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Efficacy of disinfectants against *Cronobacter* biofilm on plastic surfaces

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RESEARCH ARTICLE

Abstract

In this study, efficacy of benzalkonium chloride (BC), peroxyacetic acid (PAA) and chlorine dioxide (CD) against *Cronobacter* biofilm were evaluated on polystyrene surface. A pool of three strains of *Cronobacter* was grown to form biofilm in tryptic soy broth at 30 °C in polystyrene specimen containers. Six-days-old biofilms on containers were treated with two different concentrations (100 and 400 mg/l) of disinfectants up to 15 min at room temperature. Cell killing effectiveness and biofilm removing capacity of disinfectants were evaluated by XTT and crystal violet assays, respectively. Exposure of *Cronobacter* biofilm to BC, PAA and CD at 400 mg/l for 15 min caused reductions of 69, 73 and 51% in the level of viable cells, respectively. Treatments at 400 mg/l for 15 min were only able to remove up to 18% of the biofilm biomass. Results show that disinfectants routinely used in food industry are insufficient in removing of *Cronobacter* biofilm from plastic surfaces. Results also show that cell killing effectiveness of disinfectants is considerably restricted by the matrix of biofilm.

Keywords: biofilm, *Cronobacter*, disinfectant, plastic

1. Introduction

Cronobacter is regarded as an opportunistic human pathogen and the cause of serious infections of infants, including septicemia, meningitis and necrotising enterocolitis (Strydom *et al.*, 2012). It has been categorised as 'severe hazard for restricted populations, life threatening or substantial chronic sequelae or long duration' (ICMSF, 2002). Powdered milk and reconstituted powdered infant formula (PIF) have been the most commonly implicated products in infant *Cronobacter* infections (Beuchat *et al.*, 2009). *Cronobacter* is often isolated from the environment in milk powder and PIF manufacturing facilities (Mullane *et al.*, 2008). Milk powder is also the main ingredient of PIF. Therefore, it is suggested that *Cronobacter* has particular significance in dairy industry.

Attachment of bacterial cells to surfaces may be followed by growth, the production of exopolysaccharide (EPS), and biofilm formation (Kumar and Anand, 1998). Biofilms act as an adhesive foundation and defence barrier, and protect the embedded cells against stress conditions and antimicrobial agents (Jung *et al.*, 2013). In food industry, biofilms may be a source of recalcitrant contaminations, causing food spoilage and are possible sources of foodborne disease outbreaks (Simões *et al.*, 2010).

Several studies have shown that *Cronobacter* has the ability to persist in food processing environments (Iversen and Forsythe, 2004; Kandhai *et al.*, 2004). The persistence of *Cronobacter* has been directly related to its biofilm forming capacity. Hartmann *et al.* (2010) suggested that flagella and cellulose contribute to adhesion of *Cronobacter* to abiotic surfaces. It has been observed that *Cronobacter* strains are able to form biofilms on a range of material, including glass, stainless steel, polyvinyl chloride, polycarbonate and silicone (Iversen *et al.*, 2004).

Several authors have found that microorganisms attach more rapidly to hydrophobic surfaces such as plastics than to hydrophilic materials (Sinde and Carballo, 2000). Iversen *et al.* (2004) reported that *Cronobacter* strains were able to adhere and grow on latex, polycarbonate and silicone and to a lesser extent stainless steel. Plastic materials are widely

used to make gaskets, conveyer belts, trays and cutting boards in the food industry (Torlak and Sert, 2013). To date, there is no published study on efficacy of disinfectants against *Cronobacter* biofilms on plastic surfaces. Therefore, in this study, benzalkonium chloride (BC), peroxyacetic acid (PAA) and chlorine dioxide (CD) were evaluated against *Cronobacter* biofilm formed on polystyrene surface. For this purpose, levels of biofilm biomass and viable cells in *Cronobacter* biofilm were quantified before and after treatments by spectrophotometric-based assays.

2. Materials and methods

Microorganisms

Three field strains of *Cronobacter* were used to form biofilm on polystyrene surface. These strains were previously isolated from milk powder samples and identified as *Enterobacter sakazakii* by VITEK 2 (bioMérieux, Marcyl l'Etoile, France) biochemically-based identification system (Gökmen *et al.*, 2010). Their biofilm forming capacity was verified using the microtiter plate assay as outlined by Djordjevic *et al.* (2002).

