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Comparison among the effectiveness of ozone, nisin and benzalkonium chloride for the elimination of planktonic cells and biofilms of *Staphylococcus aureus* CECT4459 on polypropylene

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ABSTRACT

In search for alternative disinfection methods to prevent the development of bacterial resistances, the effectiveness of benzalkonium chloride (BAC), nisin and ozone against planktonic cells and biofilms of *Staphylococcus aureus* CECT 4459 was compared. Maximum effect of each compound was determined by comparing the times of exposure at which maximum mortality was reached. A dose–response study was carried out at such exposure times and LD_{90} values (doses causing 90% of mortality) were determined. BAC and ozone were more effective against biofilms than against planktonic cells, presumably due to a higher accessibility of biofilm cells. Concerning the control of biofilms, BAC was highly effective, but the application of 250 IU/ml of nisin or 1 μg/g of ozonized water allowed to reach 99% inactivation in only 2 min. It is concluded that nisin and ozone could be good alternative disinfectants to prevent the development of bacterial resistances, but factors such as applicability, price and risk to employers and food should also be taken into account.

1. Introduction

Staphylococcus aureus is one of the most common foodborne pathogens, being occasionally found in food processing plants in which it can remain attached to surfaces, forming complex structures called biofilms (Herrera, Cabo, González, Pazos & Pastoriza, 2007; Palmer, Flint & Brooks, 2007). Biofilms allow bacteria to better resist unfavourable environmental conditions, including external stress stimuli such as the application of disinfectants (Costerton, Stewart & Greenberg, 1999; Langsrud, Sidhu, Heir & Holck, 2003; McLandsborough, Rodríguez, Pérez-Conesa & Weiss, 2006).

Benzalkonium chloride (BAC) is a disinfectant widely used in the food industry belonging to the quaternary ammonium compound (QACs) group. The development of resistance to QACs has been detected in some bacteria from food industry environments. Thus, a recent study has documented that 25 out of 191 staphylococci isolates had increased MIC values for BAC (Heir, Sundheim & Holck, 1995). Similarly, the most persistent strains of *Listeria monocytogenes* in three out of four processing plants were QAC-resistant (Aase, Sundheim, Langsrud, Rørvik, 2000). In clinical environments, the use of QACs has also resulted in the appearance of bacterial resistances. Inadequate protocols of cleaning and disinfection leading to residual sub-lethal concentrations of disinfectants and biofilm formation can account for bacterial resistance to biocides (Langsrud, Sidhu, Heir & Holck, 2003).

As a result, there is a need for alternative treatments that can replace the traditional methods of disinfection. Additionally, the European environmental law is increasingly pressuring industry to replace traditional biocides with environmentally suitable alternatives (Knowles & Roller, 2001; Viera, Guiamet, de Mele & Videla, 1999).

Ozonized water and nisin are potential alternatives to traditional biocides. Ozone is a wide-spectrum biocide highly oxidant and unstable in water, all of which make it very promising as a disinfectant agent leaving no residue. (Bott, 1991; Viera, Guiamet, de Mele & Videla, 1999). Nisin is effective against several pathogens: *L. monocytogenes*: (Motlagh, Bhunia, Szostek, Hansen, Johnson & Ray, 1992), *S. aureus* (Spelhaug & Harlanger, 1989), *Bacillus cereus* (Ray & Daeschel, 1994), is non-toxic and has GRAS status. Therefore, some benefits could be expected from its presence on surfaces.

The effectiveness of BAC, ozonized water and nisin against *S. aureus* CECT 4459 biofilms formed on polypropylene surfaces was compared in the search for an alternative disinfection method to control food contamination in processing plants. The effectiveness of these compounds against planktonic cells of this strain was also determined with comparative purposes. *S. aureus* CECT 4459 was selected as previous studies had shown a high adherence of this strain in polypropylene (Herrera, Cabo, González, Pazos & Pastoriza, 2007).

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2. Materials and methods

2.1. Bacterial cultures and media

S. aureus subsp. *aureus* CECT 4459 was obtained from the Spanish Type Culture Collection (Valencia). The strain was frozen-stored in nutrient broth containing 50% glycerol (v/v) at −80 °C. Whenever required, the strain was thawed and subcultured twice in Nutrient Broth (NB: Tryptone: 10 g/l, Meat Extract: 3 g/l, NaCl: 5 g/l, pH 7.0) at 37 °C before use. Peptone water (Boente, S.L., Spain) was used as dilution medium.

2.2. Antimicrobials

Benzalkonium chloride was purchased from Roig Pharma S.A. Ozonized water was kindly provided by Peter Taboada, S.L. using the PETFROST System (Taboada, 2004). Ozone concentration in water was determined by the indigo method (Bader and Hoigné, 1981). Pure nisin was obtained from Applin and Barrett (Terrasa, Spain).

