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Effect of enterocin AS-48 singly or in combination with biocides on planktonic and sessile *B. cereus*

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Enterocin AS-48 was tested singly or in combination with biocides on a cocktail of six *Bacillus cereus* strains in planktonic state and in biofilms formed on polystyrene microtiter plates. The biocides tested were benzalkonium chloride, cetrimide, hexadecylpyridinium chloride, triclosan, chlorhexidine, polyhexamethylen guanidinium chloride and commercial sanitizers P3 oxonia and P3 topax 66. The numbers of survivors were determined after 60 min incubation with biocides or the biocide-bacteriocin combinations. Addition of enterocin AS-48 (25 mg/l) increased the inactivation of planktonic cells by the quaternary ammonium compounds, bisphenols and biguanines tested in a range of biocide concentrations from 0.25 to 2.5 g/l, and by 4 g/l polyguanine. Increased inactivation of the bacilli was also observed for the combination of enterocin AS-48 with 2.5% P3 oxonia, but not by P3 topax 66. In the sessile state, the bacilli were more resistant to biocides and also to the bacteriocin-biocide combinations. Hexadecylpyridium chloride was the most active biocide on biofilms in the single treatments. In the combined treatments with 50 mg/l bacteriocin, hexadecylpyridinium (2.5 g/l), polyhexamethylen guanidinium chloride (4 g/l) and P3 oxonia (2.5%) achieved complete inactivation of bacilli populations. P3 topax 66 showed the lowest performance among all treatments tested, either singly or in combination with bacteriocin. A cocktail of endospores was challenged with biocides and enterocin AS-48 for 60 min at temperatures of 22 °C, 40 °C, and 60 °C. Enterocin AS-48 did not significantly ($p > 0.05$) reduce viable counts or increase the lethal effect of biocides. However, treatments with 5 g/l benzalkonium chloride at 60 °C, 2.5 g/l hexadecypyridinium at 60 °C or P3 oxonia at 0.025% and 60 °C or at 0.25% at 22–60 °C achieved complete inactivation of bacterial endospores, both singly and in combination with bacteriocin. Significant reductions of viable counts (1–2 log cycles) were also obtained for some treatments with cetrimide, triclosan or polyhexamethylen guanidinium chloride, but not for chlorhexidine (up to 5 g/l) or P3 topax 66 (up to 1%). Polystyrene surfaces dosed with enterocin AS-48 (25 or 50 mg/l) remained free of detectable bacilli from 2 to 24 h after being inoculated with a cocktail of endospores, but stainless steel surfaces dosed with 50 mg/l bacteriocin did not prevent bacterial growth from endospores. Results from this study suggest that enterocin AS-48 could be applied as enhancer of biocide activity against planktonic and sessile *B. cereus* cells.

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1. Introduction

Bacillus cereus is a food-poisoning bacterium that may cause emetic and diarrhoeal syndromes, caused by different toxins (Granum & Lund, 1997; Granum, 2007). Due to its ubiquitous distribution in nature, *B. cereus* occurs frequently in a wide range of food raw materials. It has been isolated from a wide variety of foods like dairy, bakery products, rice and seafoods (Ankolekar & Labbé, 2010; Bailey & von Holy, 1993; Granum, 2007; Rahmati & Labbé, 2008). The persistent contamination of industrial food processing systems by *B. cereus* is due to the formation of endospores, which may survive pasteurization, heating, and gamma-ray irradiation (Nicholson et al., 2000), and to biofilms, which are highly resistant to cleaning and disinfection procedures (Peng, Tsai, & Chou, 2002; Ryu & Beuchat, 2005).

Biofilm formation by *B. cereus* has been described on different substrates such as stainless steel, plastic materials of different

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compositions, borosilicate glass and glass wool (Auger et al., 2009; Elhariry, 2008; Hesham & Elhariry, 2011; Houry, Briandet, Aymerich, & Gohar, 2010; Jan et al., 2011; Lindsay, Brözel, & Von Holy, 2006; Orgaz, Lobete, Puga, & Jose, 2011; Padegar & Singh 2012; Peng, Tsai, & Chou, 2001; Ryu & Beuchat 2005) and also on biotic surfaces such as cabbage and lettuce surfaces (Hesham & Elhariry, 2011). Since biofilm formation enhances bacterial tolerance to environmental factors (Abee, Kovács, Kuipers, & Van der Veen, 2011; Costerton, Stewart, & Greenberg, 1999; Van Houdt & Michiels, 2010), different approaches have been proposed to combat biofilms, based on physico-chemical agents, enzymes, or bacteriophages (de Carvalo, 2012). A few studies have explored the possibility of using bacteriocins or their producer strains against biofilm-forming bacteria (Ammor, Tauveron, Dufour, & Chevallier, 2006; Bower, McGuire, & Daeschel, 1995; Caballero Gómez, Abriouel, Grande, Pérez Pulido, & Gálvez, 2012, 2013; Guerrieri et al., 2009; Kumar, Parvathi, George, Krohne, & Karunasagar, 2009; Leriche, Chassaing, & Carpentier, 1999; Minei et al., 2008; Winkelströter et al., 2011; Zhao, Doyle, & Zhao, 2004). Interestingly, bacteriocins could improve the bactericidal effect of biocides on bacterial biofilms (Caballero Gómez et al., 2012, 2013; Lobos, Padilla, & Padilla, 2009). Enterocin AS-48 improved inactivation of *Listeria monocytogenes* and *Staphylococcus aureus* both in planktonic state as well as in biofilms (Caballero Gómez et al., 2012, 2013). Enterocin AS-48 is a cyclic antimicrobial peptide produced by strains of enterococci (Abriouel, Lucas, Ben Omar, Valdivia, & Gálvez, 2010; Maqueda et al., 2004). Previous studies have shown the effectiveness of enterocin AS-48 against vegetative cells of *B. cereus* in different food systems such as rice, rice pudding, vegetable sauces, or inoculated on the surface of vegetables (Muñoz et al., 2004; Grande et al., 2006, 2007; Cobo Molinos et al., 2008). The purpose of the present study was to investigate the capacity of enterocin AS-48 to potentiate the activity of biocides against planktonic or sessile *B. cereus* cells and its possible effects on endospores from this bacterium.

