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ARTICLE

Removal of pathogenic bacterial biofilms by combinations of oxidizing compounds

Gabriela María Olmedo, Mariana Grillo-Puertas, Luciana Cerioni, Viviana Andrea Rapisarda, and Sabrina Inés Volentini

Abstract: Bacterial biofilms are commonly formed on medical devices and food processing surfaces. The antimicrobials used have limited efficacy against the biofilms; therefore, new strategies to prevent and remove these structures are needed. Here, the effectiveness of brief oxidative treatments, based on the combination of sodium hypochlorite (NaClO) and hydrogen peroxide (H_2O_2) in the presence of copper sulfate ($CuSO_4$), were evaluated against bacterial laboratory strains and clinical isolates, both in planktonic and biofilm states. Simultaneous application of oxidants synergistically inactivated planktonic cells and prevented biofilm formation of laboratory *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, *Klebsiella pneumoniae*, and *Staphylococcus aureus* strains, as well as clinical isolates of *Salmonella enterica* subsp. *enterica*, *Klebsiella oxytoca*, and uropathogenic *E. coli*. In addition, preformed biofilms of *E. coli* C, *Salmonella* Typhimurium, *K. pneumoniae*, and *Salmonella enterica* exposed to treatments were removed by applying 12 mg/L NaClO, 0.1 mmol/L $CuSO_4$, and 350 mmol/L H_2O_2 for 5 min. *Klebsiella oxytoca* and *Staphylococcus aureus* required a 5-fold increase in NaClO concentration, and the *E. coli* clinical isolate remained unremovable unless treatments were applied on biofilms formed within 24 h instead of 48 h. The application of treatments that last a few minutes using oxidizing compounds at low concentrations represents an interesting disinfection strategy against pathogens associated with medical and industrial settings.

Key words: biofilm, oxidative treatment, disinfection, pathogens.

Résumé : Des biofilms bactériens se forment souvent sur la surface de dispositifs médicaux et d'équipements de traitement des aliments. Puisque les antibiotiques actuels n'ont que peu d'emprise sur eux, il est nécessaire d'élaborer de nouvelles stratégies aptes à prévenir et éliminer ces structures. Dans la présente étude, on a évalué l'efficacité de brefs traitements d'oxydants, basés sur une combinaison de NaClO et de H_2O_2 en présence de $CuSO_4$, pour contrer des souches bactériennes de laboratoire et des isolats cliniques en phase planctonique ou sous forme de biofilm. L'application simultanée d'oxydants a inactivé les cellules planctoniques de manière synergique tout en prévenant la formation de biofilm formé par des souches de laboratoire d'*Escherichia coli*, *Salmonella enterica* serovar Typhimurium, *Klebsiella pneumoniae* et *Staphylococcus aureus* ainsi que des isolats cliniques de *Salmonella enterica* subsp. *enterica*, *Klebsiella oxytoca* et *E. coli* uropathogène. En outre, des biofilms préformés d'*E. coli* C, *Salmonella* Typhimurium, *K. pneumoniae* et *Salmonella enterica* exposés aux traitements ont été éliminés en appliquant 12 mg/L de NaClO, 0,1 mmol/L de $CuSO_4$ et 350 mmol/L de H_2O_2 pendant 5 min. On a eu besoin de 5 fois plus de NaClO pour venir à bout de *K. oxytoca* et *Staphylococcus aureus* et l'isolat clinique d'*E. coli* était impossible à éliminer à moins de raccourcir la durée de formation de ses biofilms (24 h au lieu de 48 h). L'application d'une durée de quelques minutes de traitements à base de substances oxydantes à faibles concentrations représente une stratégie attrayante pour lutter contre les pathogènes associés aux installations médicales et industrielles. [Traduit par la Rédaction]

Mots-clés : biofilm, traitement oxydatif, désinfection, pathogènes.

