances and phenotypic distribution by flow cytometry,  
171 aliquots of 500  $\mu\text{L}$  for each sample were stained with SYBR Green I (Life Technologies) solution  
172 (1%) for 15 mins in the dark. Counts were set to a minimum of  $5 \times 10^6$  events within the three  
173 designed gates (Corno et al., 2013). The correct identification and gate-assignment in the cytograms  
174 were confirmed by a preliminary check of twelve samples by epifluorescence microscopy on 4'-6-  
175 diamidino-2-phenylindole (DAPI) stained bacteria (Corno et al., 2014). Gate design and events  
176 enumeration were performed by the Accuri C6 resident analysis software (BD Biosciences). The  
177 number of large clusters (e.g., clusters composed by at least 10 aggregated cells) resulted to be  
178 negligible in every sample, thus further analyses focussed only on the abundances, the phenotype,  
179 and the relative proportions of single/dividing cells (namely single cells) and of small aggregates  
180 composed by 3-9 cells. The number of cells in at least 100 small clusters, often organized in chains  
181 of cells, per sample was counted in epifluorescence microscopy.

182

### 183 *Molecular analyses*

184 For each of the 37 subsamples (including the inoculum, the 12 replicates after the disinfection  
185 treatment at the starting phase of the regrowth experiment, and the 24 replicates after the regrowth  
186 experiment under two conditions) up to 70 mL were filtered on 0.22  $\mu\text{m}$  polycarbonate filters and  
187 stored at  $-20^{\circ}\text{C}$  until processing for the DNA extraction. Each filter was cut in two sections and one  
188 of them was processed for the DNA extraction using a commercial kit (Ultra Clean Microbial DNA  
189 Isolation Kit, MoBio Laboratories) following the manufacture's instruction with some modification,  
190 keeping the other half as a back-up in case of problems with the following pipeline. The bacterial  
191 lysis was carried out by adding to the lysis solution of the kit 2.5  $\text{mg L}^{-1}$  of lysozyme (Sigma-

192 Aldrich) and then homogenized (two cycles of 6000 rpm for 30 seconds, using the Precellys 24  
193 homogenizer, Bertin technologies). The DNA extracts (tenfold diluted) were analysed for the  
194 abundance of 23SrDNA and of *ermB* genes (primer sequences are reported in Supplementary Data,  
195 Table S1) by qPCR using the RT-thermocycler CFX Connect (Bio-Rad). Standard calibration  
196 curves for each gene were carried out by qPCR assays as described in Di Cesare et al. (Di Cesare et  
197 al., 2015), but changing the annealing temperatures (60 and 55°C for 23SrDNA and *ermB*,  
198 respectively) and decreasing the cycles from 35 to 30 for the analysis of 23SrDNA. The inhibition  
199 of the qPCR was analysed by the dilution method as described by Di Cesare et al. (2013), and no  
200 inhibition was observed. The averages of the efficiency and of  $R^2$  considering all the runs were 89.3  
201 and 0.996 respectively. The limit of detection (LOD) for the two tested genes were determined as  
202 described in Bustin et al. (2009): the LOD expressed as copy  $\mu\text{L}^{-1}$  of 23SrDNA and *ermB* were 703  
203 and 545 respectively. The specificity of the amplicons was evaluated by the melting curve analysis  
204 using the PRECISION MELT ANALYSIS Software 1.2 built in CFX MANAGER Software 3.1  
205 (Bio-Rad) and by electrophoresis run. The relative abundance of ARG was expressed as the  
206 proportion of copy number of *ermB* on copy number of 23SrDNA.

207

### 208 *Data analysis*

209 The measurements used as response variable in each model obtained from experiment 2 were CFU  
210  $\text{mL}^{-1}$ , cell  $\text{mL}^{-1}$ , proportion between small cluster/total cell numbers, and *ermB*/23SrDNA copy  
211 number. For all models with CFU  $\text{mL}^{-1}$  and cell  $\text{mL}^{-1}$ , a natural logarithm transformation of the data  
212 was used to account for the ln-behavioural response of these metrics and to improve model fit; all  
213 other metrics were kept with their original values. As a preliminary test, Linear Models (LMs) were  
214 used to assess whether the strength of the disinfection treatment had any effect on each of the five  
215 metrics at the end of the disinfection treatment, before starting the tests on regrowth. For each  
216 metric a full model was then tested to assess whether the final values obtained were affected by (1)  
217 the strength of the disinfection treatment (continuous variable at 0, 7.5 and 25  $\text{mg L}^{-1} \text{ min}$ ), by (2)