2. Materials and methods

2.1. Bacterial strains and inoculum preparation

B. cereus strains B47, B70, CRG5, ERG1 and LWL1 (all of them from food sources) and *B. cereus* CECT 148T (type strain, Spanish Type Culture Collection CECT) were cultivated on trypticase soy broth (TSB, Scharlab, Spain) or trypticase soy agar (TSA, Scharlab) and stored at 4 °C for routine use or as stocks in TSB supplemented with 30% glycerol at −80 °C. For preparation of inocula, bacilli were grown in TSB for 18 h at 37 °C. One ml cultures from each strain were mixed in a 50 ml sterile plastic tube to prepare the cocktail of strains.

2.2. Antimicrobials

Enterocin AS-48 was obtained from cultured broths of the producer strain *Enterococcus faecalis* A-48-32 after concentration by cation exchange chromatography as described elsewhere (Abriouel, Valdivia, Martínez-Bueno, Maqueda, & Gálvez, 2003). Bacteriocin concentrates were filtered through 0.22 μm pore size low protein binding filters (Millex GV; Millipore Corp., Belford, MA, USA) under sterile conditions. Bacteriocin concentrates were diluted 20–50-fold in biocide solutions in order to achieve the desired final bacteriocin concentrations of 25 or 50 μg/ml.

The commercial sanitizers P3 oxonia (25–35% hydrogen peroxide, 0.83–2.5 N acetic acid, and 0.26–0.66 N peracetic acid) and P3 topax 66 (2–5% sodium hydroxide, 2–5% sodium hypochlorite, and 2–5% alkylamine) were from ECOLAB (Barcelona, Spain). Poly-(hexamethylen guanidinium) hydrochloride (PHMG) solution (containing 7.8% of PHMG, by weight) was a kind gift of Oy Soft Protector Ltd (Espoo, Finland). These commercial solutions are widely used for sanitation in the food industry. Benzalkonium chloride (BC), cetrimide (CT), hexadecylpyridinium chloride (HDP), triclosan (TC) and chlorhexidine (CH) were from Sigma–Aldrich (Madrid, Spain). Benzalkonium chloride commercial solution contained 50% (wt/v) of the active compound. Triclosan was dissolved (10% wt/v) in 96% ethanol. The remaining biocides were dissolved aseptically in sterile distilled water at final concentrations of 5% (CH and HDP) or 10% (CT), and stored at 4 °C for a maximum of 7 days.

2.3. Biocide treatment of planktonic cells

A cocktail of the six *B. cereus* strains prepared from overnight cultures as described in Section 2.1 was inoculated in duplicate Eppendorf test tubes containing 1 ml TSB to achieve a final cell density of ca. 5.5×10^5 CFU/ml. TSB broth was previously warmed at 30 °C and supplemented or not with biocides and/or enterocin AS-48 (25 mg/l). After inoculation, TSB broths were incubated at 30 °C for 60 min. Then, 0.5 ml of triple-strength D/E neutralizing broth (Difco, Barcelona) was added, followed by vortexing and centrifugation (12.000 × g, 10 min). Supernatants were discarded, and the sediments were resuspended in 1.5 ml sterile saline solution by vortexing, followed by centrifugation as above. The resulting cell pellets were resuspended in 1 ml sterile saline solution, serially diluted in the same diluent and plated on TSA for viable cell counting. Three replicate experiments of the duplicate samples were performed.

2.4. Biocide treatment of sessile cells

The cocktail of *Bacillus* strains obtained from overnight cultures was inoculated (0.1% vol/vol) in duplicate onto diluted TSB broth (5.0 g/l) to achieve a cell density of ca. 5.5×10^5 CFU/ml. Inoculated broths were distributed (200 μl per well) on U-shaped 96-well polystyrene microtiter plates (Beckton Dickinson Labware, Franklin Lakes, NJ). Microtiter plates were incubated at 30 °C for 24 h. Then, the cultured broths were discarded and the biofilms formed on the microtiter plates were washed with 200 μl of sterile saline solution to remove loosely associated bacterial cells. Solutions containing biocides, enterocin AS-48 or biocide/bacteriocin combinations were added to the wells and the plates were further incubated at 30 °C for 60 min. After treatments, the biocidal solutions were removed and the wells were washed with 200 μl of D/E Neutralizing broth (Difco, Barcelona) followed by 200 μl of phosphate buffered saline (PBS) (Merck, Darmstadt, Germany). Biofilms were resuspended in 200 μl PBS by sonication for 1 min in a sonicator bath (Mod 3510, Branson; Danbury, CT, USA) followed by pipetting vigorously for 30 s (Caballero Gómez et al., 2012). Removal of biofilm cells was confirmed by the crystal violet staining method described by Djordjevic, Wiedmann, & McLandsborough (2002). For each treatment, samples from two wells were pooled together and vortexed, followed by serial dilution in sterile saline solution and surface plating in triplicate on TSA. Viable cell counts obtained after 24 h incubation at 37 °C were used to calculate the average numbers of viable cells per well. To determine the number of spores in biofilm, suspensions (400 μl) were heated at 80 °C for 10 min, serially diluted in sterile saline solution and plated on TSA for viable counts as described above. Three replicate experiments (with two wells per replicate) were performed.

2.5. Effect on bacterial endospores

Cultures grown in TSB for 18 h were surface spread on a solid sporulation medium consisting of nutrient agar (NA, Oxoid, Madrid) supplemented with 0.05 g/l of $MnSO_4$ (NAMS agar) and incubated for 4 days at 37 °C to obtain at least 90–95% spores (Beuchat, Clavero, & Jaquette, 1997). Spores were collected with a sterile cotton swab and resuspended in sterile distilled water (3 ml per plate). The pool of spores collected from the different plates was centrifuged at 5000 × g for 15 min at 4 °C, washed two times with sterile distilled water by repeated centrifugation, and finally resuspended in sterile distilled water (6–7 log CFU/ml, as determined by plating on TSA) and stored in Eppendorf tubes at −20 °C until use. Cocktails of endospores from the different strains were treated in duplicate with biocides and/or enterocin AS-48 for 1 h at temperatures of 22 °C, 40 °C or 60 °C to compare the effect of mild heat on the efficacy of treatments. After treatments, samples were brought to ambient temperature under running tap water, mixed with 0.5 ml of triple-strength D/E neutralizing broth (Difco, Barcelona) followed by vortexing and centrifugation (12,000 × g, 10 min). Supernatants were discarded, and the sediments were resuspended in 1.5 ml sterile saline solution by vortexing, followed by centrifugation as above. The resulting cell pellets were resuspended in 1 ml sterile saline solution, serially diluted in the same diluent and plated on TSA for viable cell counting. Three replicate experiments were performed.

