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Experimental approach for a possible integrated protocol to determine sanitizer activity against both planktonic bacteria and related biofilms

Raffaella Campana[a,*], Eleonora Ciandrini[b], Wally Baffone[a]

[a] Department of Biomolecular Science, Division of Toxicological, Hygiene and Environmental Science, University of Urbino Carlo Bo, Urbino, Italy
[b] Department of Translational Research and New Technologies in Medicine and Surgery, University of Pisa, Italy

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ABSTRACT

The persistence of pathogenic bacteria in industrial settings is linked to biofilm embedded bacteria resistance to antimicrobial and disinfectant methods effective against planktonic cells. We proposed an experimental approach to evaluate sanitizers effectiveness against both planktonic microorganisms and related biofilms as possible integration of the official EN 1276 procedure. Firstly, the efficacy of three chemicals sanitizers was tested on planktonic cells of *Escherichia coli* O157:H7 ATCC 35150, *Staphylococcus aureus* ATCC 43387, *Pseudomonas aeruginosa* ATCC 9027, *Enterococcus faecalis* ATCC 29212 and *Candida albicans* ATCC 14053 using the suspension test indicated by EN 1276 in both dirty and clear simulated conditions (0.3% or 0.03% of bovine serum albumen). The sanitizers were tested against the related biofilms developed on stainless steel for 48 h at room temperature. The sanitizers (SANI 626, SUPERIG, IGIEN 155) reached 5-logarithmic reduction at the manufacture's recommended concentrations after 30 s and 5 min against planktonic microorganisms but, sometimes, the organic load interfered with their activity. The same concentrations tested with the proposed protocol weren't effective against biofilms and a log reduction > 3 was reached using higher concentrations of the sanitizers and 15 min of contact, with the exception of IGIEN 155. The efficacy of a disinfectant/sanitizer is assessed against planktonic microorganisms and bacteria adhered to surface, while those embedded in biofilms are not taken into consideration. The proposed protocol could be used to evaluate the effectiveness of a sanitizer also against microorganisms organized in biofilms, in order to give to the users more detailed information on its activity.

1. Introduction

Disinfectants are chemical agents used to inactivate all recognized pathogenic microorganisms on inanimate objects (Centers for Disease Control and Prevention, USA, 2008). The European standard (EN 14885, 2015) specifies the laboratory methods to be used for testing the activity of products, i.e. chemical disinfectants and antiseptics, in order to support claims that they have specific properties appropriate to their intended application. Differently from antibiotics that act on specific structures or metabolic processes in microbial cells, disinfectants have non-specifically activity against multiple targets (Meyer & Cookson, 2010) and are generally applied on surfaces in stables, abattoirs, food industry and retail shops (Cerf et al., 2010). The commercialized disinfectants for food processing environment included alcohol-based products, hypochloric solutions, aldehydes, peracetic acid, hydrogen peroxide, chlorhexidine digluconate, polyhexamethylene biguanides (PHMB) and quaternary ammonium compounds (QACs) (Abdallah et al., 2014; Campana & Baffone, 2017; Coughlan et al., 2016). However, microorganisms may survive to biocide treatments on contaminated surfaces, as reported by several investigations on the persistence of microorganisms after cleaning and disinfection of food surfaces (Giaouris et al., 2014), medical (Abdallah et al., 2014) and domestic environments (Cooper et al., 2008). The resistance of microorganisms to disinfectants is frequently associated with bacteria organized in a three-dimensional structure known as biofilm (Singh et al., 2017). Notably, a biofilm consists of surface-colonizing microbes associated in a slimy matrix composed of extracellular polymeric substances (EPS) that they produce; a biofilm can be developed both on abiotic and biotic surfaces as its structural complexity confers to microbial cells a reduced susceptibility to disinfectants (Srey et al., 2013). In food-processing environment, some conditions can promote the attachment and the following biofilm formation, such as flowing water, organic matter raw materials and suitable surfaces. Moreover, it was observed that attachment occurs most readily on surfaces that are rougher, more hydrophobic and coated by surface conditioning films (Oulahal et al., 2008; Patel et al., 2007). Nevertheless, surfaces less

* Corresponding author at: Department of Biomolecular Science, Division of Toxicological, Hygiene and Environmental Science, Via S. Chiara 27, 61029 Urbino, Italy.
E-mail address: raffaella.campana@uniurb.it (R. Campana).