Introduction

In clinical and industrial settings, bacteria are generally found forming biofilms (Costerton et al. 1995; Danese et al. 2000). Biofilms are defined as complex cell assemblages enclosed in an adherent matrix exhibiting channels and pillars that allow nutrient exchange and waste elimination (Costerton et al. 1995, 1999; Massol-Deya et al. 1995; Davey and O'Toole 2000). The biofilm's complex structural matrix effectively protects it from chemical sanitizers, rendering the structure more resistant to aqueous antimicrobial agents than their planktonic counterparts are (McLean et al. 2004). Diverse hospital-acquired infections have been associated with biofilm growth on medical devices, such as catheters, probes, and endoscopes (Fletcher and Bodenham 1999; Wagenlehner and Naber 2000). Biofilm formation by pathogenic bacteria on food contact surfaces can cause cross-contamination of final products and foodborne diseases (Brooks and Flint 2008). Bacteria of the genera *Klebsiella*, *Salmonella*, *Escherichia*, and *Staphylococcus* are important opportunistic pathogens associated with both hospital-acquired infections and foodborne diseases (Brisse and Verhoef 2001; Ibarra and Steele-Mortimer 2009; David and Daum 2010; Naves et al. 2010). Several strategies have been developed in the last decades to eradicate biofilms from food contact surfaces and medical devices, including physical and chemical methods or a combination of them (Schwach and Zottola 1984; Gibson et al. 1999; Chen and Stewart 2000; Chmielewski and Frank 2003; Cha et al. 2012).

Disinfectants are chemical agents used in medical, industrial, and domestic environments to inactivate pathogenic microorganisms that are contaminating inanimate objects (Rutala and Weber

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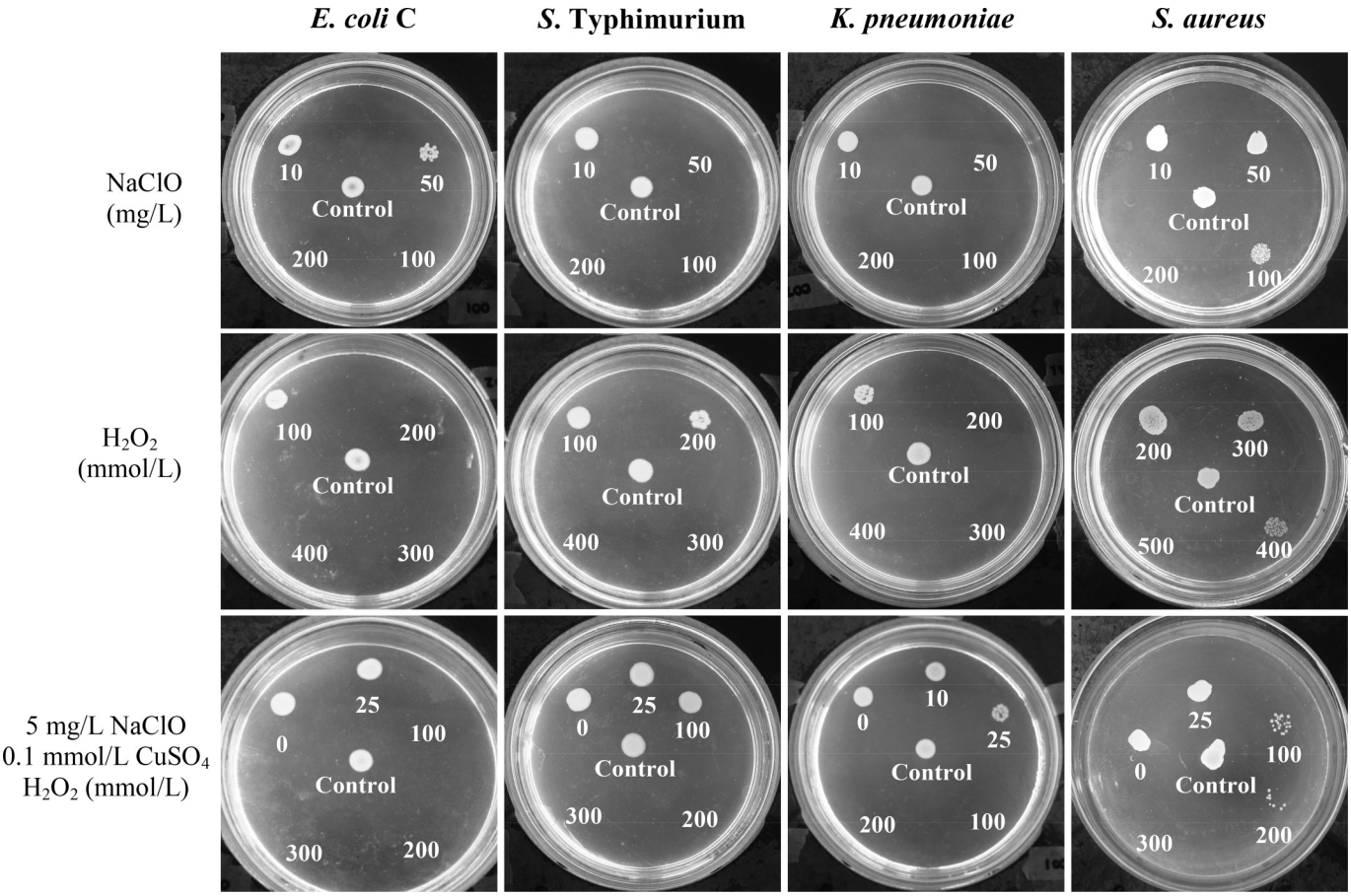
Fig. 1. Effect of oxidizing compounds on bacterial growth. Bacterial suspensions were treated for 2 min with sterile distilled water (control), or with the indicated sodium hypochlorite (NaClO), hydrogen peroxide (H_2O_2), and copper sulfate ($CuSO_4$) concentrations used alone or in combination. Plates are representative of 3 independent experiments.