2.6. Bacteriocin adsorption experiments

Stainless steel coupons (1.5 by 4.0 cm, type 304 with a no. 4 finish) were sonicated in distilled water for 2 min, immersed in 70% ethanol for 10 min, rinsed with sterile distilled water and then dried in a biosafety cabinet for 4 h and sterilized by autoclaving. Coupons were immersed in bacteriocin solutions (50 mg/l in distilled water) for 90 min at 22 °C followed by rinse with 5 ml sterile distilled water. The coupons were placed into stomacher bags containing a cocktail of endospores (ca. 10^6 spores/ml in 5 ml of diluted TSB) and incubated at 37 °C for 24 h. At desired intervals of incubation (0, 2, 4, 6, 10 and 24 h), coupons in duplicate were removed and washed with sterile saline solution (2 × 5 ml). Coupons were placed in sterile stomacher bags containing 2 ml sterile saline solution and sonicated for 30 s. Then, coupon surfaces were swabbed thoroughly with a sterile cotton swab to recover attached cells and endospores. Total viable counts from the obtained suspensions were determined by serial dilution and plating on TSA.

Wells of 96-well polystyrene microtiter plates were incubated with 200 μl of bacteriocin solutions (25 or 50 mg/l in distilled water) for 90 min at 22 °C. Then, bacteriocin solutions were removed and the wells were washed three times with 200 μl of sterile distilled water. A cocktail of endospores (ca. 10^6 spores/ml in 200 μl of diluted TSB) was added to the wells and incubated at 37 °C for 24 h. Wells were washed with sterile saline solution (2 × 200 μl). Adhered cells and endospores were recovered by sonication and pipetting as described in Section 2.4, and total viable counts were determined by plating on TSA.

2.7. Statistical analysis

All experiments were carried out in triplicate (with duplicate samples per trial), and the average data ± standard deviations were determined with Excel programme (Microsoft Corp., USA). A *t*-test was performed at the 95% confidence interval with Statgraphics Plus version 5.1 (Statistical Graphics Corp, USA), in order to determine the statistical significance of data.

3. Results

3.1. Effect of biocidal treatments on planktonic Bacillus cereus cells

Survival of vegetative cells exposed to biocides without bacteriocin decreased progressively as biocide concentration increased, especially in the concentration range of 2.5–5 g/l (Fig. 1, Fig. 2). Triclosan and chlorhexidine were the most effective, achieving complete inactivation of the bacterial population when tested at a final concentration of 2.5 g/l. The commercial solution P3 topax 66 was the less effective, with only partial inactivation of the bacilli. Treatment with enterocin AS-48 alone (25 mg/l) significantly ($p < 0.05$) reduced the population of bacilli by approx. 1.1 log cycles. Bacteriocin addition in combination with biocides significantly ($p < 0.05$) increased inactivation of bacilli compared to the single biocide treatment for at least one or more concentrations of benzalkonium chloride, cetrimide, hexadecylpyridinium chloride, triclosan, chlorhexidine, PHMG, and P3 oxonia, but not for P3 topax 66 (Figs. 1 and 2). All the combinations of AS-48 and cetrimide, hexadecylpyridinium chloride and triclosan, and also some of the combinations with benzalkonium chloride, chlorhexidine and P3 oxonia, achieved total inactivation of the bacterial population while the single biocide treatment did not. The combinations of enterocin AS-48 and 0.25 g/l hexadecylpyridinium chloride or triclosan were the most efficient in terms of biocide concentration among all combinations tested.

3.2. Effect of biocidal treatments on sessile Bacillus cereus cells

Total counts in biofilms formed on microtiter plates consisted of >99.0% of vegetative cells (heat sensitive) and <1.0% of spores (heat resistant). In the sessile state, *B. cereus* cells were much more resistant to biocides than vegetative cells, and none of the biocides tested was able to completely inactivate bacterial populations at the highest concentrations tested (Figs. 3 and 4). Benzalkonium chloride and cetrimide reduced viable counts in the biofilms by only 1.2–1.4 log cycles when tested at a final concentration of 5 g/l (Fig. 3A, B). Hexadecylpyridinium chloride was the most effective of all biocides tested, achieving a reduction of 3.3 log cycles at 2.5 g/l (Fig. 3C) followed by P3 oxonia commercial solution (2.5 log cycles at 2.5% of the commercial solution, equivalent to approximately 1100 ppm peracetic acid; Fig. 4C). Remarkably, biocides that had strong antimicrobial activity on planktonic cells, such as triclosan had a very low activity on sessile cells (with a reduction of only 1.2 log cycles at 10 g/l; Fig. 3D). Similar low activity was detected for chlorhexidine (2 log cycles reduction at 10 g/l; Fig. 4A). Combinations of biocides and enterocin AS-48 were tested at two bacteriocin concentrations of 25 and 50 mg/l. The single bacteriocin treatment decreased viable cell counts in the biofilms non-significantly ($p > 0.05$) by 0.5–0.7 log cycles (Figs. 3 and 4). Furthermore, a bacteriocin concentration of 25 mg/l did not enhance significantly the activity of any of the biocides tested. However, a higher bacteriocin concentration (50 mg/l) significantly ($p < 0.05$) increased microbial inactivation for several biocide concentrations except for P3 topax 66 (Figs. 3 and 4). Greatest inactivation was observed for highest concentrations of biocides tested, especially for the combinations of bacteriocin and cetrimide (Fig. 3B), hexadecylpyridinium chloride (Fig. 3C), poly-(hexamethylen guanidinium) hydrochloride (Fig. 4B) and P3 oxonia (Fig. 4C).

