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Inhibition of planktonic and sessile *Salmonella enterica* cells by combinations of enterocin AS-48, polymyxin B and biocides

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ABSTRACT

Enterocin AS-48 was tested against planktonic *Salmonella enterica* UJ3197 in combination with polymyxin B and/or biocides. Enterocin AS-48 added at 50 mg/l or 100 mg/l in combination with 2 mg/l polymyxin B caused partial or complete growth inhibition of *S. enterica* UJ3197 at 24 h incubation. At 2 mg/l, polymyxin B did not enhance significantly the inhibitory effect of biocides against strain UJ3197, except for 25 mg/l cetrimide. The combination of polymyxin B (2 mg/l) and enterocin AS-48 (25 mg/l) significantly inhibited growth of strain UJ3197 in combination with 2.5 or 25 mg/l cetrimide, 25 mg/l hexadecylpyridinium chloride, 500 mg/l chlorhexidine, and also with 0.16 or 1.6 mg/l poly-(hexamethylen guanidinium) hydrochloride. Biofilms formed by four *Salmonella* strains (S62, S64, UJ3197, UJ3198) on polystyrene microtiter plates were treated with biocides singly or in combinations with polymyxin B (2 mg/l), enterocin AS-48 (25 or 50 mg/l) or both. Sessile salmonellae showed an increased tolerance to all biocides tested compared to planktonic cells. At the concentration tested, polymyxin B did not enhance the bactericidal activity for any of the biocides on sessile salmonellae. Enterocin AS-48 improved the activity of most biocides, but only for some of the strains and only for the highest bacteriocin concentration tested in most cases. The combinations of enterocin AS-48 and polymyxin B improved inactivation of sessile salmonellae for all biocides tested, although the degree of inactivation was highly dependent on strain and biocide. The combination of polymyxin B (2 mg/l) and enterocin AS-48 (50 mg/l) improved significantly ($p < 0.05$) the inactivation of all *Salmonella* strains in sessile state by the biocides benzalkonium chloride, cetrimide, triclosan, hexachlorophene, chlorhexidine, poly-(hexamethylen guanidinium) hydrochloride, and P3-oxonia.

1. Introduction

In 2008, salmonellosis was the second most often reported zoonotic disease in humans in the EU, accounting for 131,468 confirmed human cases, of which 3833 occurred in Spain (EFSA, 2010). *Salmonella* was the most frequently reported cause of foodborne outbreaks (35.4% of all outbreaks). The common reservoir of *Salmonella* is the intestinal tract of a wide range of domestic and wild animals, and for that reason a variety of foodstuffs including both food of animal and plant origin can be sources of infection. Transmission often occurs when organisms are introduced in food preparation areas and are allowed to multiply in food, e.g. due to inadequate storage temperatures, inadequate cooking or cross contamination of ready-to-eat (RTE) food.

Biofilm formation plays an important role in the attachment and colonization of *Salmonella enterica* onto biotic and abiotic surfaces (Collignon & Korsten, 2010; Iturriaga, Tamplin, & Escartín, 2007; Joseph, Otta, Karunasagar, & Karunasagar, 2001; Lapidot, Romling, & Yaron, 2006; Vestby, Moretro, Langsrud, Heir, & Nesse, 2009). Biofilm formation has serious implications in industrial, environmental, public health and medical situations (Gilbert, McBain, & Rickard, 2003; Hall-Stoodley, Costerton, & Stoodley, 2004). The occurrence of biofilms in food-processing environments can cause post-processing contamination leading to lowered shelf-life of products and transmission of diseases (Jessen & Lammert, 2003). Sessile micro-organisms are more difficult to mechanically remove from food-contact surfaces and are more resistant to disinfectants compared with planktonic forms (Gilbert, Das, Jones, & Allison, 2001; Morton, Greenway, Gaylarde, & Surman, 1998). Hence there is great interest in development of treatments that will efficiently inactivate sessile bacteria, including *S. enterica*.