2010; Bridier et al. 2011). There is a wide variety of disinfectants available with different properties and usage areas, e.g., oxidants, detergents, and alcohols (Møretrø et al. 2012). Among oxidizing agents, hypochlorite has been recognized as an effective disinfectant because of its low production cost and antimicrobial activity (Rutala and Weber 1997); however, it reacts with organic matter, producing a complex mixture of undesirable disinfection by-products (Ferraris et al. 2005). Hydrogen peroxide has been demonstrated to be active against a wide range of microorganisms, such as bacteria, yeasts, viruses, and fungi (Block 2001). It has been reported that in-use solutions are stable and harmless to the environment, but they should be carefully handled to avoid skin, eye, and respiratory tract irritation (Park et al. 2013).

A previous report testing oxidant-based sanitizers demonstrated that peroxide was more effective than hypochlorite against preformed biofilms of *Listeria monocytogenes*, *Salmonella enterica* serovar Typhimurium, and *Salmonella enterica* subsp. *enterica* serovar Worthington (Harkonen et al. 1999). Moreover, sanitation with chlorine was not sufficient to remove other bacterial biofilms (Schwach and Zottola 1984).

Peroxides can produce hydroxyl radicals (HO•) through the Fenton and Haber–Weiss reactions, in which transition metals (e.g., iron or copper) participate as catalysts in the formation of free radicals (Halliwell and Gutteridge 1992), producing deleterious effects on cells (Rossoni and Gaylarde 2000). In agreement, earlier work in our laboratory showed that copper acts as a mediator of hydroperoxide-induced damage in *Escherichia coli*, with the consequent loss of bacterial viability (Rodríguez-Montelongo et al. 1995, 2006). Furthermore, the combination of sodium hypochlorite (NaClO), hydrogen peroxide (H_2O_2), and copper sulfate ($CuSO_4$) synergistically inhibits growth of *Penicillium digitatum*, *Penicillium italicum*, *Geotrichum citri aurantii*, and *Penicillium expansum*, causal agents of fungal fruit diseases (Cerioni et al. 2009, 2010, 2013).

As a first step towards finding a suitable method for the elimination of pathogen-associated biofilms from inert surfaces, the aim of this work was to evaluate the effect of combinations of oxidizing compounds (NaClO and H_2O_2) and copper on planktonic cells and biofilms of laboratory strains and clinical isolates.