218 the regrowth tests (two levels, limiting or favorable), by (3) the interaction between the two levels  
219 (whether the disinfection treatment had a different effect depending on the test), and by (4) the  
220 starting values that the metric had before the regrowth tests, but after the disinfection treatment,  
221 simply referred to as 'starting' for brevity in the following. Given that the regrowth tests originated  
222 from the same disinfection treatment, they are not independent and represent pseudoreplicates.  
223 Thus, this confounding factor was accounted for by using Linear Mixed Effect Models (LMEMs)  
224 and explicitly including the pseudoreplication in the error structure of the models (Beckerman,  
225 2014). The interaction term between disinfection treatment and regrowth tests was kept in the final  
226 models only if it was significant; in case of no significant interaction, the term was removed from  
227 the final model to avoid over-parameterisation of the models (Crawley, 2012). Another variable that  
228 could affect the final values of the metrics is the values of the inoculum, but given that only one  
229 inoculum was used at the beginning of the disinfection treatments, it could not be statistically tested  
230 for its effect, but the value of the inoculum was simply reported in the graphs. Additionally, to  
231 check whether the final values of the metrics after the regrowth tests (under limiting or favorable  
232 conditions) correlated to the starting values, paired *t*-tests were performed, independently for the  
233 results of the regrowth tests. Moreover, the effects of the single variables were tested using LMs to  
234 explore the actual behaviour of the relationships when the full model was too complex to provide  
235 unambiguous inference. To analyse the changes in cells number per cluster, LMs and Tukey's  
236 Honestly Significant Difference test were performed to assess whether number of cell per small  
237 cluster changed according to the disinfection treatment (continuous variable from 0 to 25 mg L<sup>-1</sup>  
238 min) and to the regrowth test (categorical variable with three levels: starting, under limiting  
239 conditions, and under favorable conditions; in addition to inoculum). All the data were analysed  
240 together, and then separating the effect of disinfection treatment on starting, under limiting  
241 conditions, and under favorable conditions. All analyses were run in R 3.1.2 (R Core Team, 2014),  
242 with LMEMs in packages *lme4* 1.1-7 (Bates et al., 2014) and *lmerTest* 2.0-20 (Kuznetsova et al.,  
243 2014).

244

245 **Results**246 ***Experiment 1***

247 A residual PAA concentration of  $1.49 \pm 0.08 \text{ mg L}^{-1}$  was reached in the aqueous solution after 15  
248 minutes from the dosage of an initial PAA concentration of  $1.68 \text{ mg L}^{-1}$ , indicating the occurrence  
249 of a slight PAA decay. Experimental data were fitted by a first-order kinetic model ( $R^2 = 0.85$ ) and  
250 the PAA decay coefficient was estimated ( $k_{\text{PAA}} = 0.0072 \pm 0.0030 \text{ min}^{-1}$ ). Assuming that the decay  
251 coefficient can be considered as constant in the investigated range of PAA concentrations for a  
252 liquid medium not promoting PAA decay, as physiological solution, initial PAA concentrations  
253 between  $0.07$  and  $1.68 \text{ mg L}^{-1}$  were calculated for PAA doses ranging from  $1$  to  $25 \text{ mg L}^{-1} \text{ min}$ .  
254 Experimental results obtained in the experiment 1 revealed a progressive decrease of the bacterial  
255 count, measured in terms of  $\text{CFU mL}^{-1}$ , with increasing PAA dose (Supplementary Data, Figure  
256 S1). A microbial inactivation of about 50% was obtained for PAA doses between  $5$  and  $10 \text{ mg L}^{-1}$   
257 min. Consequently, the intermediate dose chosen for the experiment was between  $5$  and  $10 \text{ mg L}^{-1}$   
258 min, namely  $7.5 \text{ mg L}^{-1} \text{ min}$ . Thus, the two selected doses,  $7.5$  and  $25 \text{ mg L}^{-1} \text{ min}$ , represent two  
259 different operating conditions at very low and low doses for a disinfection process.

260

261 ***Experiment 2***

262 Microbial inactivation obtained at two selected PAA doses was in agreement with results of the  
263 experiment 1 (Supplementary Data, Figure S1). In detail, average log-inactivation values  
264 corresponding to  $0.53 \text{ log}$  and  $2.37 \text{ log}$  were obtained for  $7.5$  and  $25 \text{ mg L}^{-1} \text{ min}$  PAA doses,  
265 respectively.