3.3. Effects of biocidal treatments on bacterial endospores

A cocktail of *B. cereus* endospores not activated to germinate was treated for 1 h at temperatures of 22 °C, 40 °C or 60 °C without antimicrobials, or with different biocides, enterocin AS-48, or

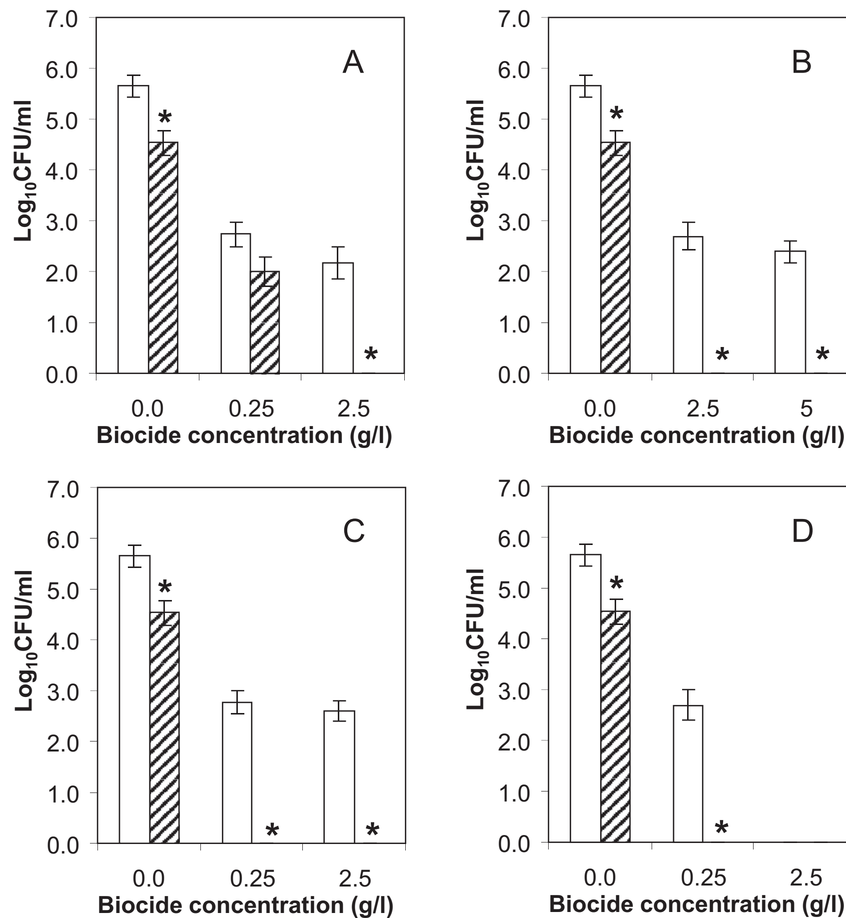

Fig. 1. Effect of biocides applied singly (open bars) or in combination with enterocin AS-48 (25 mg/l; striped bars) on the viability of a cocktail of *B. cereus* vegetative cells. The biocides tested were benzalkonium chloride (A), cetrimide (B), hexadecylpyridium chloride (C), and triclosan (D). Asterisks denote statistically significant reductions ($p < 0.05$) of viable cell concentrations in the combined treatments compared to the single biocide treatment.

biocide-bacteriocin combinations. Incubation of endospore suspensions at 40 or 60 °C did not significantly ($p > 0.05$) reduce viable counts. Similarly, challenged with enterocin AS-48 did not cause any significant ($p > 0.05$) decrease in viability (Table 1). Similarly, the effect of biocides did not increase significantly ($p > 0.05$) by addition of enterocin AS-48 when biocide and biocide-bacteriocin treatments were compared, achieving reductions in viable counts of less than 0.8 or 0.5 log cycles in most cases compared to the single biocide treatments. Many of the biocide treatments applied (either singly or in combination with bacteriocin or both) reduced viable counts significantly ($p < 0.05$) compared to the controls without antimicrobials, or achieved complete inactivation of bacterial endospores. Treatment with benzalkonium chloride at 60 °C achieved complete inactivation of endospore populations (for both single and combined treatments). In addition, single and combined treatments with this biocide also reduced viable counts significantly ($p < 0.05$) at lower temperatures of 22 or 40 °C, e.g. by 1.6–2 log cycles at 10 g/l or by 1–1.8 log cycles in most treatments at 5 g/l. For cetrimide, significant reductions of viable counts (1.1–1.6 log cycles) were observed only in treatments carried out at 60 °C. Hexadecypyridinium chloride achieved complete inactivation of bacterial endospores at the highest concentration tested (2.5 g/l) and 60 °C, and also reduced viable counts significantly (by 1–2.4 log cycles) for the rest of treatments (both single and combined). Triclosan only reduced viable counts significantly (1.5 log cycles; $p < 0.05$) when tested at 0.25 g/l, 60 °C, and combined with bacteriocin. None of the chlorhexidine treatments reduced the viability of endospores significantly, even at 5 g/l and 60 °C. Poly-(hexamethylen guanidinium) hydrochloride only reduced viable counts significantly (by 1–1.2 log cycles; $p < 0.05$) when tested at 0.2 g/l in combination with bacteriocin, both a 40 °C and 60 °C. P3 oxonia was highly effective in that it achieved complete inactivation of endospore populations in all treatments at 0.25% and also in treatments with 0.025% biocide solution at 60 °C. This biocidal preparation also reduced viable counts significantly when tested at 0.025% and 40 °C, both singly and in combination with bacteriocin. In contrast, the reductions of viable counts obtained for the commercial solution P3 topax 66 were not significant ($p > 0.05$) for any of the treatments tested.

