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(Article begins on next page)

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

Andrea Turolla[a,1], Raffaella Sabatino[b,c,1], Diego Fontaneto[d], Ester M. Eckert[d], Noemi Colinas[d], Gianluca Corno[d,*], Barbara Citterio[e], Francesca Biavasco[f], Manuela Antonelli[a], Alessandro Mauro[c,g], Gianmarco Mangiaterra[f], Andrea Di Cesare[d]

[1] Both authors contributed equally to this work


[a] Department of Civil and Environmental Engineering (DICA) – Environmental Section, Politecnico di Milano, Piazza Leonardo da Vinci 32, 20133 – Milano, Italy

[b] Laboratory of Metabolic Research – [c] Laboratory of Clinical Neurobiology, Ospedale San Giuseppe, IRCCS Istituto Auxologico Italiano, Strada Cadorna 90, 28988 – Piancavallo di Oggebbio, Italy

[d] Microbial Ecology Group, National Research Council – Institute of Ecosystem Study (CNR-ISE), Largo Tonolli 50, 28922 – Verbania, Italy

[e] Department of Biomolecular Sciences, Biotechnology Section, University of Urbino "Carlo Bo", via Arco d'Augusto 2, 61032 – Fano, Italy

[f] Department of Life and Environmental Sciences, Polytechnic University of Marche, via Brecce Bianche, 60131 – Ancona, Italy.

[g] Department of Neuroscience "Rita Levi Montalcini", University of Turin, via Cherasco 15, 10126 – Turin, Italy

*Corresponding author.* Microbial Ecology Group, National Research Council – Institute of Ecosystem Study (CNR-ISE), Largo Tonolli 50, 28922, Verbania, Italy. Tel.: +39 0323518321; fax: +39 0323556513.

*E-mail address:* g.corno@ise.cnr.it (G. Corno)

1

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 $L^{-1}$ 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.

## Introduction

17

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 (Morroni 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;

3

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.,

4

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 (*erm*B) 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 (*erm*B), 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 *erm*B carrying donor strain *E. faecium* 6767/7 and the recipient *E. faecium* 64/3,

5

94  rifampin- and fusidic acid-resistant and carrying the ERY low-level resistance gene *msr*C (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 $mL^{-1}$ (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%, $H_2O_2$ 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 $L^{-1}$ 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 ($PAA_0$) of 1.68 mg $L^{-1}$ 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 $k_{PAA}$ was estimated.

6

$$PAA_t = PAA_0 \cdot e^{-k_{PAA}t} \tag{1}$$

119 Then, initial PAA concentrations required for applying PAA doses ($CT_{PAA}$) ranging from 1 to 25

120 mg L$^{-1}$ 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.

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

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 mg

136 L$^{-1}$ 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 $L^{-1}$.

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 $L^{-1}$ min, four at 25 mg $L^{-1}$ 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

8

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 µL of the dilutions (up to $10^{-8}$)

169 of the inoculum and of the samples on brain-hearth infusion agar plates incubated at 37°C for 24 h.

170 For the measurement of bacterial abundances and phenotypic distribution by flow cytometry,

171 aliquots of 500 µL 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 $5\times10^6$ 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 µm polycarbonate filters and

187 stored at -20°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 mg $L^{-1}$ of lysozyme (Sigma-

9

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 *erm*B 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 *erm*B,

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 $\mu L^{-1}$ of 23SrDNA and *erm*B 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 *erm*B 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 $mL^{-1}$, cell $mL^{-1}$, proportion between small cluster/total cell numbers, and *erm*B/23SrDNA copy

211 number. For all models with CFU $mL^{-1}$ and cell $mL^{-1}$, 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 mg $L^{-1}$ min), by (2)

10

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 $L^{-1}$

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

**Results**

*Experiment 1*

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

*Experiment 2*

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

*Bacterial CFU counts*

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

12

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$^{-1}$ values were

272    obtained under limiting conditions than under favorable conditions (Figure 2A); moreover, CFU

273    mL$^{-1}$ 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$^{-1}$ values slightly diminished from the starting values (paired *t*-

277    test: t = -3.8, p = 0.0028), whereas under favorable conditions CFU mL$^{-1}$ 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$^{-1}$ was 21.8x10$^6$ 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$^{-1}$ 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$^{-1}$ 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 x10$^6$ cell mL$^{-1}$ 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

13

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: $t = -8.4$, $p < 0.0001$)

300  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).

