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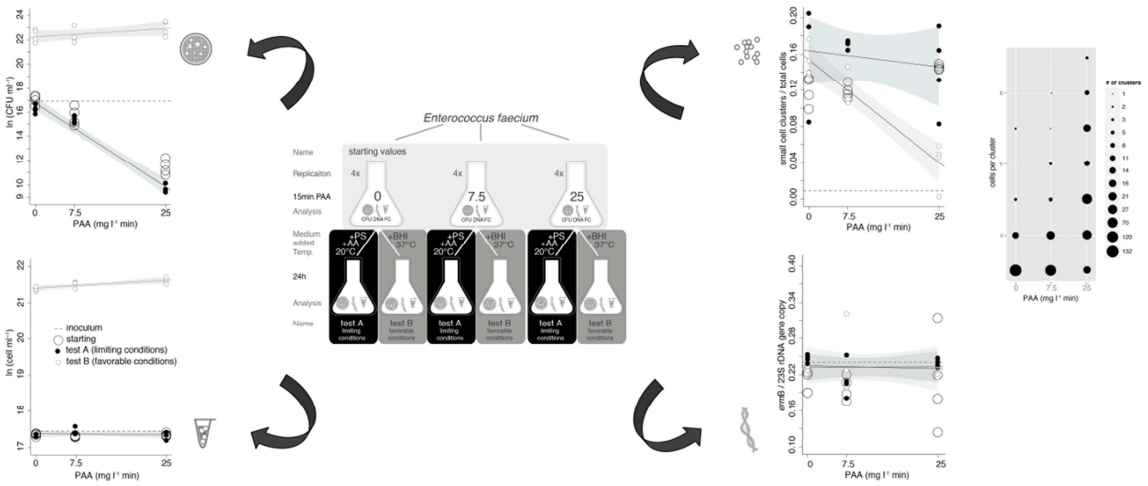

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Defence strategies and antibiotic resistance gene abundance in enterococci under stress by exposure to low doses of peracetic acid

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Abstract

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

Introduction

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

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

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

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

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

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

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

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

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

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

Material and methods

Bacterial strain

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

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

Inoculum preparation

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

Experimental design

The experimental design consisted of two parts. In the first one, referred to as "experiment 1", the response of the strain *E. faecium* 64/3-67/7E to PAA was investigated in order to determine the doses of a PAA commercial solution (VigorOx WWT II, Peroxychem, PAA 15%, H_2O_2 23%, AA 16%) to be used in the following steps. In the second part, referred to as "experiment 2", the response of a population of *E. faecium* 64/3-67/7E to the disinfection treatment and during two tests of regrowth in low (heavy limiting conditions, test A) and rich (favorable conditions, test B) medium (Figure 1).The aim of "experiment 1" was to select two doses for the subsequent experiments, within the range of what is expected as low doses of PAA, as previously discussed, in order to determine a strong (order of logs) and weak (about 50%) inactivation of FIB. The upper limit for the investigated interval of doses in experiment 1 was defined *a priori* as 25 mg L^{-1} min and several lower doses were assessed. Decay and microbial inactivation tests were performed using 100 mL aliquots of the inoculum. As for decay tests, the decrease of residual PAA concentration over time (t) at an initial PAA concentration (PAA_0) of 1.68 mg L^{-1} was evaluated in triplicate replicates. Results were fitted with a first-order kinetic model, as reported in Equation 1, and the decay coefficient k_{PAA} was estimated.

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

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

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

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

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

For the experiment under limiting conditions (test A) the subsamples were incubated with shaking at 20°C for 24 h in physiological solution 0.9% (80 mL for each subsample) supplemented with different AA concentrations (as carbon source) to reach the final AA concentration of 2 mg L^{-1}. Specifically, the amount of AA added for each subsample was calculated by considering the amount already present in the flasks after the disinfection treatment, derived from PAA decay, to avoid the unbalance of carbon content in different subsamples. For the experiment under favorable conditions (test B), the subsamples were incubated at 37°C for 24 h in BHI (80 mL for each subsample).

A total of 37 subsamples were processed for bacterial count, size distribution of cell clusters, and molecular analyses. These represented: 3 subsample of the inoculum, 12 subsamples after the disinfection treatment (four at 7.5 mg L^{-1} min, four at 25 mg L^{-1} min, plus four controls), and 24 subsamples of the four replicate for each the three groups of PAA dose after the tests under limiting and under favorable conditions.