266

267 ***Bacterial CFU counts***

268 After the regrowth tests, the bacterial CFU counts showed significant differences between the  
269 limiting and the favorable conditions, the strength of disinfection was important, but with its effect

270 being different between the two regrowth conditions (Table 1). PAA disinfection had a significantly  
271 overall negative effect on bacterial count (Table 1); significantly lower CFU mL<sup>-1</sup> values were  
272 obtained under limiting conditions than under favorable conditions (Figure 2A); moreover, CFU  
273 mL<sup>-1</sup> values under limiting conditions diminished with increasing strength of disinfection (LM:  $t = -$   
274 5.39,  $p = 0.0003$ ), whereas under favorable conditions they remained stable regardless of the  
275 strength of the disinfection (LM:  $t = 1.92$ ,  $p = 0.08$ ).

276 Under limiting conditions, CFU mL<sup>-1</sup> values slightly diminished from the starting values (paired  $t$ -  
277 test:  $t = -3.8$ ,  $p = 0.0028$ ), whereas under favorable conditions CFU mL<sup>-1</sup> values significantly  
278 increased from the starting values (paired  $t$ -test:  $t = 9.5$ ,  $p < 0.0001$ ) (Figure 2A).

279 As for the inoculum, average CFU mL<sup>-1</sup> was  $21.8 \times 10^6$  with its ln value of 16.9 within the range of  
280 the negative control (Figure 2A).

281

#### 282 *Bacterial cell count*

283 Cell counts resulted significantly higher under favorable than under limiting conditions, and the  
284 disinfection treatment had no effect on cell abundances (Table 1, Figure 2B), even if the significant  
285 interaction term in the model (Table 1) points to the fact that the effect of disinfection treatment  
286 may have differential responses under favorable or limiting conditions. Indeed, under limiting  
287 conditions, cell mL<sup>-1</sup> values did not significantly change from the starting values (paired  $t$ -test:  $t =$   
288 1.1,  $p = 0.291$ ), whereas under favorable conditions cell mL<sup>-1</sup> values increased from the starting  
289 values (paired  $t$ -test:  $t = 114.9$ ,  $p < 0.0001$ ) (Figure 2B).

290 The average cell abundance of the inoculum was  $36 \times 10^6$  cell mL<sup>-1</sup> with its ln value of 17.4 within  
291 the range of the values of the disinfection treatments (Figure 2B).

292

#### 293 *Bacterial clusters*

294 Almost no large clusters were found, and thus we focused only on small clusters. The relative  
295 abundance of small clusters was significantly affected by the interaction between the regrowth tests



296 (limiting or favorable conditions) and the strength of the disinfection treatment (Table 1),  
297 suggesting a differential effect of the strength of disinfection between the two growing conditions.  
298 In details, whereas overall no difference was present in the values between the two growing  
299 conditions, the disinfection treatment had a negative effect under favorable conditions (LM:  $t = -$   
300  $8.4$ ,  $p < 0.0001$ ) but no effect under limiting conditions (LM:  $t = -0.6$ ,  $p = 0.541$ ) (Figure 2C).  
301 Overall the relative abundance of small clusters did not change from the starting values, neither  
302 under limiting conditions (paired  $t$ -test:  $t = 1.6$ ,  $p = 0.144$ ), nor under favorable conditions (paired  $t$ -  
303 test:  $t = -1.4$ ,  $p = 0.182$ ).  
304 The value of the relative abundance of small clusters of the inoculum was  $0.009$ , much lower than  
305 the values after the disinfection treatment (Figure 2c).

#### 307 *Cell number in clusters*

308 The number of cells per small cluster was significantly influenced by the disinfection treatment  
309 (ANOVA summary of LM:  $F = 153.5$ ,  $p < 0.0001$ ), by differences among the three types of  
310 experiment (starting values, limiting, and favorable conditions) ( $F = 539.4$ ,  $p < 0.0001$ ), and by the  
311 interaction between the two levels ( $F = 26.3$ ,  $p < 0.0001$ ).  
312 Regarding the three types of experiment, the number of cells per small cluster ranged from 3 to 9 in  
313 all three. Yet, under limiting conditions (mean $\pm$ st.dev.  $6.3\pm 2.0$ ) it was significantly higher than  
314 under favorable conditions ( $3.9\pm 1.3$ ) and in starting values ( $3.5\pm 1.0$ ) (Tukey's HSD tests: all  $p <$   
315  $0.0001$ ), and it was higher under favorable conditions than in the starting values ( $p = 0.001$ ).  
316 Including the inoculum values ( $3.7\pm 1.0$ ) in the analysis, no differences in cell numbers were found  
317 between inoculum, starting values and under favorable conditions, whereas under limiting  
318 conditions it was significantly higher than in all the others (all  $p < 0.0001$ , Figure 3A).  
319 Analysing the composition of small clusters separately for each step of the experiment (starting  
320 values, limiting, and favorable), the positive relationship with the strength of the disinfection  
321 treatment was supported for all the experiments, but the  $R^2$  values were moderately high only under

322 favorable conditions (LM: adjusted  $R^2 = 0.43$ , Figure 3B, Figure S2 in Supplementary Data),  
323 whereas they were very low under limiting conditions (adjusted  $R^2 = 0.01$ ) and for the starting  
324 values (adjusted  $R^2 = 0.08$ ).