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While many bacteriocins have been investigated for inactivation of foodborne pathogens in foods in planktonic state, only a few studies have explored the possibility of using bacteriocins or their producer strains against sessile bacteria (Ammor, Tauveron, Dufour, & Chevallier, 2006; Bower, McGuire, & Daeschel, 1995; Guerrieri et al., 2009; Kumar, Parvathi, George, Krohne, & Karunasagar, 2009; Leriche, Chassaing, & Carpentier, 1999; Minei, Gomes, Ratti, D'Angelis, & De Martinis, 2008; Winkelströter, Gomes, Thomaz, Souza, & De Martinis, 2011; Zhao, Doyle, & Zhao, 2004). Nisin and sakacin 1 reduced the adherence of *Listeria monocytogenes* to silica and stainless steel surfaces, respectively (Bower et al., 1995; Winkelströter et al., 2011). The antimicrobial activity of bacteriocins can be improved by combination with other antimicrobial substances, but still few studies have investigated the potential for synergy between bacteriocins and disinfectants or biocides. The *Pseudomonas* bacteriocin PsVP- 10 was shown to act synergistically with chlorhexidine and triclosan on *Streptococcus mutans* and *Streptococcus sobrinus* in planktonic state as well as in biofilms (Lobos, Padilla, & Padilla, 2009). Another bacteriocin named enterocin AS-48 improved the inactivation of sessile *L. monocytogenes* cells in combination with a variety of biocides (Caballero Gómez, Abriouel, Grande, Pérez Pulido, & Gálvez, 2012). Enterocin AS-48 is a cyclic antimicrobial peptide of great interest for application in food preservation and food sanitation (Abriouel, Lucas, Ben Omar, Valdivia, & Gálvez, 2010; Maqueda et al., 2004). AS-48 has been thoroughly investigated for its antimicrobial activity against foodborne pathogens both in liquid cultures and in foods (Abriouel et al., 2010). However, activity of bacteriocins including enterocin AS-48 on Gram-negative bacteria is limited because of the permeability barrier imposed by the outer cell membrane. Polymyxins are cationic detergent-like cyclic peptides active on Gram-negative bacteria by means of their detersive activity, which disrupts the bacterial outer membrane and the cytoplasmic membrane (Horton & Pankey, 1982). Therefore, polymyxins could improve the activity of bacteriocins on Gram-negative bacteria. In a recent study, it was shown that polymyxin E activity on *Escherichia coli* increased in combination with the bacteriocins nisin or pediocin PA-1 (Naghmouchi, Belguesmia, Baah, Teather, & Drider, 2011). The purpose of the present study was to investigate if polymyxin B could potentiate the effect of enterocin AS-48 against *S. enterica* and if the combination of polymyxin B and enterocin AS-48 could be useful to enhance the antimicrobial activity of biocides against planktonic and sessile *Salmonella* cells.

2. Materials and methods

2.1. Bacterial strains and growth conditions

S. enterica serovar Enteritidis strains UJ3449 and UJ3197 were isolated from Spanish omelette and grilled pork, respectively, and were both implicated in domestic outbreaks of human salmonellosis. *S. enterica* strains S62 and S64 were isolated from chicken hamburger and mayonnaise, respectively. Strains were propagated at 37 °C in Brain Heart Infusion Broth (BHI, Scharlab, Barcelona) or BHI agar slants and stored at 4 °C for routine use or in glycerol (30% in distilled water) at −80 °C. For preparation of inocula, strains were grown overnight (18 h) in BHI broth.

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. Polymyxin B solution (1 mg/ml in water) was from Fluka (Madrid, Spain).

The commercial sanitizer P3-oxonia (25–35% hydrogen peroxide, 0.83–2.5 N acetic acid, and 0.26–0.66 N peracetic acid) was 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). Benzalkonium chloride (BC), cetrimide (CT), hexadecylpyridinium chloride (HDP), triclosan (TC), hexachlorophene (CF) and chlorhexidine (CH) were from Sigma–Aldrich (Madrid, Spain). Benzalkonium chloride commercial solution contained 50% (wt/v) of the active compound. Triclosan and hexachlorophene were dissolved (10% wt/v) in 96% ethanol. The remaining biocides were dissolved aseptically in sterile distilled water at final concentrations of 1.0–10.0% (depending of each particular case), and stored at 4 °C for a maximum of 7 days.