3.4. Effect of adsorbed bacteriocin on population recovery from endospores

Stainless steel coupons dosed with enterocin AS-48 (50 mg/l) had no inhibitory effect on adherence and outgrowth when immersed in broth inoculated with a cocktail of bacterial endospores, since viable counts of recovered cells and endospores from controls and bacteriocin-treated coupons did not differ significantly ($p > 0.05$) both at time 0 or during further incubation (Fig. 5A). Nevertheless, when endospore suspensions were inoculated in polystyrene microtiter plates previously dosed with bacteriocin concentrations of 25 or 50 mg/l, no viable cells were obtained after 2 h incubation and for at least 24 h for the two bacteriocin concentrations tested (Fig. 5B). These results indicate

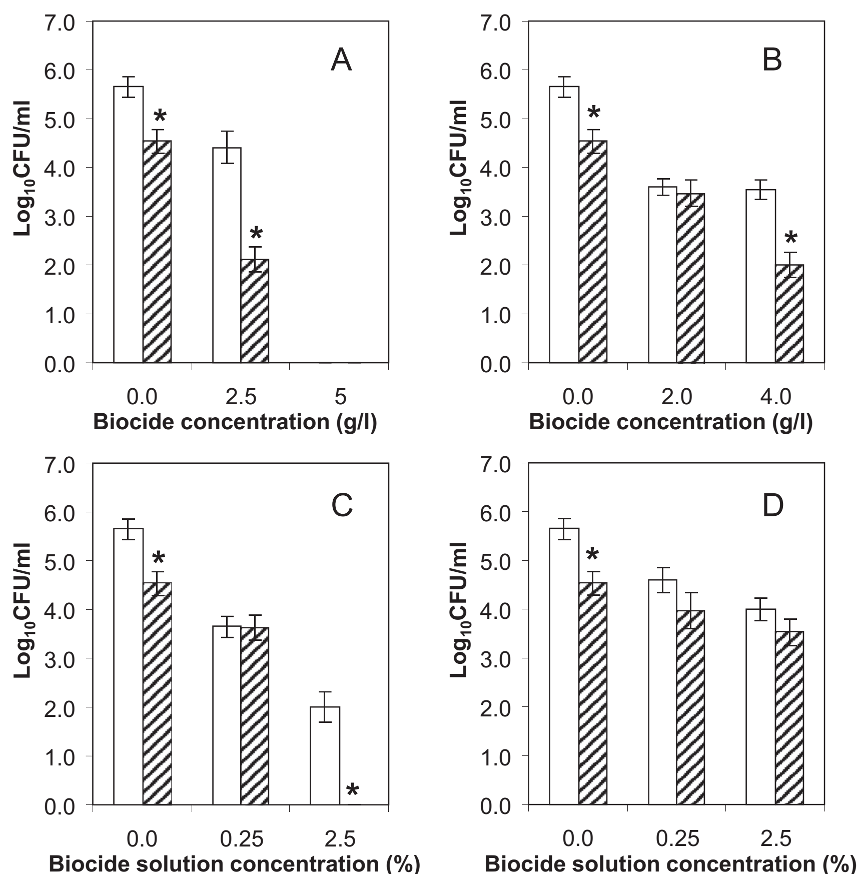

Fig. 2. Effect of biocides applied singly (open bars) or in combination with enterocin AS-48 (25 mg/l; striped bars) on the viability of a cocktail of *B. cereus* vegetative cells. The biocides tested were chlorhexidine (A), poly-(hexamethylen guanidinium) hydrochloride (B) or the commercial sanitizers P3 oxonia (C) and P3 topax 66 (D). Asterisks denote statistically significant reductions ($p < 0.05$) of viable cell concentrations in the combined treatments compared to the single biocide treatment.

clear differences in adsorption of enterocin AS-48 to polystyrene compared to stainless steel coupons.

4. Discussion

Previous studies have shown that enterocin AS-48 kills vegetative cells of *B. cereus* in the planktonic state when tested in cultured broths as well as in food systems (Abriouel, Maqueda, Gálvez, Martínez-Bueno, & Valdivia, 2002; Cobo Molinos et al., 2008; Grande et al., 2006, 2007; Muñoz et al., 2004). The strong bactericidal activity shown by AS-48 on endospore-forming bacteria could open new possibilities for disinfection in combination with biocides, like for example reducing the concentration of biocides (which are generally obtained by chemical synthesis) while adding a natural product (the bacteriocin). Results from the present study provided new information on the activity of different biocides against *B. cereus*. Biocides such as benzalkonium chloride, chlorhexidine and polyhexametylenebiguanide are used for sanitizing the surfaces of utensils and instruments in the food industry at working concentrations that may range from 0.01% to 0.2% of the active ingredient (Ueda & Kuwabara, 2007). Yet, there are scarce reports in the scientific literature on the effects of biocides on *B. cereus* vegetative cells. Ceragioli et al., (2010) determined that treatment of *B. cereus* ATCC 14579 planktonic cells for 60 min with 5 mg/l benzalkonium chloride reduced viable cell counts by approximately 1.5 log cycles, while 0.2 mM hydrogen peroxide or 100 mg/l peracetic acid achieved reductions of about 2.5 log cycles. *B. cereus* strains were weakly susceptible to Biseptine, an antiseptic composed of chlorhexidine digluconate, benzalkonium chloride and benzylic alcohol (Reverdy, Martra, & Fleurette, 1997). In another study, benzalkonium chloride diluted in 70% alcohol (1:5000) was considered to be the best disinfectant in vitro for human sclera artificially contaminated with *B. cereus* (Lucci, Yu, & Höfling-Lima, 1999). Our results also indicated that enterocin AS-48 in combination with biocides improved inactivation of planktonic cells of a cocktail of *B. cereus* strains at concentrations where the single bacteriocin addition only decreased viable cell counts by less than one log cycle. Interestingly, this synergism occurred at very low biocide concentrations in the case of triclosan and hexadecylpyridinium chloride. Triclosan and other biocides are widely used in industry (Cerf, Carpentier, & Sanders, 2010). Triclosan is now incorporated in a wide range of products, such as dishcloths, food boxes, toothbrushes, washing-up liquid and hand-washing gels, plastics, chopping boards, chopsticks, pizza cutters, food storage containers, garbage bags, or kitty litter, among others (Yazdankhah et al., 2006). Triclosan-impregnated storage boxes inhibited growth of *B. cereus* (Braid & Wale, 2002). Several bacteriocins have been inmmobilized in different types of supports such as films or particles (Gálvez, Abriouel, López, & Ben Omar, 2007). The efficacy of films or packages containing enterocin AS-48 and biocides deserves to be investigated in future studies.