A cocktail of three strains, prepared by mixing an equal volume of cultures grown in tryptic soy broth (TSB; Lab M, Bury, UK), was used as inoculum in the biofilm formation.

Biofilm formation

Biofilm formation was performed in 100 ml sterile flat bottom polystyrene specimen containers (Gosselin, Hazebrouck cedex, France) according to Harvey *et al.* (2007). Containers, filled with 19 ml of uninoculated TSB and 1 ml inoculum, were incubated at 30 °C for 6 days. Every 48 h during the incubation period, spent medium were pipetted from containers and replaced with same volume of fresh TSB. At the end of incubation, contents of the containers were poured off; containers were washed four times with 50 ml of phosphate-buffered saline (PBS) and remained for 30 min to dry at 30 °C.

Disinfectant treatments of *Cronobacter* biofilm

Concentrated solutions of BC (10%), PAA (5%) and CD (2%) were kindly supplied by Dez-Kim Chemicals (Istanbul, Turkey). On the day of the evaluation, they were diluted with sterile distilled water to obtain working concentrations of 100 and 400 mg/l.

Containers were filled with 100 ml volumes of working concentrations of disinfectants. Control containers were filled with the same volume of sterile distilled water instead of disinfectants. Treatments were performed as duplicate for three different exposure times (1, 5 and 15 min) at room temperature. After treatments, contents of the containers were poured off, and containers were washed with 50 ml of PBS.

Quantification of initial biofilm level

Levels of biofilm biomass and viable cells within biofilm were quantified by crystal violet staining (Harvey *et al.* 2007) and XTT (Chaieb *et al.*, 2011) assays, respectively. In viable bacteria cells, XTT is reduced to a coloured water soluble formazan derivative by enzymes of the respiratory chain localised in the cytoplasmic membrane. Therefore, XTT assay can discriminate viable and dead cells (Peeters *et al.*, 2008). Crystal violet is a basic dye, which binds to negatively charged surface molecules and polysaccharides in the extracellular matrix (Li *et al.*, 2003).

Crystal violet staining assay

An aqueous 1% crystal violet solution (20 ml) was added to each container and incubated for 45 min at room temperature. Then, crystal violet solution was poured off; containers were washed four times with 50 ml of sterile distilled water and air dried at 30°C for 30 min. Twenty ml of 95% ethanol was added to containers and remained for 30 min to destain. Concentration of crystal violet in the destaining solution was determined by measuring the optical density (OD) value at 595 nm using a spectrophotometer (Thermo Scientific, Waltham, MA, USA).

XTT assay

XTT solution was prepared by dissolving XTT (Sigma-Aldrich, St. Louis, MO, USA) in PBS in a concentration of 1 mg/ml and filtered. Menadione (Sigma-Aldrich) was dissolved in acetone in a concentration of 0.4 mM. Prewashed containers were filled with 18 ml of PBS and 2 ml of the XTT-menadione solution (12.5:1 v/v). Then, containers were incubated at 37 °C in the dark for 3 h. At the end of incubation, reduction of XTT was determined by measuring the OD value at 492 nm.

Statistical analysis

Three independent trials were conducted. The percentage reduction (PR) values were calculated using OD values obtained from spectrophotometric-based assays prior to the statistical data treatment. Differences in the mean PR values were assessed by one-way analysis of variance using statistical software (SPSS Inc., Chicago, IL, USA). Means were compared using the Duncan grouping test at 5% significant level.

3. Results and discussion

Disinfectants tested in this study were selected based on differences in their antibacterial mechanisms and intended usages. In a comprehensive survey study conducted in the UK (Holah *et al.*, 2002) quaternary ammonium compounds including BC were reported as most commonly used disinfectants in the food industry. However, it should be noted that BC is not recommended for use in dairy processing facilities because the residues may inhibit starter cultures (Chmielewski and Frank, 2003). PAA, one of the commonly employed sanitizers in the dairy and beverage industries, maintains its antimicrobial activity with an organic load (Li *et al.*, 2003). CD has received similar focus as PAA for control of bacterial biofilm since it maintains activity in the presence of high levels of organic matter (Cruz and Fletcher, 2012).