325

### 326 *Relative abundance of ermB*

327 The disinfection treatment and the regrowth experiment had no effect on the relative abundance of  
328 *ermB* (Table 1).

329 After the regrowth tests, the ARG relative abundance did not change in relation to disinfection  
330 treatment nor between the limiting and favorable conditions (Figure 2D, Table 1). The abundance of  
331 *ermB* did not change from the starting values, neither under limiting conditions (paired *t*-test:  $t =$   
332  $1.9$ ,  $p = 0.086$ ) nor under favorable conditions (paired *t*-test:  $t = 1.7$ ,  $p = 0.108$ ).

333 The proportion of *ermB* per 23SrDNA copy number in the bacterial inoculum was of 0.24, without  
334 significant differences to the values measured in the disinfection treatments before the start of the  
335 regrowth experiments.

336

### 337 **Discussion**

338 The results of this study clarify the defence strategies of *E. faecium* under the stress exerted by low  
339 doses of PAA, focusing not only on the efficiency in bacterial inactivation due to disinfection, but  
340 also on the direct response of the strain to the stress, and on its survival chances under limiting  
341 conditions (test A) and under favorable conditions (test B) after the exposure to PAA. Our results  
342 revealed several important aspects.

343 The use of sub-inhibitory low PAA doses, up to  $10 \text{ mg L}^{-1} \text{ min}$ , in which minimal inactivation  
344 values are reached, was followed by a sudden increase in the inactivation efficiency. Such bacterial  
345 response has been already reported and the so-called 'S-model' defined to provide an effective  
346 description of inactivation kinetics (Antonelli et al., 2013). Thus, in the view of minimizing the  
347 PAA dosage to reduce the ecotoxicity of the disinfected effluents and the operational costs, the

348 dose-response curve at low PAA doses based on an appropriated methodological approach was  
349 estimated, accounting for PAA decay. Moreover, low PAA doses can result in a strong and long-  
350 lasting inactivation of FIB, so that the microbiological quality of the effluent can be ensured over  
351 time, as required in case of reclaimed effluent reuse (Antonelli et al., 2006). Counting by flow  
352 cytometry did not evidence any variation in cell abundance ( $\text{cell mL}^{-1}$ ) under the limiting conditions  
353 (room temperature, scarcity of easily biodegradable substrate) imposed by the experimental design,  
354 possibly because not distinguishing active and inactive bacterial cells, anyway present in the  
355 sample. Indeed, the results from plate count ( $\text{CFU mL}^{-1}$ ) are limited to actively growing cells and  
356 thus evidenced that cell damage was irreversible and the decline of cultivable bacterial cells  
357 continued even in the absence of bacteriostatic agents, as also previously reported by Antonelli et  
358 al. (2006). Otherwise, as evidenced by the test under favorable conditions, the non-negligible  
359 residual (not inactivated) bacterial population could grow considerably in a favorable environment,  
360 suggesting that such favorable conditions for proliferation must be strictly avoided in WWTPs.

361 The proportion between small clusters and total cells increased during the experiment, regardless of  
362 the disinfection treatment and of the regrowth tests. This is in agreement with what was observed in  
363 previous studies where aggregation, or the selection towards small clusters, was a common response  
364 of bacterial communities when exposed to a stress such as UV light exposure (Kollu and Örmeci,  
365 2015), predation (Corno and Jurgens, 2006), antibiotic pressure (Corno et al., 2014), and chemical  
366 disinfection (Di Cesare et al., 2016). However, a significant increase of the relative abundance of  
367 small clusters as a consequence of the disinfection treatment was not observed. Indeed, even in  
368 correspondence of the highest PAA dose, no changes in bacterial phenotype were detected,  
369 although the further decrease in  $\text{CFU mL}^{-1}$  observed under limiting conditions in comparison to the  
370 starting values (Figure 2A) supports the occurrence of relevant stress conditions for the bacteria,  
371 even at such low doses of PAA as the ones tested in our experiments.