One of the issues to be addressed in disinfection is biofilm formation. The capacity of *B. cereus* to form biofilms on different kinds of surfaces is well known. However, only a few studies have determined the increased tolerance of sessile *B. cereus* vegetative cells to biocides. Peng, Tsai, & Chou (2002) compared the tolerance

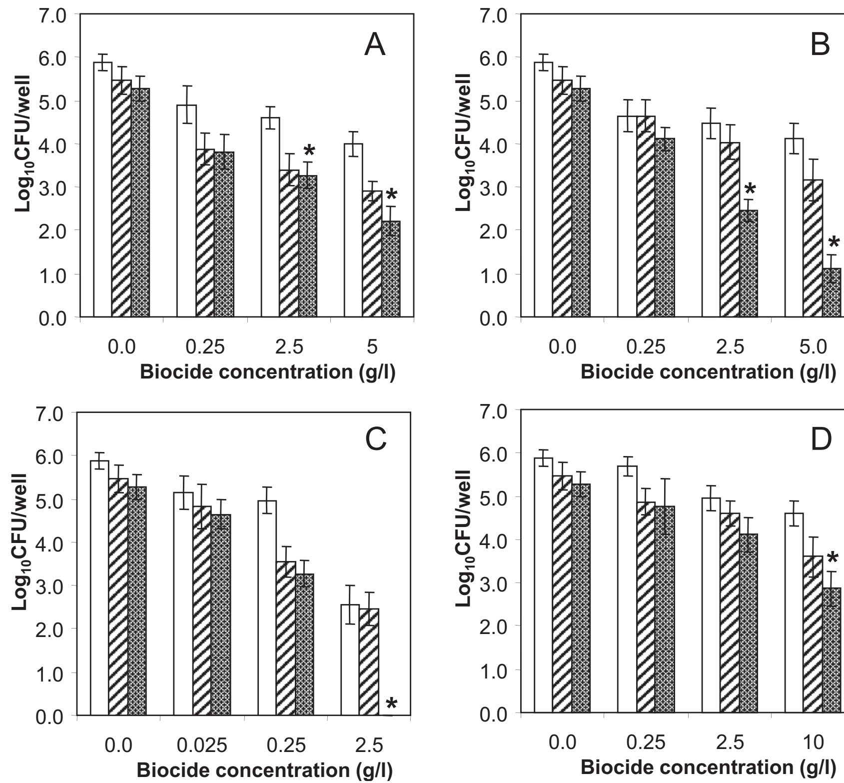

Fig. 3. Effect of biocides applied singly (open bars) or in combination with enterocin AS-48 at 25 mg/l (striped bars) or 50 mg/l (shaded bars) on the viability of 24-h biofilms formed by a cocktail of *B. cereus* vegetative cells on polystyrene microtiter plates. The biocides tested were benzalkonium chloride (A), cetrimide (B), hexadecylpyridium chloride (C), and triclosan (D). Asterisks denote statistically significant reductions ($p < 0.05$) of viable cell concentrations in the combined treatments compared to the single biocide treatment.

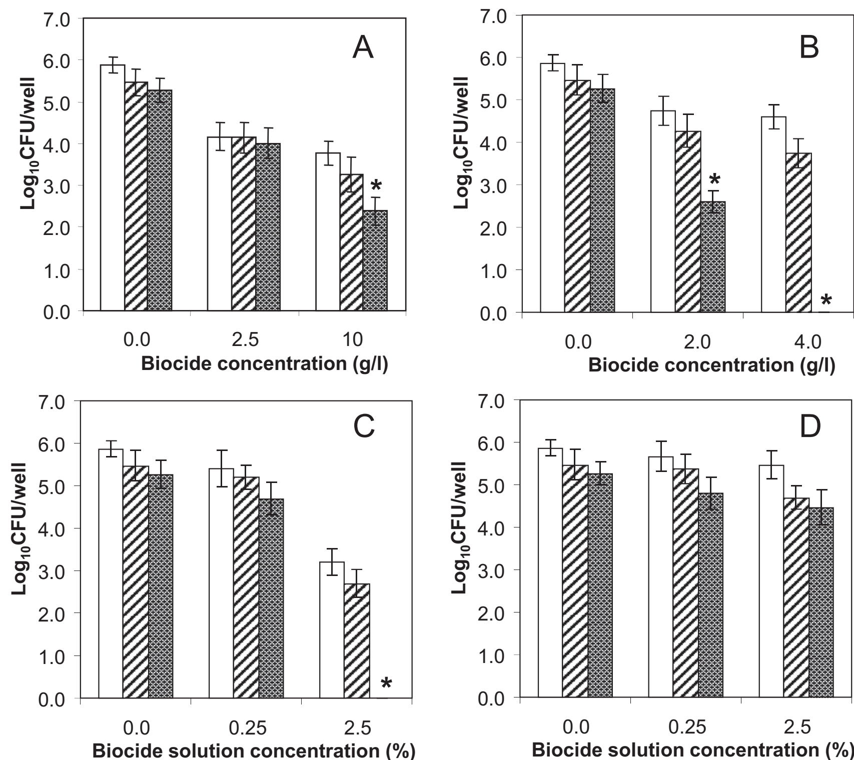

Fig. 4. Effect of biocides applied singly (open bars) or in combination with enterocin AS-48 at 25 mg/l (striped bars) or 50 mg/l (shaded bars) on the viability of 24-h biofilms formed by a cocktail of *B. cereus* vegetative cells on polystyrene microtiter plates. The biocides tested were chlorhexidine (A), poly-(hexamethylen guanidinium) hydrochloride (B) or the commercial sanitizers P3 oxonia (C) and P3 topax 66 (D). Asterisks denote statistically significant reductions ($p < 0.05$) of viable cell concentrations in the combined treatments compared to the single biocide treatment.

Table 1
Effect of treatments with biocides singly or in combination with enterocin AS-48 on the viability of *Bacillus cereus* endospores.