2.3. Effect of antimicrobial peptides polymyxin B and enterocin AS-48 and biocides on growth of S. enterica

S. enterica UJ3197 cells (ca. 1.0×10^7 CFU/ml) were inoculated onto Trypticase Soy Broth (TSB, Scharlab, Barcelona) supplemented or not with polymyxin B, enterocin AS-48, biocides or their combinations. After inoculation, broths were distributed (200 μl/well) in triplicate on U-shaped 96-well polystyrene microtiter plates (Becton Dickinson Labware, Franklin Lakes, NJ), which were covered with a tight fitting lid and incubated at 30 °C. At desired intervals of incubation (0, 5, 10, and 24 h), the optical density of wells was recorded at 650 nm in a microplate reader iMark TM (BioRad, Madrid) after shaking for 30 s.

Polymyxin B was tested singly at 2, 5 or 10 mg/l or at 2 mg/l in combination with enterocin AS-48 (50 or 100 mg/l) in order to establish possible interactions between the two antimicrobial peptides.

In preliminary trials, biocides were tested singly at four different concentrations each in order to approximate the range of inhibitory concentrations. The minimum inhibitory concentration (MIC) was determined as the minimum concentration of biocide that completely inhibited growth at 24 h incubation. In order to determine if biocide activity on planktonic *Salmonella* could be potentiated by polymyxin B (2 mg/l) or polymyxin B (2 mg/l) plus enterocin AS-48 (at final concentrations of 12.5 or 25 mg/l), the antimicrobial peptides were tested in TSB broths in combination with biocides at concentrations below or close to their MIC as follows: BC, 1.25, 12.5, 125 mg/l; CT, 2.5, 25, 250 mg/l; HDP, 10, 25, 250 mg/l; TC, 1, 2, 2.5 mg/l; CF, 2.5, 25, 250 mg/l; CH, 25, 250, 500 mg/l; PHMG, 0.016, 0.16, 1.6 mg/l; P3-oxonia, 0.025, 0.1, 0.25%, v/v. Bacterial growth in the bacteriocin/biocide containing broths was determined in microtiter plates incubated at 30 °C as described above.

2.4. Determination of bactericidal effects on sessile Salmonella

Bacterial suspensions of *Salmonella* strains (ca. 1.0×10^7 CFU/ml) prepared in diluted TSB broth (6.0 g/l) were distributed (200 μl/well) on 96-well polystyrene microtiter plates (Becton Dickinson Labware). The plates were incubated at 30 °C for 24 h to allow biofilm formation. 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. Aqueous solutions containing polymyxin B (2 mg/l), enterocin AS-48 (25 or 50 mg/l), biocides (which were tested at three different concentrations in a range of 5–20 g/l. depending on each biocide), or their 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 Dey/Engley 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 rigorously for 30 s. Removal of biofilm cells was confirmed by the crystal violet staining method described by Djordjevic, Wiedmann, and McLandsborough (2002). For each treatment, samples from two wells were pooled together and vortexed, followed by serial dilution in sterile saline solution and 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 expressed as $\log_{10}$ CFU/ml.

2.5. Statistical analysis

All experiments were carried out in duplicate (two experiments 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. Inhibition of planktonic salmonellae by enterocin AS-48 in combination with polymyxin B

The inhibitory effect of enterocin AS-48 singly or in combination with polymyxin B was studied on *S. enterica* UJ3197 inoculated in TSB. The bacteriocin had no inhibitory effect on bacterial growth at concentrations of 50 mg/l, and only a weak inhibition was observed at 100 mg/l (Fig. 1). Polymyxin B had strong inhibitory effects at concentrations of 5 or 10 mg/l (data not shown). At 2 mg/l, polymyxin B significantly inhibited growth of *S. enterica* UJ3197 for the first 10 h of incubation, but not in the subsequent hours (Fig. 1). For that reason, a concentration of 2 mg/l polymyxin B was selected for combined tests with enterocin AS-48. The combination of 50 mg/l AS-48 and polymyxin B resulted in growth inhibition that was significantly higher ($p < 0.05$) at 24 h compared to polymyxin B alone, while the combination of 100 mg/l enterocin AS-48 and polymyxin B resulted in complete inhibition of growth (Fig. 1).