306

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±st.dev. 6.3±2.0) it was significantly higher than

314  under favorable conditions (3.9±1.3) and in starting values (3.5±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±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

14

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     *erm*B (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     *erm*B 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 *erm*B 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 mg $L^{-1}$ 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

15

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 (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 (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 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

16

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 *erm*B 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 β-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 $l^{-1}$ min)

395 can efficiently inactivate *E. faecium*, and that PAA disinfection did not affect the *erm*B 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

17

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 *erm*B.

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$^{-1}$ 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$^{-1}$ min PAA dose.

416   Magnification 100x. The white arrows point at some small clusters.

417

418   **Acknowledgements**

424

425   **References**

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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-hearth

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$^{-1}$ 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 (*erm*B /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

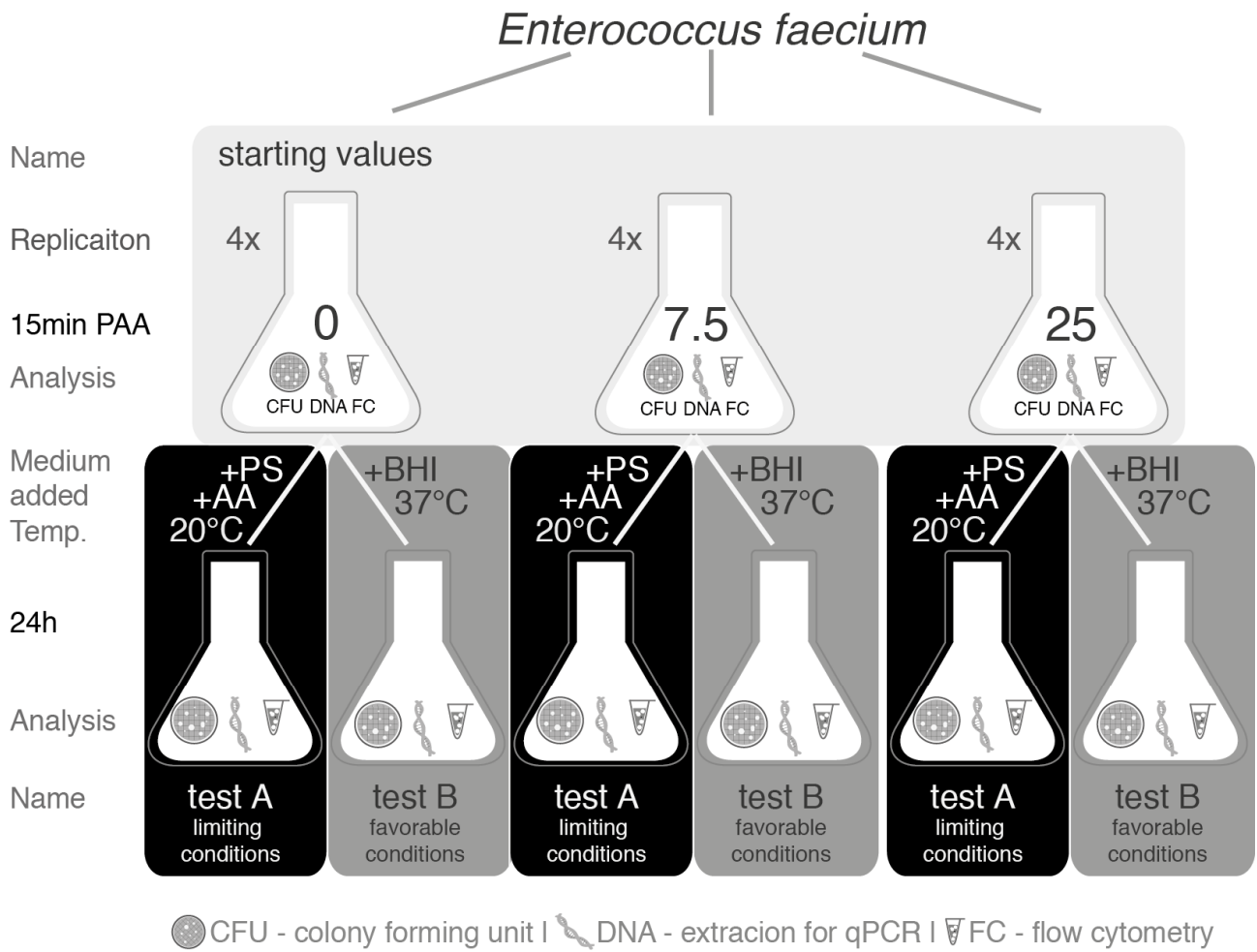554 the circles is proportional to number of clusters for each number of cells.