Materials and methods

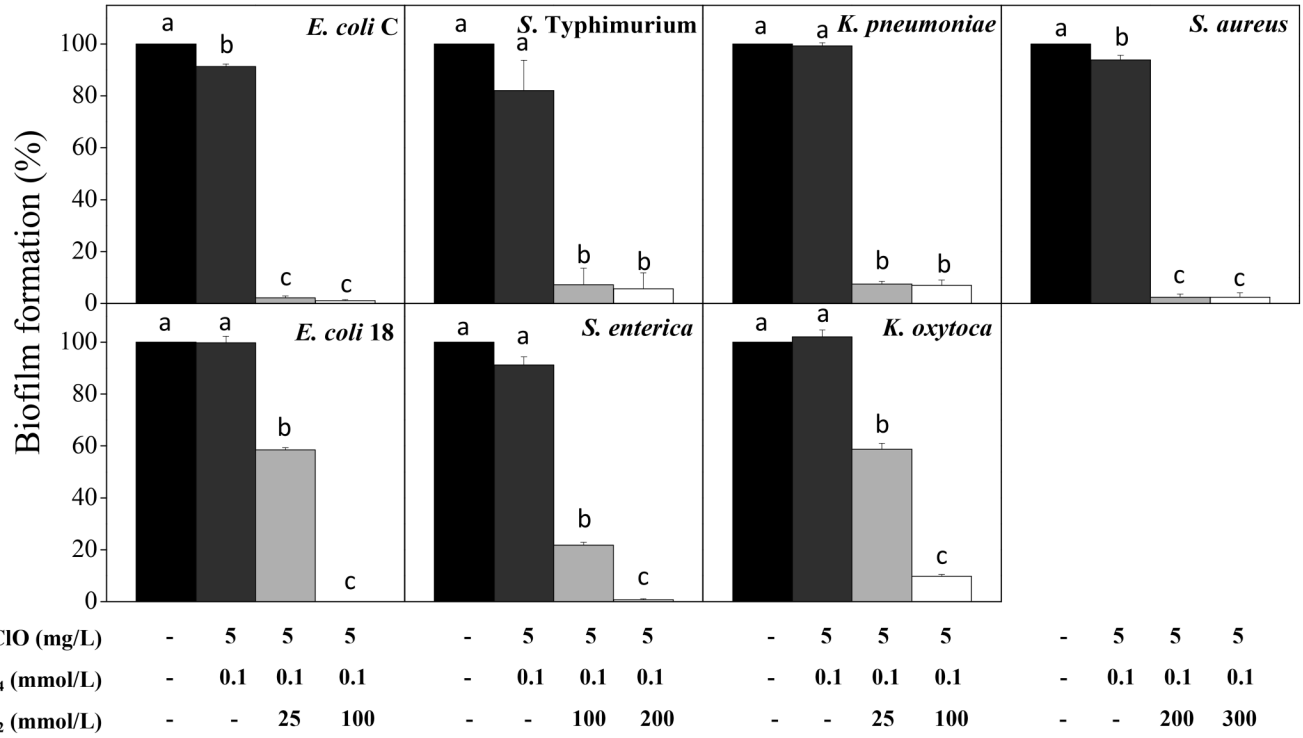
Chemical solutions

Disinfectants used in this study were NaClO (55 000 mg/L total chlorine) and H_2O_2 (30%, Cicarelli, Santa Fe, Argentina). Both disinfectants were diluted in sterile distilled water (pH 5) and used immediately. A solution of 1 mol/L $CuSO_4$ at pH 5 (Merck, Darmstadt, Germany) was sterilized using a filter of 0.22 μm.

Bacterial strains, growth conditions, and preparation of bacterial suspensions

Seven strains were tested: laboratory strains *E. coli* C (ATCC 13706), *Salmonella* Typhimurium (ATCC 14028), *Klebsiella pneumoniae* (ATCC 700603), and *Staphylococcus aureus* (ATCC 25923), and clinical isolates *E. coli* 18 (human prostatitis isolate from the Clinical Microbiology Laboratory-Hospital Clinic, Barcelona, Spain), *Salmonella enterica* (ATCC 4931, human gastroenteritis isolate), and *Klebsiella oxytoca* (ATCC 13182, pharyngeal tonsil isolate). Cells were grown at 37 °C with agitation for 24 h in either Luria–Bertani (LB) broth

Fig. 2. Effect of oxidative treatments (OT) on biofilm formation. Bacterial suspensions were exposed to OT with the indicated sodium hypochlorite (NaClO) and hydrogen peroxide (H_2O_2) concentrations in the presence of 0.1 mmol/L copper sulfate ($CuSO_4$), for 2 min. After incubation, cells were subjected to biofilm-forming conditions for further 24 or 48 h, and the biofilm was quantified with the crystal violet method (see Materials and methods). Absorbance values (at 595 nm) for controls were as follows: 1.0–1.4 for *Escherichia coli* C, *Klebsiella pneumoniae*, and *Staphylococcus aureus*; 0.3–0.4 for *Salmonella* Typhimurium, *E. coli* 18, *Salmonella enterica*, and *Klebsiella oxytoca*. Results are expressed as the mean percentage of formed biofilm compared with that of controls (black bars) of 3 independent experiments. Different letters above bars indicate significant differences between treatments for each strain, according to Tukey's test with a *p* value of ≤0.05.



(laboratory strains) or yeast extract – casamino acids (YESCA) (clinical isolates). Cultures were diluted in sterile distilled water to obtain bacterial suspensions of OD_{560} = 0.1 (approximately 10^8 cfu/mL).

Determination of lethal concentration (LC) of oxidizing compounds

LC was defined as the lowest oxidant concentration applied to planktonic cells that is required to kill 100% of bacterial population after 2 min of exposure at 25 °C. Laboratory strains suspensions were incubated with sterile water (control) or with different concentrations of NaClO or H_2O_2. In all cases, pH value after the addition of the sanitizer agents was 5. Cells were centrifuged at 2000*g* for 10 min and the supernatants were discarded. Pellets were washed once and resuspended to the original volume with sterile distilled water. Aliquots of 5 μL were spotted onto LB agar plates before incubation at 37 °C for 24 h.