2.3. Inoculum preparation

Once activated, the culture was centrifuged, and then the precipitated cells were resuspended in phosphate buffer saline (PBS, pH 7.4, 2 mM KCl, 5 mM Na_2PO_4, 1.8 mM KH_2PO_4, 0.13 M NaCl) and the suspension was standardized by adjusting the absorbance at 700 nm to a value of 0.100, which corresponds to a cell density of 10^8 CFU/ml. This cell suspension was used directly as inoculum in experiments with sessile cells or used as 10-fold dilution in experiments with planktonic cells, to achieve approximately the same number of cells in both cases according with a previous study (Herrera, Cabo, González, Pazos & Pastoriza, 2007).

2.4. Experimental systems

In the case of sessile cells, a 3 ml aliquot of the inoculum was pipetted into a circular contour (50 mm diameter) drawn on each polypropylene coupon (60 mm × 60 mm) with a correction pen (Tipp-Ex). Coupons had been previously sterilized by overnight exposure to ultraviolet light. Coupons were then incubated at 25 °C during 14 h, time at which *S. aureus* CECT 4459 had reached maximum levels of adherence at this temperature (Herrera, Cabo, González, Pazos & Pastoriza, 2007). Coupons were then rinsed with 10 ml of PBS for 30 s to release non-adhered cells, and immediately used in antimicrobial assays.

In the case of planktonic cells, an aliquot (2.5 or 0.5 ml) of the inoculum (10-fold diluted) was added in each tube, which was immediately used in antimicrobial assays.

2.5. Antimicrobial assays

In the case of sessile cells, 3 ml aliquots of the antimicrobials (ozonized water, nisin or BAC) were added at various doses.

In the case of planktonic cells, 2.5 ml of ozonized water or nisin, or 0.5 ml of BAC were added in each tube at various doses. Doses and time of exposure were as follows:

- With nisin: 25, 50, 100, 250 and 500 IU/ml at 5, 10, 20, 30, 60, 90 and 120 min.
- With BAC: 5, 10, 25, 50 and 500 mg/L at 2, 5, 10, 20 and 30 min.
- With ozone: 0.62,1 and 1.12 μg/g in the case of planktonic cells, and 0.54, 0.83, 1.07, 2.05 and 2.81 μg/g in the case of biofilms, with times of 2, 5, 10, 15, 20 and 30 min in both systems.

Several blanks with distilled water instead of antimicrobial were included in each experimental series. All experiments were carried out at 25 °C.

After being applied, BAC and nisin had to be neutralised. In contrast, the high decay rate of the ozone made neutralization unnecessary. BAC was neutralized by adding to a ratio 9:1 (v/v), a solution comprising 10 ml of a buffer containing 34 g/l KH_2PO_4 adjusted to pH 7.2 with NaOH, 3 g/l soybean lecithin, 30 ml/l Tween 80, 5 g/l $Na_2S_2O_3$ and 1 g/l L-histidine. This solution was applied during 1 min at room temperature, which was considered sufficient by preliminary experiments. Nisin was neutralized by culturing surviving cells in Nutrient Agar previously adjusted to pH 9. The lack of effect of this pH on the growth of *S. aureus* CECT 4459 was checked in preliminary experiments.

After neutralization, in the sessile system, adhered cells were collected by thoroughly rubbing with three moistened swabs and resuspended by vigorously vortexing swabs in 9 ml of peptone water.

The number of surviving cells in both systems was determined by incubation on nutrient agar (NA) at 37 °C for 24 h. Percentage mortality was calculated according to Eq. (1)

$$M\ (\%) = 100^{*}\left(\frac{\mathrm{NTVC/NVC}}{\mathrm{NTVC}}\right), \quad \text{where :} \tag{1}$$

- *M*: mortality.
- NTVC: number of total viable cells (CFU).
- NVC: number of viable cells in each experimental condition.

2.6. Determination of the LD_{90} value

LD_{90} values were obtained by adjusting experimental data to a logistic equation modified by Cabo et al. (1999) as follows:

$$M\ (\%) = \frac{K}{1 + be^{-rD}}, \quad \text{where} \tag{2}$$

- *M*: mortality (%).
- *D*: Dose of antimicrobial.
- *K*: Maximum inhibition value.
- *r*: Specific inhibition coefficient (dimensions: inverse of the dose).

$$b = \left(\frac{K}{Mo} - 1\right)$$

Mo = Mortality (%) at dose zero.

Reparametrizing Eq. (2) to obtain the lethal dose 90 (LD_{90}), i.e. the dose that kills ninety percent of the initial population the following expression is obtained:

$$K^{*}0.9 = \frac{K}{1 + b^{*}e^{-rLD90}}, \tag{3}$$

From which the next equation is obtained:

$$b = 0.11^{*}e^{rLD90} \tag{4}$$

Substituting the value of *b* into the Eq. (3) and subtracting the ordinate at the origin as described in Cabo, Murado, González and Pastoriza (1999), the following equation is obtained, from which the LD_{90} value can be easily determined by fitting to experimental values:

$$M\ (\%) = K\frac{1}{1 + 0.011e^{rLD90}} - \frac{1}{0.11e^{rLD90} + 1} \tag{5}$$