Residual PAA concentration

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

Bacterial count and phenotype

Bacterial abundance was evaluated both by plate count and by flow cytometry. Bacterial phenotype 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 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 5×10^{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 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-

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 μL^{-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)

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

244

245 **Results**

246 ***Experiment 1***

247 A residual PAA concentration of 1.49±0.08 mg L^{-1} was reached in the aqueous solution after 15
248 minutes from the dosage of an initial PAA concentration of 1.68 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_{PAA} = 0.0072±0.0030 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 mg L^{-1} were calculated for PAA doses ranging from 1 to 25 mg L^{-1} min.
254 Experimental results obtained in the experiment 1 revealed a progressive decrease of the bacterial
255 count, measured in terms of 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 mg L^{-1}
257 min. Consequently, the intermediate dose chosen for the experiment was between 5 and 10 mg L^{-1}
258 min, namely 7.5 mg L^{-1} min. Thus, the two selected doses, 7.5 and 25 mg L^{-1} 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 log and 2.37 log were obtained for 7.5 and 25 mg L^{-1} 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

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

Under limiting conditions, CFU mL^{-1} values slightly diminished from the starting values (paired *t*-test: $t = -3.8$, $p = 0.0028$), whereas under favorable conditions CFU mL^{-1} values significantly increased from the starting values (paired *t*-test: $t = 9.5$, $p < 0.0001$) (Figure 2A).

As for the inoculum, average CFU mL^{-1} was 21.8×10^6 with its ln value of 16.9 within the range of the negative control (Figure 2A).

Bacterial cell count

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

The average cell abundance of the inoculum was 36×10^6 cell mL^{-1} with its ln value of 17.4 within the range of the values of the disinfection treatments (Figure 2B).

Bacterial clusters

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

(limiting or favorable conditions) and the strength of the disinfection treatment (Table 1), suggesting a differential effect of the strength of disinfection between the two growing conditions. In details, whereas overall no difference was present in the values between the two growing conditions, the disinfection treatment had a negative effect under favorable conditions (LM: $t = -8.4$, $p < 0.0001$) but no effect under limiting conditions (LM: $t = -0.6$, $p = 0.541$) (Figure 2C).

Overall the relative abundance of small clusters did not change from the starting values, neither under limiting conditions (paired t-test: $t = 1.6$, $p = 0.144$), nor under favorable conditions (paired *t*-test: $t = -1.4$, $p = 0.182$).

The value of the relative abundance of small clusters of the inoculum was 0.009, much lower than the values after the disinfection treatment (Figure 2c).

Cell number in clusters

The number of cells per small cluster was significantly influenced by the disinfection treatment (ANOVA summary of LM: $F = 153.5$, $p < 0.0001$), by differences among the three types of experiment (starting values, limiting, and favorable conditions) ($F = 539.4$, $p < 0.0001$), and by the interaction between the two levels ($F = 26.3$, $p < 0.0001$).

Regarding the three types of experiment, the number of cells per small cluster ranged from 3 to 9 in all three. Yet, under limiting conditions (mean±st.dev. 6.3±2.0) it was significantly higher than under favorable conditions (3.9±1.3) and in starting values (3.5±1.0) (Tukey's HSD tests: all $p < 0.0001$), and it was higher under favorable conditions than in the starting values ($p = 0.001$). Including the inoculum values (3.7±1.0) in the analysis, no differences in cell numbers were found between inoculum, starting values and under favorable conditions, whereas under limiting conditions it was significantly higher than in all the others (all $p < 0.0001$, Figure 3A).

Analysing the composition of small clusters separately for each step of the experiment (starting values, limiting, and favorable), the positive relationship with the strength of the disinfection 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 *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

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

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

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

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

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

Conclusions

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

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

The authors declare no competing financial interest.

Description of Supplementary Data

Table S1. Primers pairs used to detect and/or quantify 23SrDNA and *erm*B.

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

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

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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.

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

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	

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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	