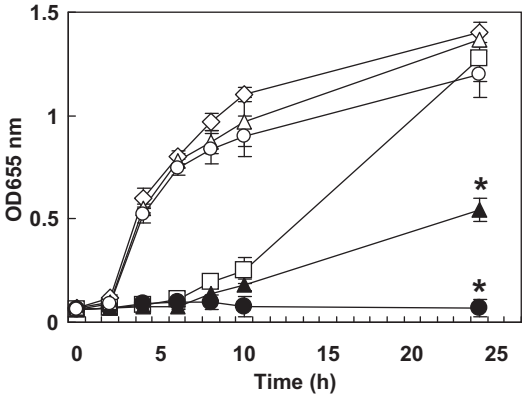

Fig. 1. Effect of enterocin AS-48 and polymyxin B singly or in combination on growth of *Salmonella enterica* UJ3197. Enterocin AS-48 was tested at 50 mg/l (△) or 100 mg/l (○) singly (open symbols) or in combination (closed symbols) with polymyxin B (2 mg/l). Polymyxin B added singly (□). Control cultures (◇). Asterisks denote statistically significant ($p < 0.05$) growth inhibition at 24 h.

3.2. Inhibition of planktonic salmonellae by combinations of enterocin AS-48, polymyxin B and biocides

Enterocin AS-48 (12.5 or 25 mg/l) and polymyxin B (2 mg/l) were tested against planktonic *S. enterica* UJ3197 in TSB in combination with different biocides. Polymyxin B (without enterocin AS-48) only improved growth inhibition significantly ($p < 0.05$) for the biocide cetrimide at 25 mg/l. For the combinations containing polymyxin B, enterocin AS-48 and biocide, growth inhibition was observed for all QAC (Fig. 2). For BC, greatest growth inhibition was obtained at 12.5 mg/l biocide in combination with polymyxin B and 25 mg/l enterocin AS-48, but it was not statistically significant ($p > 0.05$) compared to the single biocide treatment (Fig. 2A). For CT at 25 mg/l, significant growth inhibition ($p < 0.05$) was observed for all the combinations tested (polymyxin B and its combinations with enterocin AS-48), while at a ten-fold lower biocide concentration a significant growth inhibition ($p < 0.05$) was observed in combination with polymyxin B and 25 mg/l enterocin AS-48 (Fig. 2B). In the case of HDP, significant growth inhibition ($p < 0.05$) was observed only for the combinations of polymyxin B and enterocin AS-48 at 12.5 or 25 mg/l (Fig. 2C).

Growth inhibition of *S. enterica* UJ3197 by bis-phenols did not increase remarkably in combination with polymyxin B and enterocin AS-48 (Fig. 2D, E). A partial, non-significant ($p > 0.05$) growth inhibition was observed for 2 mg/l TC in combination with polymyxin B and 25 mg/l enterocin AS-48 compared to the single biocide addition (Fig. 2D). The combinations of polymyxin B and enterocin AS-48 with CF did not enhance growth inhibition significantly ($p > 0.05$), and it could also be observed that this biocide required ten-fold higher concentrations for inhibition of *S. enterica* UJ3197 when applied singly compared with TC (Fig. 2E).

Growth inhibition by chlorhexidine at 500 mg/l increased significantly ($p < 0.05$) for the combinations of polymyxin B and enterocin AS-48 at concentrations of 12.5 and 25 mg/l (Fig. 2F). Growth inhibition by the polyguanine PHMG was enhanced significantly ($p < 0.05$) only by combination with polymyxin B and 25 mg/l enterocin AS-48, both at 0.16 and 1.6 mg/l biocide (Fig. 2G). The inhibitory effect of the commercial solution P3-oxonia was enhanced non-significantly ($p > 0.05$) in the combinations of biocide (0.025% or 0.1%) and polymyxin B plus 25 mg/l enterocin AS-48 (Fig. 2H).

3.3. Inactivation of sessile Salmonella by enterocin AS-48 and polymyxin B in combination with biocides

Enterocin AS-48 (25 or 50 mg/l) was tested singly or in combination with polymyxin B (2 mg/l) and biocides on biofilms formed by *S. enterica* strains S62, S64, UJ3197 and UJ3198. Without biocides, neither polymyxin B, enterocin AS-48 nor the combinations of polymyxin B and enterocin AS-48 had any bactericidal effect on any of the *Salmonella* strains tested (Fig. 3A). The single treatment with benzalkonium chloride (10 g/l) reduced viable counts by 2.8–3.9 log cycles, depending on strain. Polymyxin B and BC did not reduce significantly the numbers of viable cells for any of the strains, but the combination of BC and enterocin AS-48 (50 mg/l) significantly reduced the numbers of viable cells for strains S62 and UJ3198 (Fig. 3B). The combination of BC, polymyxin B and 25 mg/l AS-48 significantly reduced the number of viable cells for strains S62 and S64 compared to the single biocide treatment, while the combination of BC, polymyxin B and 50 mg/l AS-48 significantly