Application of oxidative treatments on cell suspensions

Bacterial suspensions were incubated for 2 min with 5 mg/L NaClO and different concentrations of H_2O_2, at pH 5 and 25 °C. In all cases, copper was used as a catalyst; hence, it was added at a concentration of 0.1 mmol/L. Cells were centrifuged, washed once with sterile distilled water, and resuspended in the original volume of fresh LB or YESCA medium. After treatments, viability (cfu/mL) and biofilm formation capacity were evaluated.

Evaluation of biofilm formation after oxidative treatments

Treated laboratory strains resuspended in LB were added into 96-well microtiter plates (polystyrene; Orange Scientific, USA) and incubated under static conditions for 24 h at 30 °C. Treated clinical isolates resuspended in YESCA medium were incubated in the same conditions for 48 h. Biofilm formation was quantified by crystal violet (CV) staining (O'Toole and Kolter 1998). Briefly, after removing the unattached cells and rinsing the plates 2 times with sterile distilled water, 200 μL of 0.1% crystal violet solution were added to each well. The plates were incubated at room temperature for 30 min in dark. Then, wells were rinsed again 2 times with water, and CV-stained attached cells were solubilized with 200 μL of 95% ethanol. Absorbance was measured at 595 nm (SpectraMax Plus384 Absorbance Microplate Reader, USA). In all cases, water-treated cells were included as positive controls, and media without cells as negative controls.

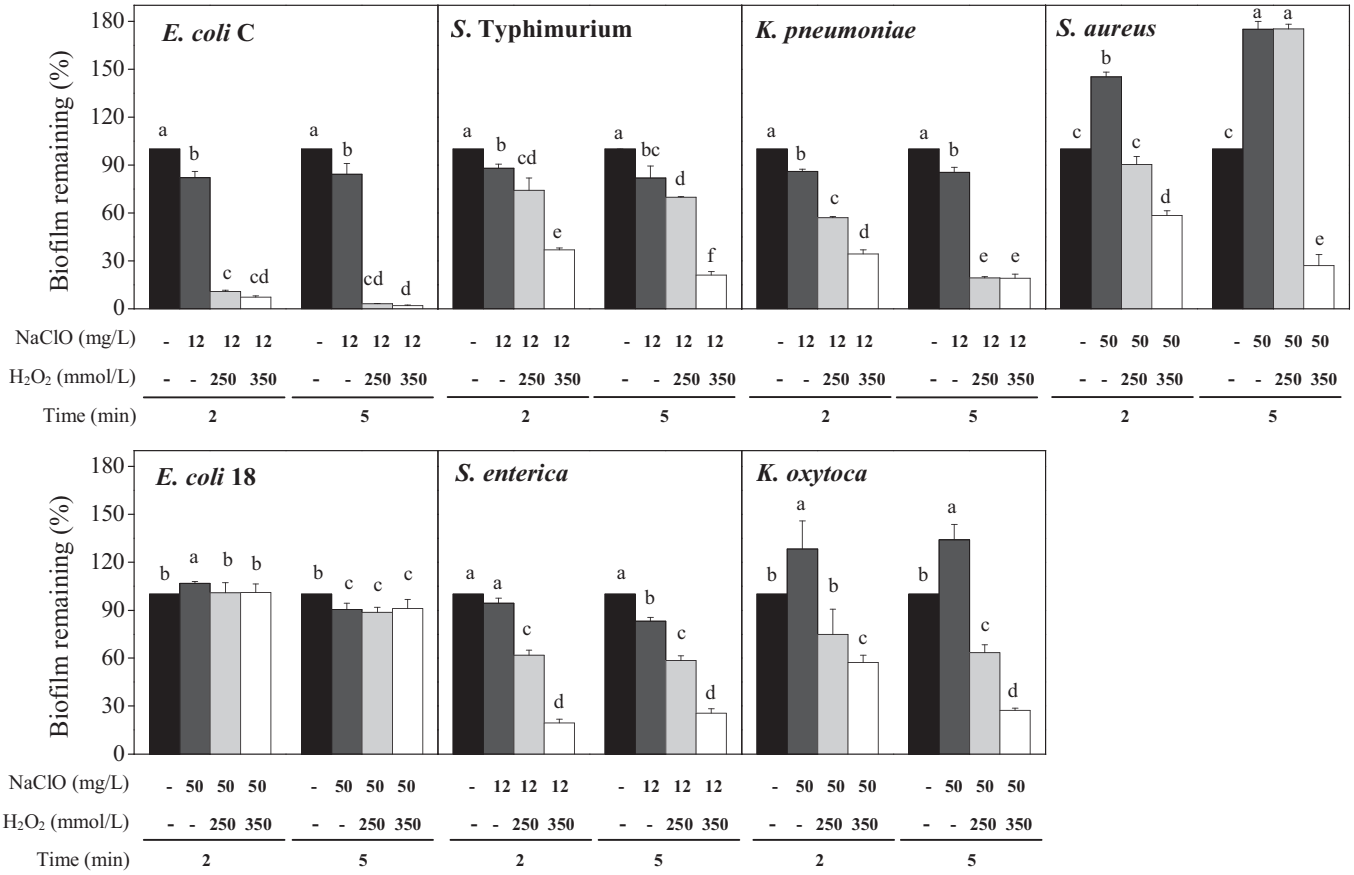
Application of oxidative treatments on preformed biofilms