555

24

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

| CFU mL$^{-1}$ | estimate | standard error | t | p | |
|---|---|---|---|---|---|
| (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 | |
| Cell mL$^{-1}$ | estimate | standard error | t | P | |
| (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 | |
| relative abundance of small clusters | estimate | standard error | t | p | |
| (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 | |

| ermB/23SrDNA | estimate | standard error | t | p | |
|---|---|---|---|---|---|
| (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

26

563  **Figure 1**
564



CFU - colony forming unit l DNA - extracion for qPCR l FC - flow cytometry
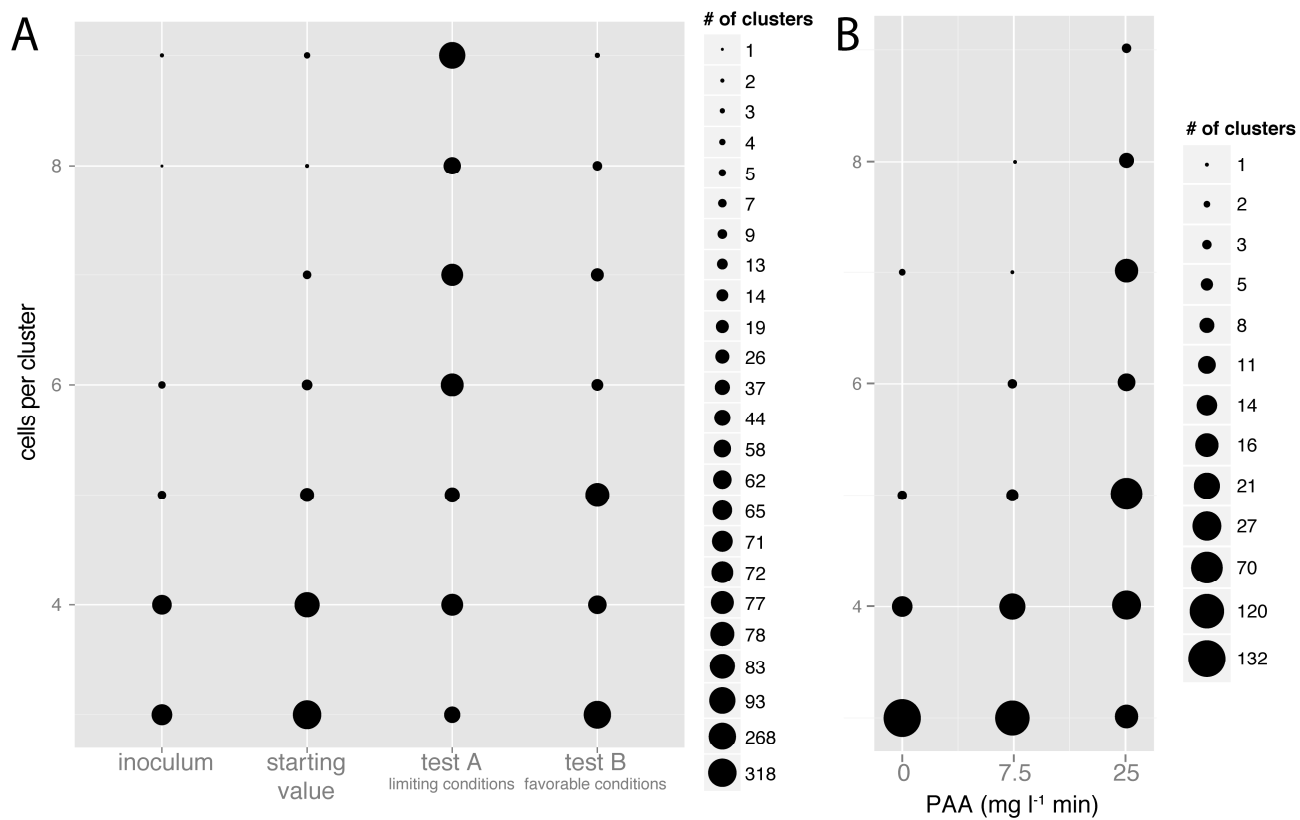
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567    **Figure 2**
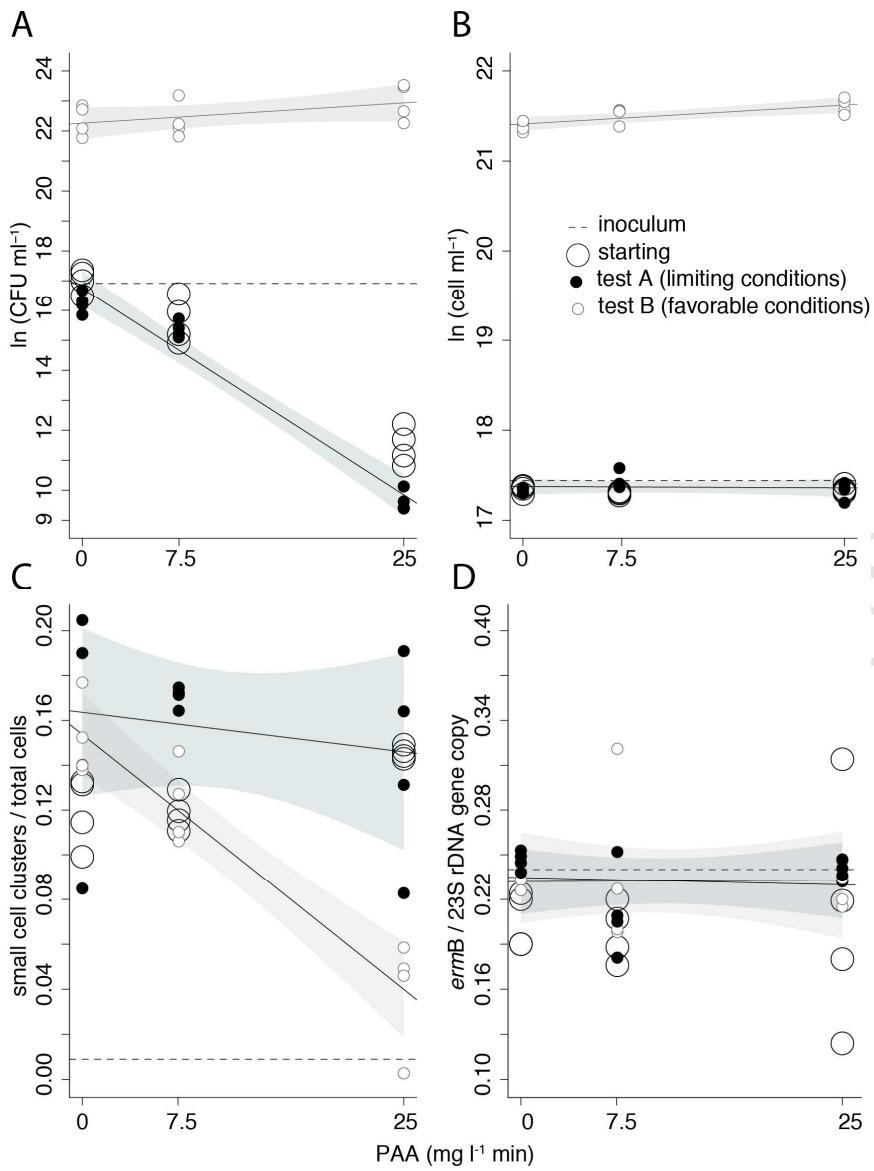


568

569    **Figure 3**
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**Highlights:**

- Low doses of PAA efficiently remove enterococci

- Disinfection stress induces enterococcal phenotypic changes

- PAA does not affect ARG relative abundance