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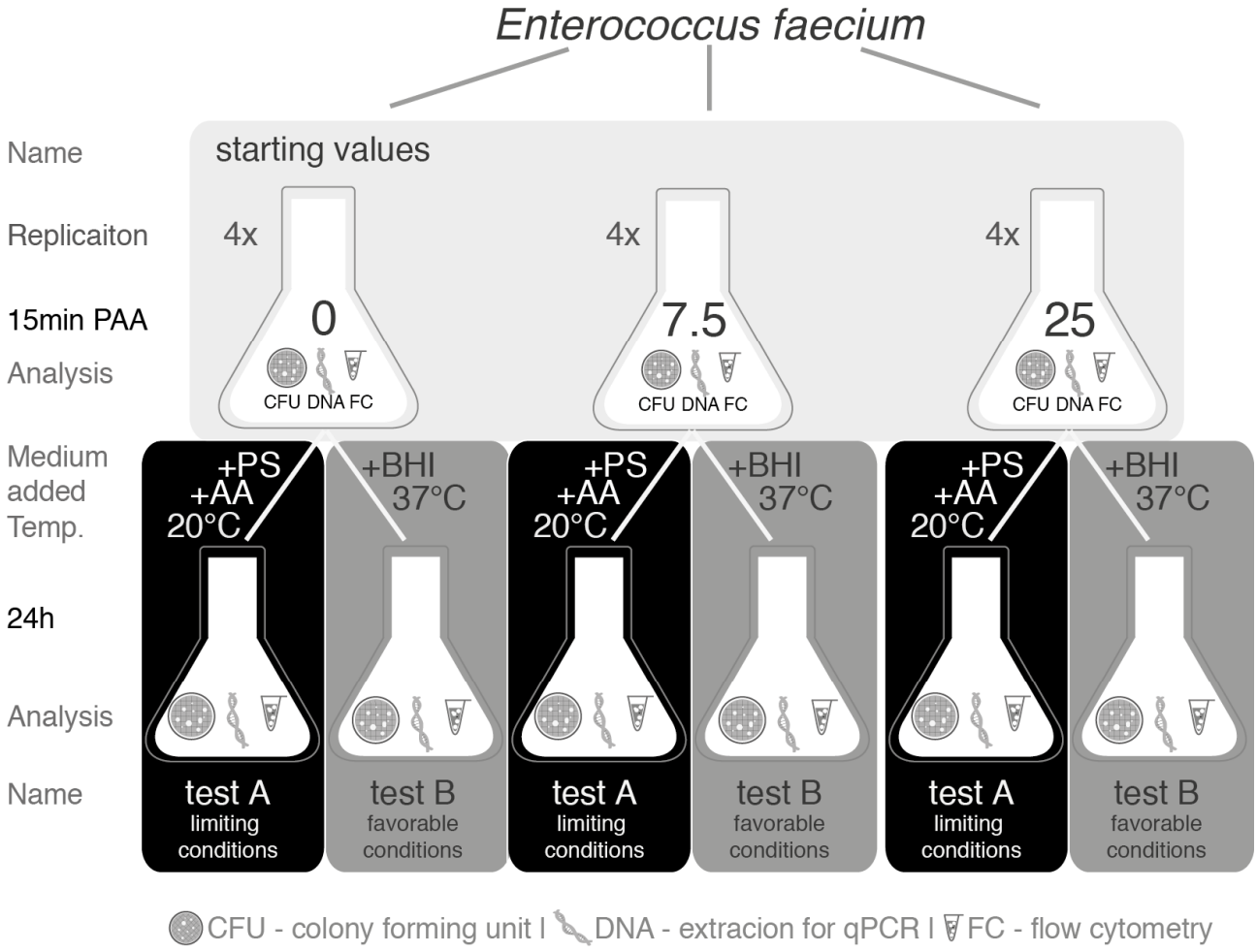
563 **Figure 1**
564