To allow biofilm formation, bacterial suspensions were incubated in static conditions at 30 °C in polystyrene plates. Clinical isolates were incubated in YESCA medium for 48 h and laboratory strains in LB medium for 24 h. Subsequently, spent media were discarded and biofilms were treated at 25 °C with oxidizing solutions containing NaClO and H_2O_2 (pH 5) at different concentrations and contact times, in the presence of 0.1 mmol/L $CuSO_4$. The controls were performed with sterile water. After treatments, solutions were removed, wells were rinsed once with sterile distilled water, and fresh media were added. Plates were incubated for further 24 or 48 h before quantifying biofilms by CV staining (O'Toole and Kolter 1998), as described above.

Fractional inhibitory concentration (FIC) calculation

Interactions between NaClO and H_2O_2 in treatments applied to planktonic cells were determined by the FIC index, calculated as follows: (LC of NaClO, tested in combination) / (LC of NaClO, tested alone) + (LC of H_2O_2, tested in combination) / (LC of H_2O_2, tested alone). Interaction is defined as synergistic when the FIC

Fig. 3. Effect of oxidative treatment (OT) on preformed biofilm. The biofilms formed on microtiter plates were exposed to OT with the indicated sodium hypochlorite (NaClO) and hydrogen peroxide (H_2O_2) concentrations in the presence of 0.1 mmol/L copper sulfate ($CuSO_4$), for 2 or 5 min. After incubation, cells were subjected to biofilm-forming conditions for further 24 or 48 h (see Materials and methods), and biofilm was quantified with the crystal violet method. Results are expressed as the mean percentage of biofilm remaining following OT compared with that of controls (black bars) of 3 independent experiments. Different letters above bars indicate significant differences between treatments for each strain, according to Tukey's test with a p value of ≤0.05.



value is <1, additive when the value is 1, and antagonistic when the value is >1 (Berenbaum 1978).

Statistical analysis

Assays performed with cells in planktonic state were carried out in triplicate. For biofilm determinations, 8 replicates were performed for each condition and experiments were done 3 times. Data were subjected to analysis of variance (ANOVA) followed by Tukey's test with Infostat for Windows. Differences of p value ≤0.05 were considered significant.

Results

Bacterial inactivation by oxidizing compounds

NaClO and H_2O_2 LCs were determined as shown in Fig. 1. For *E. coli* C, NaClO and H_2O_2 LCs were 100 mg/L and 200 mmol/L, respectively. For *Salmonella* Typhimurium and *K. pneumoniae*, NaClO LCs were 50 mg/L, while H_2O_2 LCs were 300 and 200 mmol/L, respectively. LCs for *Staphylococcus aureus* were 200 mg/L and 500 mmol/L for NaClO and H_2O_2, respectively.

To evaluate the potential synergistic effect of compounds, NaClO plus H_2O_2 in the presence of $CuSO_4$ were tested. The procedures in which compounds were applied in combination, i.e., different H_2O_2 concentrations combined with 5 mg/L NaClO and 0.1 mmol/L $CuSO_4$, were defined as "oxidative treatments" (OTs). In OT conditions, *E. coli* C and *K. pneumoniae* growth were completely inactivated with 100 mmol/L H_2O_2, while *Salmonella* Typhimurium required 200 mmol/L H_2O_2 and *Staphylococcus aureus* 300 mmol/L H_2O_2 (Fig. 1). The FIC index between H_2O_2 and NaClO was calculated to determine the type of interaction in lethal OT. Values <1 were obtained (i.e., 0.55 for *E. coli* C, 0.77 for *Salmonella* Typhimurium, 0.72 for *K. pneumoniae*, and 0.62 for *Staphylococcus aureus*), indicating a synergistic effect between compounds. Clinical isolates were also exposed to OTs, with *E. coli* 18 and *K. oxytoca* inactivated with 100 mmol/L H_2O_2 and *Salmonella enterica* with 200 mmol/L H_2O_2. In all cases, sublethal OTs (containing H_2O_2 concentrations of 25 mmol/L for *Escherichia* and *Klebsiella*, and 100 mmol/L for *Salmonella*) decreased cfu/mL values from 10^8 to 10^5.