372 Surprisingly, under favorable conditions the relative abundance of small clusters significantly  
373 decreases as the PAA dose increase. This seems to be in contradiction with other studies on

374 aggregation in aquatic microbes (Di Cesare et al., 2016). However, the analysis of the bacterial  
375 assemblages by epifluorescence microscopy revealed a positive correlation between cells per small  
376 cluster and the strength of the disinfection treatment (Figure 3). This could be explained by the fact  
377 that bacteria under chemical stress or nutrient limitation can modify their phenotype, as previously  
378 reported for *E. coli* showing elongated cells and a correlated increased autofluorescence under stress  
379 by antibiotics (Renggli et al., 2013); also other pathogens like *Legionella pneumophila*, *Salmonella*  
380 *typhimurium* or *Micobacterium tuberculosis* were shown to give rise to filamentous forms when  
381 inside macrophages (Justice et al., 2008). Enterococci do not show changes in cell morphology  
382 under stress, but according to our results they increase the chain length, in agreement with Giard et  
383 al. (2000). The same behaviour was also observed under limiting conditions regardless of PAA  
384 dose, suggesting this phenotypic adaptation as a physiological response of an enterococcal  
385 population to environmental stress.

386 The disinfection treatment did not affect the relative proportion of *ermB* over time (Figure 2D).  
387 This is in agreement with the fact that WWTPs are not specifically designed to remove ARGs  
388 (Zhang et al., 2015) and with recent experimental results obtained by testing the effect of advanced  
389 oxidation process on  $\beta$ -lactams resistance gene, showing that the tested ARG was unaffected by the  
390 disinfection treatment (Ferro et al., 2017).

391

## 392 **Conclusions**

393 Although bacteria are able to express multiple defence strategies in response to the stress imposed  
394 by high level of disinfectants, the present work shows that low PAA doses (below 50 mg l<sup>-1</sup> min)  
395 can efficiently inactivate *E. faecium*, and that PAA disinfection did not affect the *ermB* abundance  
396 within the studied bacterial population. Furthermore, the study highlights the need to share  
397 competences between microbiologists and engineers, because only through a holistic approach the  
398 scientific community will gain the possibility to understand complex ecological systems and to  
399 design efficient disinfection treatments. It is indeed pivotal to take into account not only the

400 efficiency of bacterial inactivation, but also the ecological countermeasures adopted by a bacterial  
401 community. These steps are fundamental for a correct estimation of the survival chances of  
402 disinfectant-treated bacteria once released into open waters. A last remark concerns the importance  
403 of adopting methodologically appropriate practices when dealing with PAA disinfection, including  
404 the actual PAA dose as reference operating parameter rather than the starting PAA concentration,  
405 due to the not negligible occurrence of PAA decay in wastewaters.

406

407 The authors declare no competing financial interest.

408

#### 409 **Description of Supplementary Data**

410 Table S1. Primers pairs used to detect and/or quantify 23SrDNA and *ermB*.

411 Figure S1. Bacterial count on plate as a function of PAA dose in the experiment 1 (dots) and in the  
412 experiment 2 (diamonds). Results are presented as CFU mL<sup>-1</sup> for each dose. In case of experiment 2  
413 mean±st.dev.is reported. IN = inoculum, B = control sample.

414 Figure S2. Comparison of small cluster composition by epifluorescence microscopy after recovery  
415 experiment in **A)** negative control and **B)** sample treated with 25 mg L<sup>-1</sup> min PAA dose.  
416 Magnification 100x. The white arrows point at some small clusters.

417

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424

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- 537

538 **Figure Captions:**

539

540 **Figure 1:** Graphical depiction of the experimental design (disinfection treatments and regrowth  
541 tests under limiting (test A) and favorable conditions (test B)). Abbreviations: BHI, brain-heart  
542 infusion broth; CFU, colony forming units; DNA, extraction for qPCR; FC, flow cytometry; PAA,  
543 Peracetic Acid; PS, physiological solution.

544

545 **Figure 2:** Effect of three doses of disinfection treatment (0, 7.5 and 25 mg l<sup>-1</sup> min) on **A**) CFU  
546 counts, **B**) cell abundance, **C**) relative abundance of small clusters (small clusters/total cells), and  
547 **D**) ARG relative abundance (*ermB* /23SrDNA gene copy), determined in the bacterial inoculum,  
548 starting values, and regrowth experiments under limiting (test A) and favorable conditions (test B).

549

550 **Figure 3:** Size distribution of small clusters by epifluorescence microscopy. Distribution of the  
551 number of cells per cluster in the small clusters considering **A**) the whole study (inoculum, starting  
552 values after the disinfection treatment, regrowth tests under limiting (test A) and favorable  
553 conditions (test B); **B**) the test under favorable conditions as a function of PAA dose. The size of  
554 the circles is proportional to number of clusters for each number of cells.