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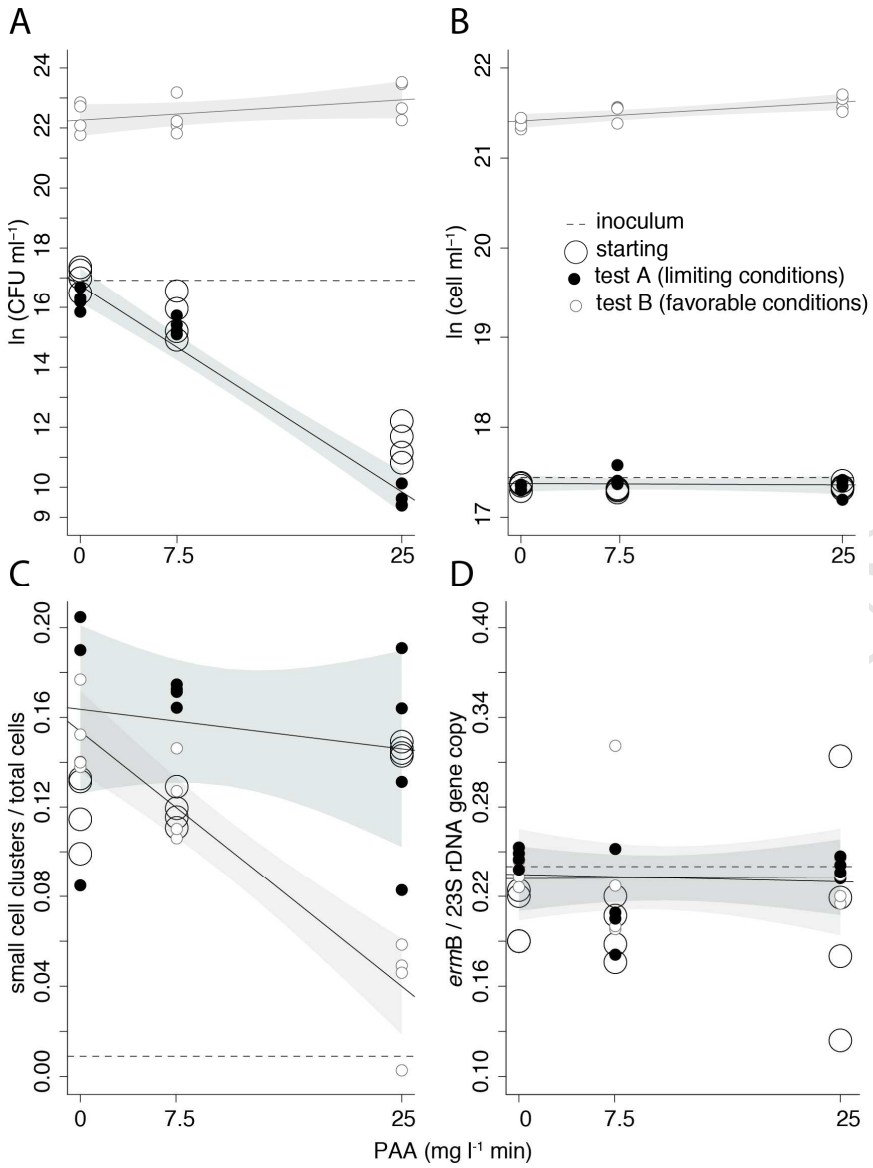
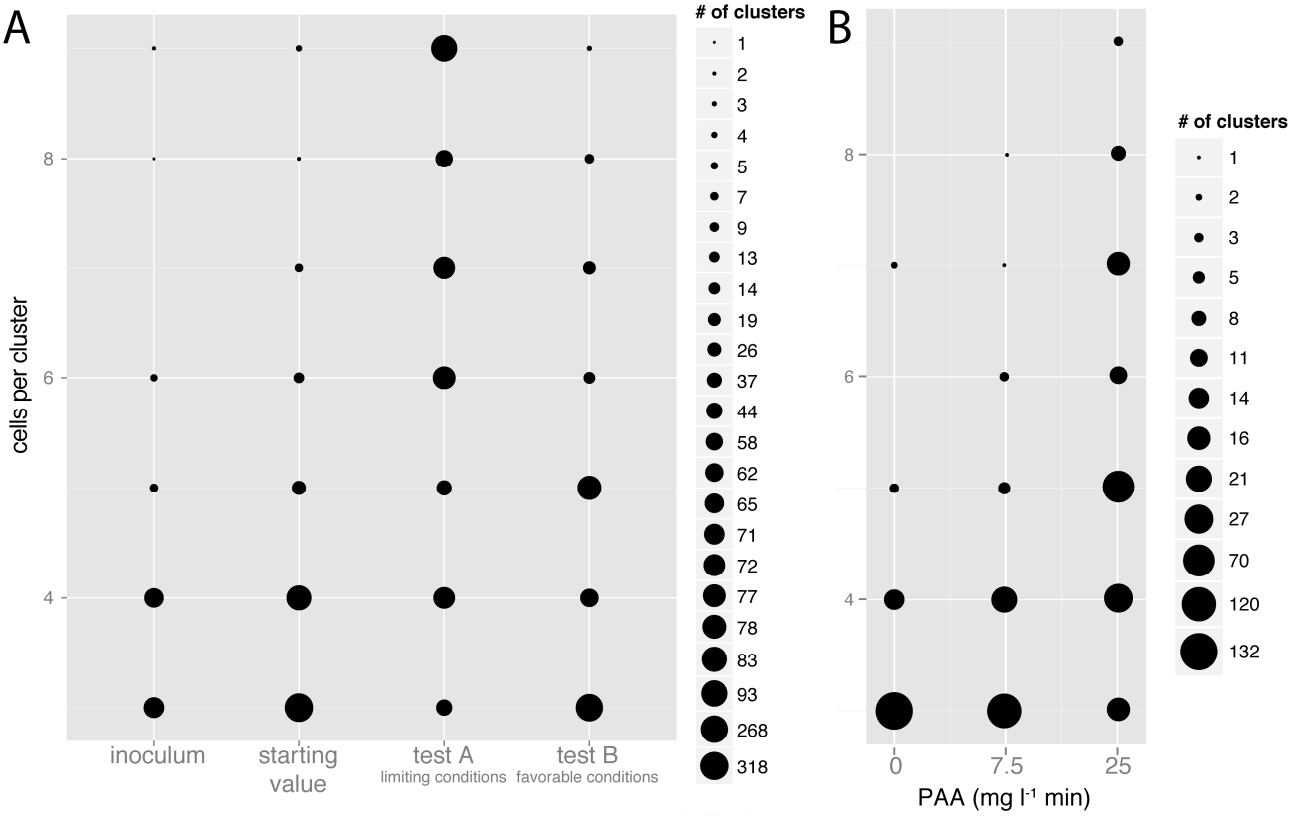
567 **Figure 2**



568

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

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