Reductions in the level of viable cells in *Cronobacter* biofilm after treatments are given Figure 1. Except for CD, treatment of containers with disinfectants for 1 min at 400 mg/l significantly (P<0.05) reduced the level of viable cells in biofilm compared to treatment with distilled water based on the OD values obtained by XTT assay. Exposure of *Cronobacter* biofilm to BC, PAA and CD at 100 mg/l for 15 min caused reductions of 39, 44 and 25% in the level of viable cells, respectively. Reductions of 69, 73 and 51% were observed when the containers were treated with BC, PAA and CD at 400 mg/l for 15 min.

Cell killing efficacy of PAA and BC in *Cronobacter* biofilm at 400 mg/l for 15 min were significantly (P<0.05) higher than that of CD. Similar finding have been reported by Belessi *et al.* (2011) for *Listeria monocytogenes* biofilm exposed to different sanitizers. In contrast with this finding, Cruz and Fletcher (2012) found that minimal effective concentration of CD for *L. monocytogenes* biofilm was significantly (P<0.05) lower than that of BC. These controversial results indicate that efficacy of sanitizers against biofilms can vary with experimental conditions and strain of microorganisms used.

The aim of disinfection is to reduce the surface population of viable cells left after cleaning and prevents microbial growth on surfaces prior to the production stage (Simões *et al.*, 2010). The reductions obtained in the level of viable cells indicated that treatments with BC, PAA or CD at manufacturers' recommended concentrations for 15 min are insufficient for complete inactivation of *Cronobacter* cells in biofilm. Previously, Kim *et al.* (2007) reported that the lethality of disinfectants to *Cronobacter* in biofilm was lower than that observed for planktonic cells. Biofilms have been reported as possessing susceptibilities towards disinfectants that are 10-100 times less than equivalent population of planktonic cells (Holah *et al.*, 2002; McDonnell and Russell, 1999).

Resistance of bacterial biofilms to disinfectants is supposed to be due essentially to potential barrier of EPS which delays or prevents disinfectant from penetration into the biofilm and from reaching target microorganisms in all parts of a biofilm (Schulte *et al.*, 2005). A study of Lehner *et al.* (2005) revealed the presence of glucose, galactose, fucose, and glucuronic acid in *Cronobacter* biofilms. Cellulose was identified as another extracellular matrix component present in the *Cronobacter* biofilms (Grimm *et al.*, 2008). Another explanation for reduced biocide penetration into biofilms is the interaction between biocide and biofilm constituents. Oxidising sanitizers, including PAA, can be neutralised and lose bactericidal activity upon contact with the surface of biofilms (Simões *et al.*, 2010). It has been reported that cationic disinfectants such as BC may be immobilised by binding to negatively charged microbial EPS (Schulte *et al.*, 2005).

Reductions in the level of biofilm biomass after treatments are given Figure 2. Treatments with disinfectants at 100 mg/l for 15 min decreased the biofilm biomass by only up

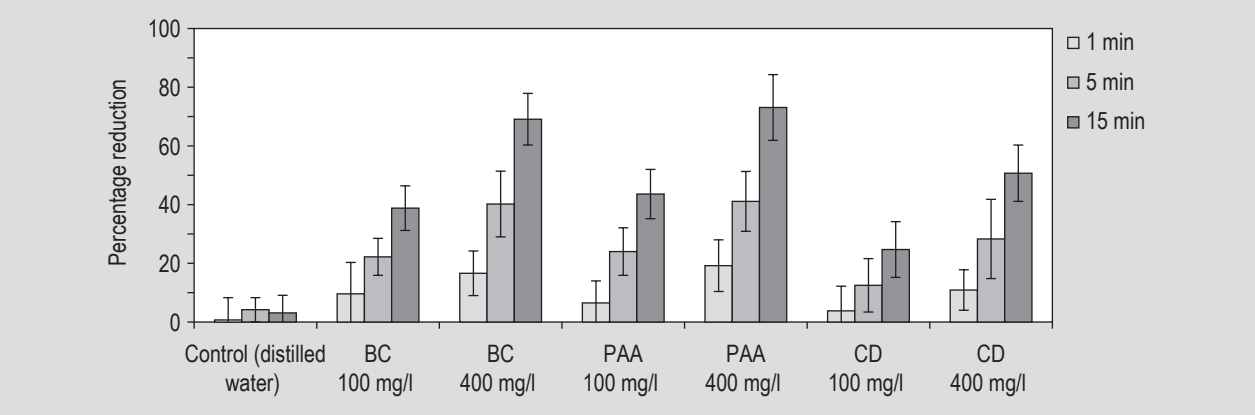

Figure 1. Effects of disinfectant treatments on level of viable cells in *Cronobacter* biofilm (error bars denote standard deviations, n=3). BC = benzalkonium chloride; PAA = peroxyacetic acid; CD = chlorine dioxide.