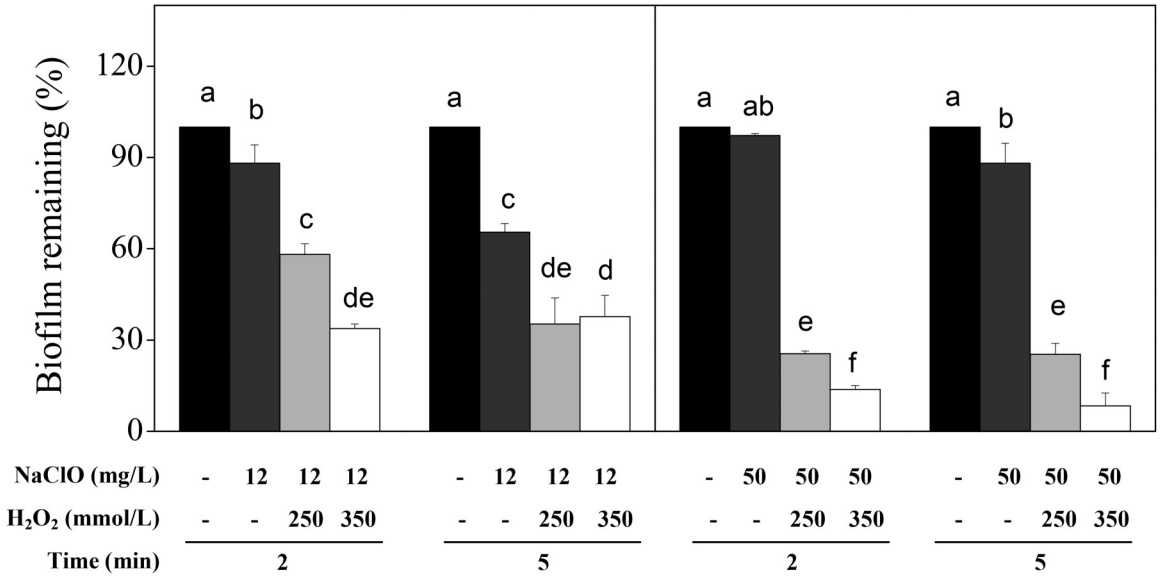
Reduced biofilm formation capacity after exposure to OTs

Cell capacity to form biofilm after OTs was determined (Fig. 2). When sublethal OT was applied, *E. coli* C and *Staphylococcus aureus* biofilm formation was completely inhibited, while *Salmonella* Typhimurium and *K. pneumoniae* biofilms were reduced by 90% compared with water-treated controls (i.e., 10% biofilm formation). For clinical isolates, sublethal treatments prevented biofilm formation by approximately 40% for *E. coli* 18 and *K. oxytoca* and by 80% for *Salmonella enterica*. After the application of lethal OTs, biofilm formation was not observed. It should be noted that treatments combining only NaClO and $CuSO_4$ did not significantly differ from the controls in most of the strains, but for *E. coli* C and *Staphylococcus aureus* the biofilm reduction was below 10%.

Removal of preformed biofilms by OTs

OTs were applied on preformed biofilms to simulate disinfection processes. For *E. coli* C, OT consisting of 12 mg/L NaClO and

Fig. 4. Oxidative treatment (OT) effect on *Escherichia coli* 18 preformed biofilms. Biofilms were formed during 24 h on microtiter plates and then exposed to OT with the indicated sodium hypochlorite (NaClO) and hydrogen peroxide (H_2O_2) concentrations plus 0.1 mmol/L copper sulfate ($CuSO_4$), for 2 or 5 min. After incubation, cells were subjected to biofilm-forming conditions for an additional 24 h, and biofilm was quantified with the crystal violet method. Results are expressed as mean percentage of biofilm remaining following OT compared with that of the controls (black bars) of 3 independent experiments. Different letters above bars indicate significant differences between treatments, according to Tukey's test with a p value of ≤0.05.