555

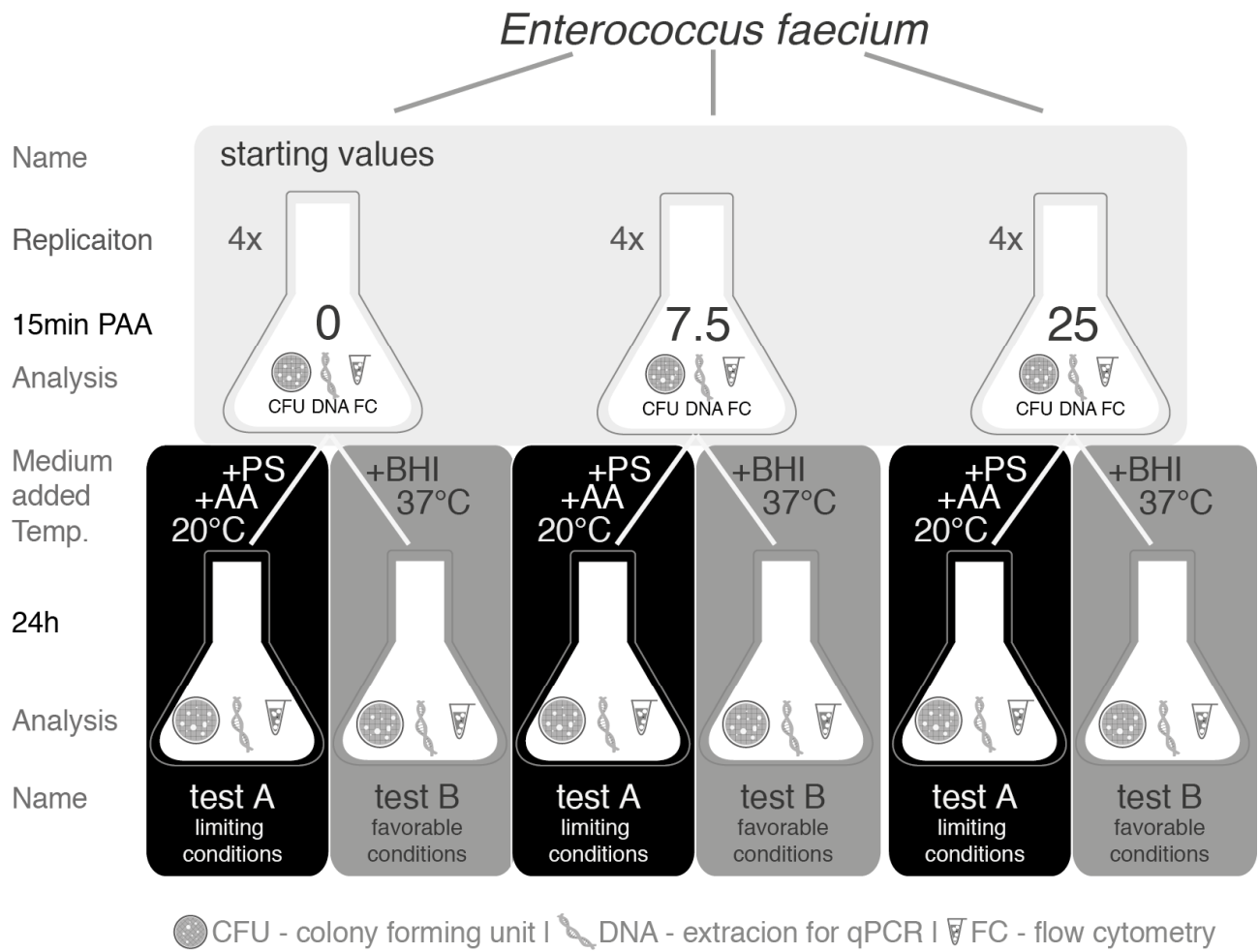
556 **Table 1.** Results of the Linear Mixed Effect Models on the four analysed metrics, in relation to  
 557 disinfection treatment, regrowth tests (under limiting (test A) or favorable conditions (test B)), their  
 558 interaction, and the effect of starting values. Estimates, standard errors, t-values, and p-values are  
 559 reported for the explicitly tested variables retained in the final models. Significance symbols are: \*  
 560 for  $p < 0.05$ , \*\* for  $p < 0.01$ , \*\*\* for  $p < 0.001$ .  
 561

