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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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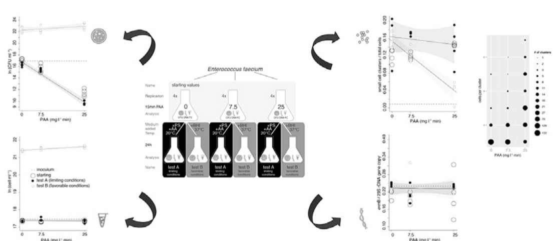
HIGHLIGHTS

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

GRAPHICAL ABSTRACT



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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.

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1. 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 min 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 min) 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 ARGs (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 number when exposed to different experimental conditions.

2. Material and methods

2.1. 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).

2.2. 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 min, 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).

2.3. 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*