350 mmol/L H_2O_2 caused a biofilm reduction of around 90%, when applied for 2 or 5 min (Fig. 3). For *Salmonella* Typhimurium, the same OT applied for 2 min led to a biofilm decrease of 60%. In this case, a contact time of 5 min improved treatment efficiency by 15%. Similar results were observed for *K. pneumoniae* biofilm. Note that after the exposure of *K. pneumoniae* biofilm to OT containing 250 mmol/L H_2O_2 for 5 min, 80% removal was achieved. In all cases, reductions below 20% were observed for treatments without H_2O_2. For *S. aureus* an OT consisting of 50 mg/L NaClO and 350 mmol/L H_2O_2 applied for 5 min was required to reduce the biofilm by 75%.

In respect to *Salmonella enterica* biofilm, treatment with 12 mg/L NaClO and 350 mmol/L H_2O_2 led to a decrease of around 80%, for both contact times. For *K. oxytoca* and *E. coli* 18, this treatment was not effective (data not shown). Therefore, an OT containing 50 mg/L NaClO was applied. This treatment achieved a reduction of 75% of *K. oxytoca* biofilm when applied for 5 min. None of the OTs produced a significant detachment on *E. coli* 18 biofilms formed during 48 h. For this reason, challenges against 24 h biofilms were performed (Fig. 4). The application of 12 mg/L NaClO and 350 mmol/L H_2O_2 resulted in a reduction of around 65% using contact times of 2 and 5 min. The removal effect on young biofilms was higher after the application of OT containing 50 mg/L NaClO, reaching reductions of between 85% and 90% (Fig. 4).

Discussion

In this work, removal of bacterial biofilms has been achieved by combining oxidizing compounds at a restrained concentration and exposure time. Removal was accomplished in a selection of Gram-positive and Gram-negative bacteria, which are able to contaminate inanimate surfaces of clinical and food industry settings.

The active principles of the treatment used here are NaClO, H_2O_2 and $CuSO_4$. When the treatment is applied to planktonic cells, the FIC index indicates that NaClO and H_2O_2 in OT acted synergistically, as it has been reported for other microorganisms (Cerioni et al. 2009). Thus, compared with individual applications, the combination proposed in OT generates a significant reduction of the oxidants effective concentrations. OTs were performed at pH 5. It has previously been reported that the highest biocide activity of NaClO solution occurs at pH below 6.5 (Fukuzaki et al. 2007). In addition, it has been reported that acidic conditions maintain high levels of dissolved copper and extend H_2O_2 lifetime and reactivity, important factors in the feasibility of Fenton-like reactions (Jung et al. 2009). Bacterial suspensions treated at sublethal conditions have reduced ability to form biofilm compared with water controls. Note that the inhibition of *E. coli* 18 and *K. oxytoca* biofilm formation was lower than that observed for the other strains, despite cfu/mL were not significantly different among all the strains after treatments. This might indicate the presence of adaptative strategies or distinctive biofilm formation features in these 2 clinical isolates.

It was previously reported that biofilms are more resistant to disinfectants than are their planktonic counterparts (Mah and O'Toole 2001; Wong et al. 2010). In agreement, our results show that planktonic cells were more easily inactivated by OT than cells enclosed in a biofilm. However, a significant reduction of bacterial biofilms was achieved with the assayed treatments. An OT consisting of 50 mg/L NaClO, 0.1 mmol/L $CuSO_4$, and 350 mmol/L H_2O_2 applied for 5 min is proposed to reduce 48 h preformed biofilms of all strains tested, except for *E. coli* 18. Compared with the concentrations used in OT, higher concentrations of NaClO (Rossoni and Gaylarde 2000), H_2O_2 (Robbins et al. 2005), or $CuSO_4$ combined with quaternary ammonium compounds (Harrison et al. 2008) have previously been reported as effective for biofilm removal. This could be a result of differences in bacteria, compound combinations, and contact times chosen for the studies.

In our conditions, results indicate that biofilms of clinical isolates are more resistant than those of laboratory strains. Although all preformed biofilms were removed by adjusting oxidant concentrations and contact times, *E. coli* 18 was only reduced when OT was challenged against a 24 h biofilm. It should be considered that a more developed biofilm may have a complex extracellular matrix, not only in thickness but also in components such as enzymes that may play a role in neutralizing toxic compounds (Stewart et al. 2000).

Oxidants involved in OTs are quickly degraded, thus the residue amount on surfaces and in water would be practically negligible. Despite the introduction of new disinfectants, several advantages of chlorine are likely to lead to its continued usage in both healthcare and food industry settings. In the same way, H_2O_2 quick and innocuous degradation assures its persistence among preferred disinfectants in the market. Thus, the OT proposed in this study is a promising safe alternative to disinfect contact surfaces, preventing and removing biofilms of different bacteria.

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