<b>CFU mL<sup>-1</sup></b>	<b>estimate</b>	<b>standard error</b>	<b>t</b>	<b>p</b>	
(intercept)	13.11	4.10	3.21	0.0105	*
disinfection treatment	-0.23	0.05	-4.08	0.0023	**
regrowth test (limiting vs favorable)	5.52	0.33	16.49	<0.0001	***
interaction disinfection:regrowth	0.30	0.02	13.63	<0.0001	***
starting values	0.21	0.23	0.89	0.3961	
<b>Cell mL<sup>-1</sup></b>	<b>estimate</b>	<b>standard error</b>	<b>t</b>	<b>P</b>	
(intercept)	30.05	9.97	3.01	0.0146	*
disinfection treatment	-0.00	0.00	-0.15	0.8844	
regrowth test (limiting vs favorable)	4.03	0.03	119.28	<0.0001	***
interaction disinfection:regrowth	0.01	0.00	4.02	0.0024	**
starting values	-0.73	0.57	-1.27	0.2353	
<b>relative abundance of small clusters</b>	<b>estimate</b>	<b>standard error</b>	<b>t</b>	<b>p</b>	
(intercept)	0.24	0.08	3	0.0146	*
disinfection treatment	0.00	0.00	0.09	0.9314	
regrowth test (limiting vs favorable)	-0.01	0.02	-0.56	0.5864	
interaction disinfection:regrowth	-0.01	0.00	-3.30	0.0079	**
starting values	-0.71	0.70	-1.02	0.3341	

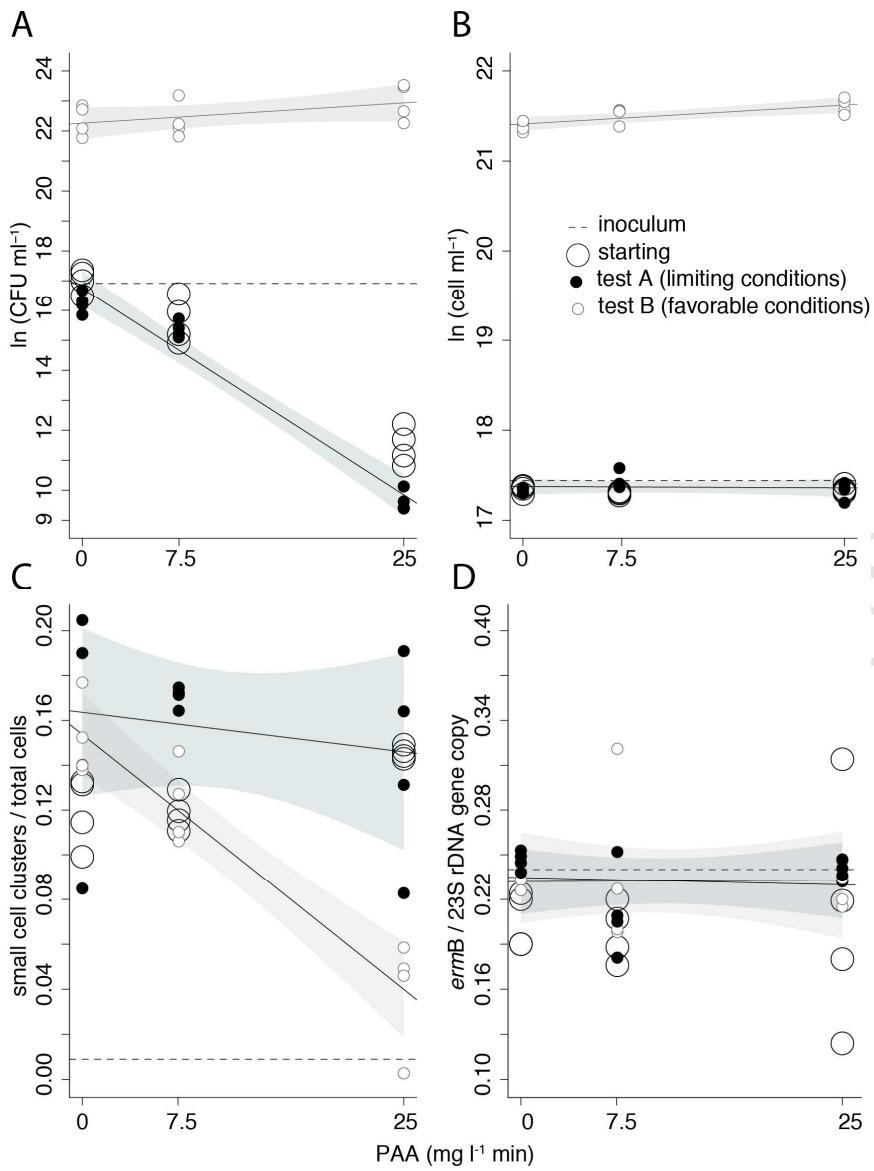
<b>ermB/23SrDNA</b>	<b>estimate</b>	<b>standard error</b>	<b>t</b>	<b>p</b>	
(intercept)	0.24	0.04	6.28	0.0001	***
disinfection treatment	-0.00	0.00	-0.09	0.9270	
regrowth test (limiting vs favorable)	0.00	0.00	0.02	0.9825	
starting values	-0.04	0.18	-0.22	0.8292	