Antimicrobial	Biocide concentration (g/l)	Treatment temperature					
		22 °C		40 °C		60 °C	
		Biocide	Biocide + AS-48	Biocide	Biocide + AS-48	Biocide	Biocide + AS-48
Benzalkonium chloride (BC)	0	6.20 ± 0.14	6.10 ± 0.21	6.17 ± 0.24	6.00 ± 0.18	6.10 ± 0.25	5.87 ± 0.27
	5	5.17 ± 0.19*	4.3 ± 0.21*	5.06 ± 0.30	4.20 ± 0.32*	–*	–*
	10	4.6 ± 0.24*	4.22 ± 0.31*	4.47 ± 0.12*	4.10 ± 0.33*	–*	–*
Cetrimide (CT)	0	6.20 ± 0.14	6.10 ± 0.21	6.17 ± 0.24	6.00 ± 0.18	6.10 ± 0.25	5.87 ± 0.27
	5	5.97 ± 0.23	5.99 ± 0.23	5.84 ± 0.36	5.90 ± 0.24	4.95 ± 0.25*	4.78 ± 0.25*
	10	5.92 ± 0.30	5.87 ± 0.16	5.81 ± 0.23	5.90 ± 0.31	4.60 ± 0.32*	4.47 ± 0.24*
Hexadecylpyridinium chloride (HDP)	0	6.20 ± 0.14	6.10 ± 0.21	6.17 ± 0.24	6.00 ± 0.18	6.10 ± 0.25	5.87 ± 0.27
	0.25	5.22 ± 0.24*	4.12 ± 0.21*	5.06 ± 0.25*	4.06 ± 0.37*	3.81 ± 0.29*	3.70 ± 0.25*
	2.5	4.12 ± 0.17*	4.22 ± 0.31*	3.95 ± 0.21*	3.80 ± 0.34*	–*	–*
Triclosan (TC)	0	6.20 ± 0.14	6.10 ± 0.21	6.17 ± 0.24	6.00 ± 0.18	6.10 ± 0.25	5.87 ± 0.27
	0.025	5.43 ± 0.33	5.57 ± 0.29	5.40 ± 0.24	5.40 ± 0.24	5.30 ± 0.28	5.04 ± 0.29
	0.25	5.34 ± 0.33	5.07 ± 0.33	5.30 ± 0.28	5.00 ± 0.24	5.09 ± 0.35	4.54 ± 0.29*
Chlorhexidine (CH)	0	6.20 ± 0.14	6.10 ± 0.21	6.17 ± 0.24	6.00 ± 0.18	6.10 ± 0.26	5.87 ± 0.27
	2.5	6.14 ± 0.19	5.44 ± 0.28	6.07 ± 0.31	5.40 ± 0.18	5.65 ± 0.21	5.07 ± 0.29
	5	6.07 ± 0.25	5.40 ± 0.28	6.04 ± 0.33	5.30 ± 0.25	5.40 ± 0.25	5.00 ± 0.32
Poly-(hexamethylen guanidinium) hydrochloride (PHMG)	0	6.20 ± 0.14	6.10 ± 0.21	6.17 ± 0.24	6.00 ± 0.18	6.10 ± 0.26	5.87 ± 0.27
	0.02	6.24 ± 0.34	5.40 ± 0.19	6.00 ± 0.32	5.30 ± 0.25	5.90 ± 0.29	5.22 ± 0.31
	0.2	6.04 ± 0.29	5.34 ± 0.36	5.78 ± 0.25	5.00 ± 0.24*	5.07 ± 0.38	4.87 ± 0.21*
P3 oxonia**	0	6.20 ± 0.14	5.97 ± 0.23	6.17 ± 0.24	5.90 ± 0.24	6.10 ± 0.25	5.84 ± 0.29
	0.025	5.80 ± 0.28	5.40 ± 0.21	5.10 ± 0.24*	4.84 ± 0.34*	–*	–*
	0.25	–*	–*	–*	–*	–*	–*
P3 topax 66**	0	6.20 ± 0.14	5.97 ± 0.23	6.17 ± 0.24	5.90 ± 0.24	6.10 ± 0.25	5.84 ± 0.29
	0.5	5.60 ± 0.21	5.40 ± 0.19	5.47 ± 0.31	5.39 ± 0.30	5.30 ± 0.27	5.00 ± 0.32
	1	5.54 ± 0.27	5.20 ± 0.34	5.47 ± 0.24	5.06 ± 0.31	5.25 ± 0.31	4.84 ± 0.33

Data are the average of two experiments carried out in duplicate each, ±standard deviation (SD).
*Statistically significant reductions ($p < 0.05$) of viable cell concentrations of treatments compared to controls without antimicrobials.
**Concentration is in % (vol/vol).

of planktonic cells of *B. cereus,* attached cells, and cells in a biofilm to treatment with sodium hypochlorite and a quaternary ammonium compound. Cells in a biofilm were most resistant to treatment with chlorine (50 μg/ml), followed by attached single cells and cells in a planktonic state. Another study (Ryu & Beuchat, 2005) showed that spores and, to a lesser extent, vegetative cells embedded in biofilm were protected against inactivation by sanitizers chlorine, chlorine dioxide, and a peroxyacetic acid–based sanitizer. Results from the present study indicated that sessile vegetative cells of *B. cereus* were more resistant to biocides, enterocin AS-48 and the combinations of bacteriocins and biocides. Nevertheless, addition of enterocin AS-48 potentiated the activity of biocides on sessile cells, reducing the populations below detectable levels at still low concentrations of hexadecylpyridinium chloride, polyhexamethylen guanidinium chloride and P3 oxonia sanitizer. These results are of great interest, since P3 oxonia is widely used for disinfection in the food industry, at concentrations that may range from 0.05% up to 5% (Blakistone, Chuyate, Kautter, Charbonneau, & Suit, 1999). Polyhexamethylen guanidinium chloride has been widely used for many years as an antiseptic in medicine and the food industry (Allen, White, & Morby, 2006; Cox, Bailey, & Berrang, 1998; Cox, Berrang, Buhr, & Bailey, 1999), and hexadecylpyridinium chloride (cetylpyridinium chloride) was approved in 2004 by the US FDA for decontaminating raw poultry at a working concentration not exceeding 0.8% by weight (Food and Drug Administration, 2004). In a previous study, application of washing treatments containing enterocin AS-48 (25 mg/l) in combination with 0.5% hexadecylpyridinium chloride significantly reduced the viability of *B. cereus* and *Bacillus weihenstephanensis* LWL1 cells in alfalfa sprouts (Cobo Molinos et al., 2008). Enterocin AS-48 also enhanced the activity of biocides on sessile *L. monocytogenes* cells, including hexadecylpyridinium and polyhexamethylen guanidinium chloride (Caballero Gómez et al., 2012). However, no synergism was observed between enterocin AS-48 and P3 oxonia, suggesting that different bacteria may respond in different ways to bacteriocins and biocides.