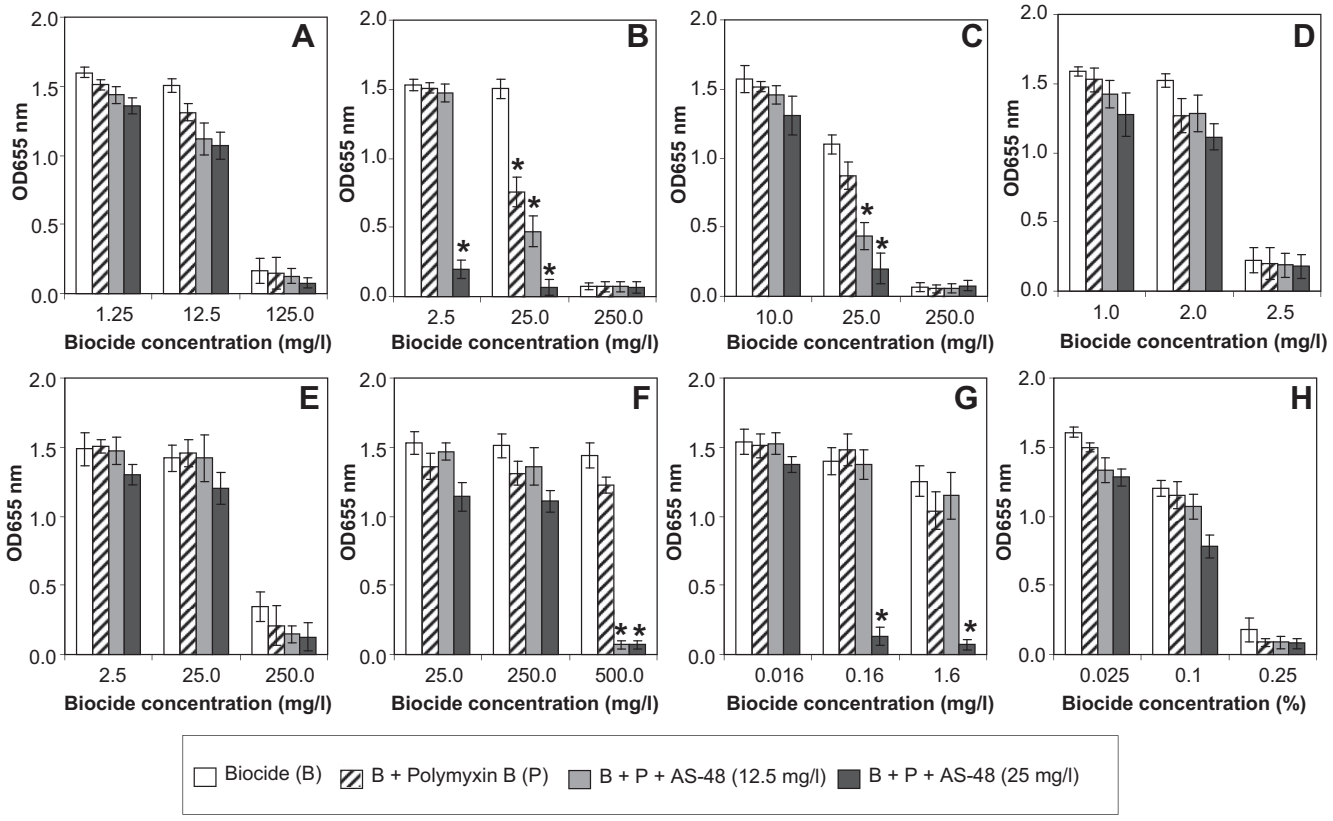

Fig. 2. Effect biocides benzalkonium chloride (A), cetrimide (B), hexadecylpyridinium chloride (C), triclosan (D), hexachlorophene (E), chlorhexidine (F), poly-(hexamethylen guanidinium) hydrochloride (G), and P3-oxonia commercial sanitizer (H) on growth of *Salmonella enterica* UJ3197, singly or in combinations with polymyxin B (2 mg/l) or polymyxin B plus enterocin AS-48 (added at 12.5 or 25 mg/l). Asterisks denote statistically significant ($p < 0.05$) growth inhibition compared to the single biocide treatment.