562

563 **Figure 1**  
564

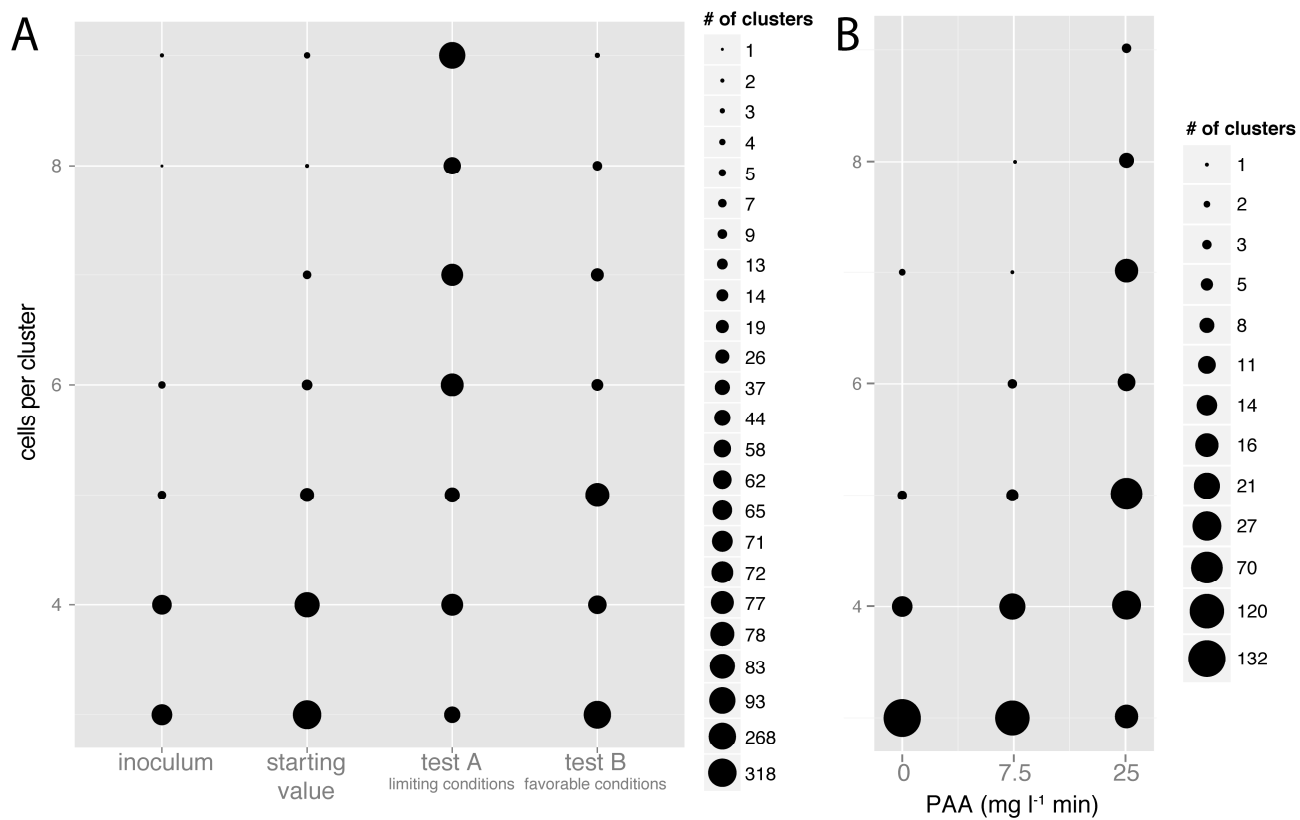


565  
566

567 **Figure 2**

568

569 **Figure 3**  
 570



571  
 572  
 573



**Highlights:**

- Low doses of PAA efficiently remove enterococci
- Disinfection stress induces enterococcal phenotypic changes
- PAA does not affect ARG relative abundance