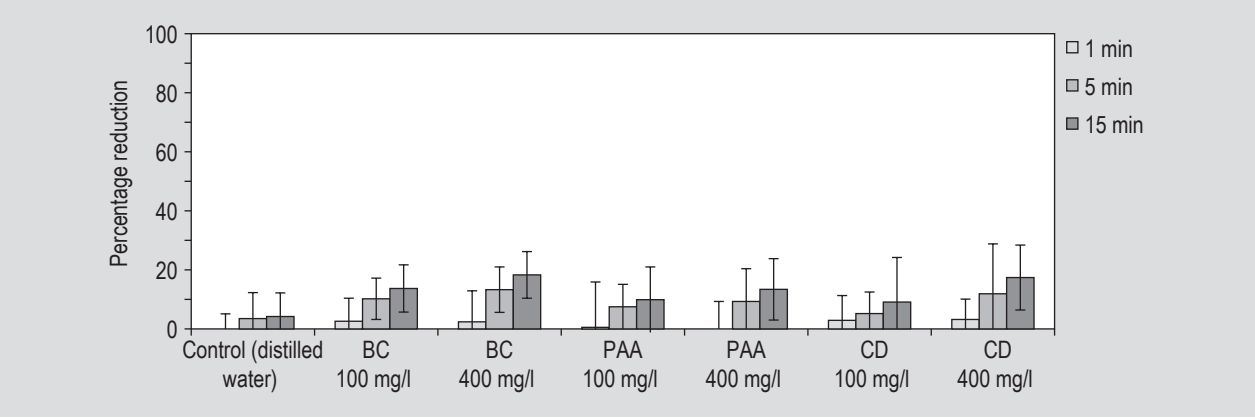

Figure 2. Effects of disinfectant treatments on *Cronobacter* biofilm biomass (error bars denote standard deviations, n=3). BC = benzalkonium chloride; PAA = peroxyacetic acid; CD = chlorine dioxide.

to 14% based on the OD values obtained by crystal violet assay. Reductions of 18, 13 and 17% were observed in the level of biofilm biomass after 15 min of treatment with BC, PAA and CD at 400 mg/l, respectively. According to these reduction values, it can be suggested that biofilm removing capacity of BC, PAA and CD can be ignorable when they used at manufacturers' recommended concentrations.

To date, there is no published data dealing with the biomass reduction effectiveness of disinfectants against *Cronobacter* biofilms. For this reason, our results can only be compared with the results of studies conducted with different microorganisms. Torlak and Sert (2013) evaluated biofilm removing capacity of BC on *L. monocytogenes* biofilm formed on plastic surface. They quantified the remained biomasses using crystal violet staining assay after 15 min contact time and found that reductions were below 30% for concentration of 400 mg/l. Pitts *et al.* (2003) demonstrated that exposure for 60 min to sodium hypochlorite at chlorine concentration of 100 mg/l was not enough to remove *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* biofilms from the plastic surface.

Generally, user-concentrations of disinfectants are determined based on simple laboratory tests measuring efficacy in suspension (Araújo *et al.*, 2011). In this study, the commercial disinfectants tested at manufacturers' recommended concentrations were clearly found to be insufficient when applied to control *Cronobacter* biofilm on polystyrene surface. This result led us to conclude that elimination of *Cronobacter* biofilms from food-contact surfaces by disinfectants is a big challenge. Efficacy of disinfectants against bacterial biofilms can be increased by increasing concentration, treatment temperature and exposure time. However, costs, practicability and environmental impacts should be taken into account in the selection disinfection process. Therefore, new control strategies are still needed to control the bacterial biofilms in food industry.

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