Bacterial endospores represent a main problem for disinfection in the food industry. Intact endospores of *B. cereus* are resistant to enterocin AS-48, but they gradually become sensitive during the course of germination and outgrowth (Abriouel, Maqueda, Gálvez, Martínez-Bueno, & Valdivia, 2002). In the present study, endospores from a cocktail of *B. cereus* strains not activated to germinate were resistant to 50 mg/l AS-48. As opposed to vegetative cells, enterocin AS-48 did not significantly increase the activity of biocides on endospores. However, promising results were obtained for some of the biocides and commercial disinfectants like P3 oxonia, benzalkonium chloride and hexadecylpyridinium chloride. The efficacy of oxonia commercial solutions on bacterial endospores has been described in previous studies, in which endospores from *B. cereus* were found to be the most resistant (Blakistone, Chuyate, Kautter Jr, Charbonneau, & Suit, 1999; Hilgren, Swanson, Diez-Gonzalez, & Cords, 2009). The results obtained in the present study for benzalkonium chloride and hexadecylpyridinium chloride on bacterial endospores treated at 60 °C are very promising, since these biocides may be used at industrial scale as part of detergent solutions for cleaning and disinfection of surfaces.

One possible strategy to avoid proliferation of *B. cereus* could be incorporation of bacteriocin into food contact surfaces, since *B. cereus* vegetative cells as well as endospores are known to adhere to stainless steel surfaces where they can germinate and form biofilms (Peng, Tsai, & Chou, 2001). In agreement with previous studies with *L. monocytogenes* (Caballero Gómez et al., 2012), enterocin AS-48 adsorbed on polystyrene surfaces completely avoided proliferation of *B. cereus* after challenge with a suspension of endospores. However, stainless steel coupons dosed with bacteriocin did not have any inhibitory effect, suggesting that the bacteriocin does not adsorb to stainless steel surfaces. Other bacteriocins (especially nisin and sakacin 1) and bacteriocin-producing

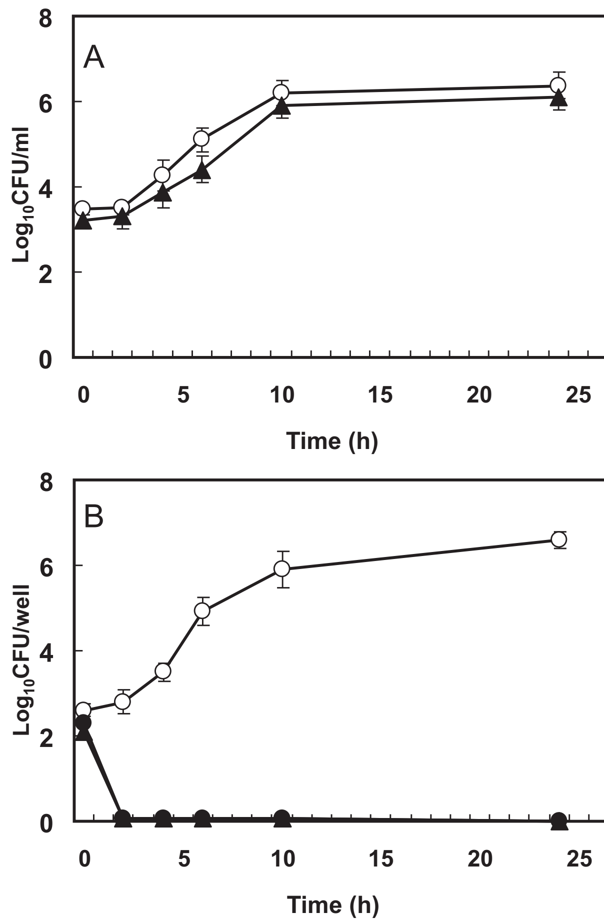

Fig. 5. Adsorption of enterocin AS-48 on stainless steel coupons (A), and polystyrene microtiter plates (B). A cocktail of *Bacillus cereus* endospores was incubated in contact with stainless steel coupons (A) previously dosed with 50 mg/l enterocin AS-48 (●), or in polystyrene microtiter plates previously dosed with AS-48 at 25 (▲) or 50 (●) mg/l. Controls without bacteriocin (○).

strains have shown to decrease adhesion of *L. monocytogenes* to surfaces, either by the release of adsorbed bacteriocin molecules or by *in situ* produced bacteriocin in biofilms of the producer strains (Bower, McGuire, & Daeschel, 1995; Winkelströter et al., 2011). The possibility of using enterocin AS-48 for surface coating thus needs further investigations.

In conclusion, results from the present study suggest new applications for enterocin AS-48 in combination with biocides against *B. cereus*. The bactericidal effect of the different combinations greatly depends on the planktonic or sessile state, as well as whether vegetative cells or endospores are targeted. Among all the combinations tested, those including P3 oxonia seem to be the most promising, since they could be applied on planktonic and sessile cells as well as on endospores. Enterocin AS-48 can be produced on by-products from dairy industry, reducing the costs of industrial production (Ananou et al., 2010). In addition, previous studies have shown that it is not toxic to eukaryotic cells (reviewed by Maqueda et al., 2004). For specific applications, different parameters such as biocidal composition, concentration of the active ingredients contact time or treatment temperature would need to be optimized.

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