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rough, such as stainless steel, are equally colonized by bacteria because the micro-topography of this material examined by canning electron microscope (SEM) (Zoltai et al., 1981) and Atomic Force Microscopy (AFM) (Arnold & Bailey, 2000) reveals cracks and crevices, which could provide a greater area for cell attachment and possible protection from chemicals cleaning (Palmer et al., 2007).

The persistence of microorganisms on food contact surfaces has the potential to transmit pathogens to food mainly by cross-contamination from raw products via hands, cleaning cloths, sponges and utensils used with foods not subjected to further cooking, contributing to the occurrence of outbreaks of foodborne illness (Giaouris et al., 2014). The activity of disinfectants, sanitizers and antiseptics is determined as indicated by the European Committee for Standardization (CEN), applying standardized tests involving different stages, such as the water dilution of the product at different concentrations, the presence of inhibitory substances that simulate the organic load, or conditions comparable to the practical use of the product. In this direction, the European standards (EN 1040, 2005; EN 1276, 2009) determine bactericidal activity of biocides based on the susceptibilities of planktonic cells, while (EN 13697, 2015) evaluate quantitatively the antimicrobial effectiveness against microbes adhered on polymeric or hydrophobic material. In any case, no official protocol takes in consideration the bacteria organized in biofilm.

For this, the aim of this study was to compare the efficacy of three chemicals sanitizers, commonly used in food industry environments, against different pathogens both in planktonic and biofilm state, in order to define a preliminary integrated protocol for determining the effectiveness of a sanitizer. The experimental design included two different phases: in the first, the efficacy of the sanitizers to reduce the bacterial growth of *Escherichia coli* O157:H7 ATCC 35150, *Staphylococcus aureus* ATCC 43387, *Pseudomonas aeruginosa* ATCC 9027, *Enterococcus faecalis* ATCC 29212 and *Candida albicans* ATCC 14053 in planktonic cultures was assessed by the official suspension test indicated by EN 1276; in the second part, the activity of the sanitizers was tested against the related bacterial biofilms developed on stainless steel surface.

2. Material and methods

2.1. Sanitizers

On the basis of our previous experience (Campana & Baffone, 2017) in which we tested the antimicrobial efficacy of several chemicals sanitizers kindly furnished by M.D. International (Fermignano, Urbino, Italy), three products, SANI 626, SUPERIG and IGIEN 155, were selected and used in this study.

The manufacturer has not revealed the complete composition of each product but gave only the active ingredients of the formulations: SANI 626 (benzalkonium chloride 2.5%; sodium hydroxide 3%), SUPERIG (benzalkonium chloride 10%) and IGIEN 155 (sodium hypochlorite 4.5%; potassium hydroxide 10%). All the sanitizers were hard water diluted and tested at the manufacturer's recommended concentrations: 1% for SANI 626 (benzalkonium chloride 0.025%; sodium hydroxide 0.03%) and SUPERIG (benzalkonium chloride 0.10%), 5% for IGIEN 155 (sodium hypochlorite 0.225%; potassium hydroxide 0.5%). All the solutions were maintained at room temperature (RT) in the dark until use.

2.2. Bacterial strains and growth conditions

Five reference human pathogens were used in this study, *E. coli* O157:H7 ATCC 35150, *S. aureus* ATCC 43387, *P. aeruginosa* ATCC 9027, *E. faecalis* ATCC 29212, *C. albicans* ATCC 14053 (American Type Culture Collection, Manassas, United States). All the strains were routinely maintained in Tryptic Soy Agar (TSA) (Oxoid, Milan, Italy) at 37 °C, while stock cultures were keep at −80 °C in Nutrient broth (Oxoid) with 15% of glycerol.

2.3. Determination of chemicals sanitizers' activity by quantitative suspension test

The evaluation of chemicals sanitizers' activity on target bacteria was performed by the quantitative suspension test, according to the standard procedure EN 1276:2009 described by Sandle (Sandle, 2017) and reported in testing protocol of Eurofins on the efficacy and stability studies of disinfectants biocides (Eurofins, n.d).

Briefly, each sanitizer was distributed in sterile tubes containing 8 mL of sterile water in order to reach the final manufacturer's recommended concentration (1% for SANI 626 and SUPERG, 5% for IGIEN 155). Then, bovine serum albumen (BSA, Sigma) was added to simulate dirty and clean conditions (final concentration of BSA 0.3 and 0.03% respectively). At this point, one mL of each bacterial overnight cultures, grown in TSB at 37 °C and adjusted by spectrophotometer to about 10^6 cell/mL, was added and left in contact for 30 s, 5 and 15 min at 20 ± 1 °C. Control samples were added by inoculating one mL of each bacterial overnight cultures in 9 mL of sterile distilled water. In this way, for each microorganism, three series of sterile tubes were prepared and subdivided as follow: three experimental tubes, one for each time of contact (30 s, 5 and 15 min), with 0.3% of BSA (simulated dirty condition); three experimental tubes, one for each time of contact (30 s, 5 and 15 min), with 0.03% of BSA (simulated clean condition); three control tubes, one for each time of contact (30 s, 5 and 15 min), with sterile water. At each time point, 1 mL from each tube was transferred to another correspondent series of tubes containing 9 mL of neutralizing buffer (polysorbate 80, 3% *v*/v, saponin 3% *w/v* and lecithin 0.3% *w/v*) (Sigma) for 2 min; then, one mL aliquot from each tube was diluted in physiological saline solution, plated in triplicate onto TSA and incubated at 37 °C for 24 h. At the end of incubation, the colony forming units (CFU) were enumerated. Control samples were treated with 9 mL of sterile distilled water instead of sanitizer and enumerated as above. Previous experiments were carried out to verify that the neutralizing solution has no antibacterial effect on microbial strains but neutralized the antimicrobial effect of each sanitizer, as request by EN 1276 (data not shown).

Each experiment was carried out two times in duplicate.

2.4. Biofilm formation

Stainless steel type 304 coupons (2x2x0.8 cm) were firstly cleaned with acetone to remove grease, treated with HCl 5 N for 15 min and cleaned in a detergent solution (ethanol 70%). At final, the coupons were double rinsed with deionized water, air-dried and sterilized by autoclave for 15 min at 121 °C.

All the strains were grown in Tryptic Soy Broth (TSB, Oxoid) at 37 °C for 24 h to obtain a bacterial suspension at the end of the logarithmic phase. The optical density of each suspension (OD_{610} nm) was adjusted to about 0.13–0.15 correspondent to 10^6–10^7 CFU/mL and used for assembling biofilms on stainless steel coupons as described by Campana et al. (Campana et al., 2017) with several modifications. Briefly, the sterilized coupons were placed in sterile Petri dishes containing 2 mL of bacterial suspension and 18 mL of 1:10 diluted TBS, and then incubated at RT for 48 h under static condition. After this period, the coupons were aseptically removed and washed in sterile PBS to eliminate the unbound bacteria; fresh TSB medium (10 mL) was added and the incubation was prolonged at the same conditions described above. This procedure was repeated every 24 h to allow biofilm formation. To enumerate the number of viable cells in biofilm after incubation, the coupons were PBS washed again and the adherent bacteria were removed from the upper side of each coupon by swabbing with sterile cotton swab. Swabs were then transferred into sterile 15 mL tube containing 1 mL of physiological saline and shaken for 2 min by vigorous vortexing. The bacterial suspensions were serially diluted in