reduced the concentration of survivors in biofilms for all strains. Cetrimide (20 g/l) reduced viable counts by 1.6–3.8 log cycles (Fig. 3C). The efficacy of treatments with CT increased significantly for 50 mg/l enterocin AS-48 only in strains S62 and UJ3197 (Fig. 3C). Significant reductions of viability ($p < 0.05$) were observed for CT in combination with AS-48 (25 as well as 50 mg/l) for all the strains. Hexadecylpyridinium chloride (20 g/l) reduced viable counts by 2.6–4.2 log cycles (Fig. 3D). The activity of HDP was potentiated significantly ($p < 0.05$) only for the combinations including polymyxin B and AS-48 and only for strain 64 (Fig. 3D).

Triclosan (5 g/l) reduced viable counts of sessile salmonellae by 3.7–5.2 log cycles (depending on strain) when tested singly (Fig. 4A). The effect of treatment with TC improved significantly ($p < 0.05$) in combination with 50 mg/l AS-48 for strains S64 and UJ3108 (Fig. 4A). For the combinations of TC with polymyxin B and AS-48, viable counts were reduced significantly for strains S64, UJ3197 and UJ3198 for 25 mg/l bacteriocin, or for all strains in the treatments with 50 mg/l bacteriocin. For strains S62, S64 and UJ3197, no viable salmonellae were detected for most of the combined treatments with biocide, polymyxin B and bacteriocin. Hexachlorophene (20 g/l) was in general less effective than TC against sessile salmonellae (Fig. 4B), reducing viable count by 1.4–2.7 log cycles. The activity of CF was potentiated significantly ($p < 0.05$) in combination with 50 mg/l AS-48 for strains S64 and UJ3198. The combination of CF, polymyxin B and 25 mg/l AS-48 reduced viable counts significantly ($p < 0.05$) for three strains (S62, S64, UJ3198), and increasing the bacteriocin concentration to 50 mg/l reduced viable counts significantly ($p < 0.05$) for all strains.

The single treatment with chlorhexidine (20 g/l) reduced viable counts of sessile salmonellae by 1.9–3.3 log cycles (Fig. 5A). The activity of chlorhexidine on sessile salmonellae was potentiated by enterocin AS-48 only in strains S64 and UJ3197 (Fig. 5A). The differences in strain sensitivity to the combined treatments including chlorhexidine, polymyxin B and enterocin AS-48 were remarkable, with significant reductions of viable counts for the two bacteriocin concentrations tested in strains S64, UJ3197 and UJ3198, while strain S62 was significantly inactivated only for the highest bacteriocin concentration in the combined treatments compared to the single biocide treatment. In contrast, strain S64 seemed to be the most sensitive, since no viable salmonellae were detected for the highest bacteriocin concentration tested. Poly-(hexamethylen guanidinium) hydrochloride (16 g/l) reduced viable counts by 1.8–2.6 cycles when tested singly (Fig. 5B). The activity of PHMG was only potentiated by combinations including polymyxin B and AS-48 (Fig. 5B). The observed reductions of viable counts in sessile salmonellae were statistically significant ($p < 0.05$) in most strains (except for 25 mg/l AS-48 in strain UJ3197). Inactivation of salmonellae by the commercial sanitizer P3-oxonia tested at 2% (2.2–3.4 log reductions) increased significantly ($p < 0.05$) only for 50 mg/ml bacteriocin in the case of strain UJ3198 (Fig. 5C). All the combined treatments including P3-oxonia, polymyxin B and enterocin AS-48 reduced viable counts of strain 3198 significantly, but only the treatments including 50 mg/l bacteriocin reduced viable counts significantly for the rest of the strains.

4. Discussion

In a previous study, we showed that enterocin AS-48 could improve the activity of biocides against planktonic as well as sessile *L. monocytogenes* cells (Caballero Gómez et al., 2012). Therefore, it would be interesting to determine if similar effects could be demonstrated on *S. enterica*. Being a Gram-negative bacterium,

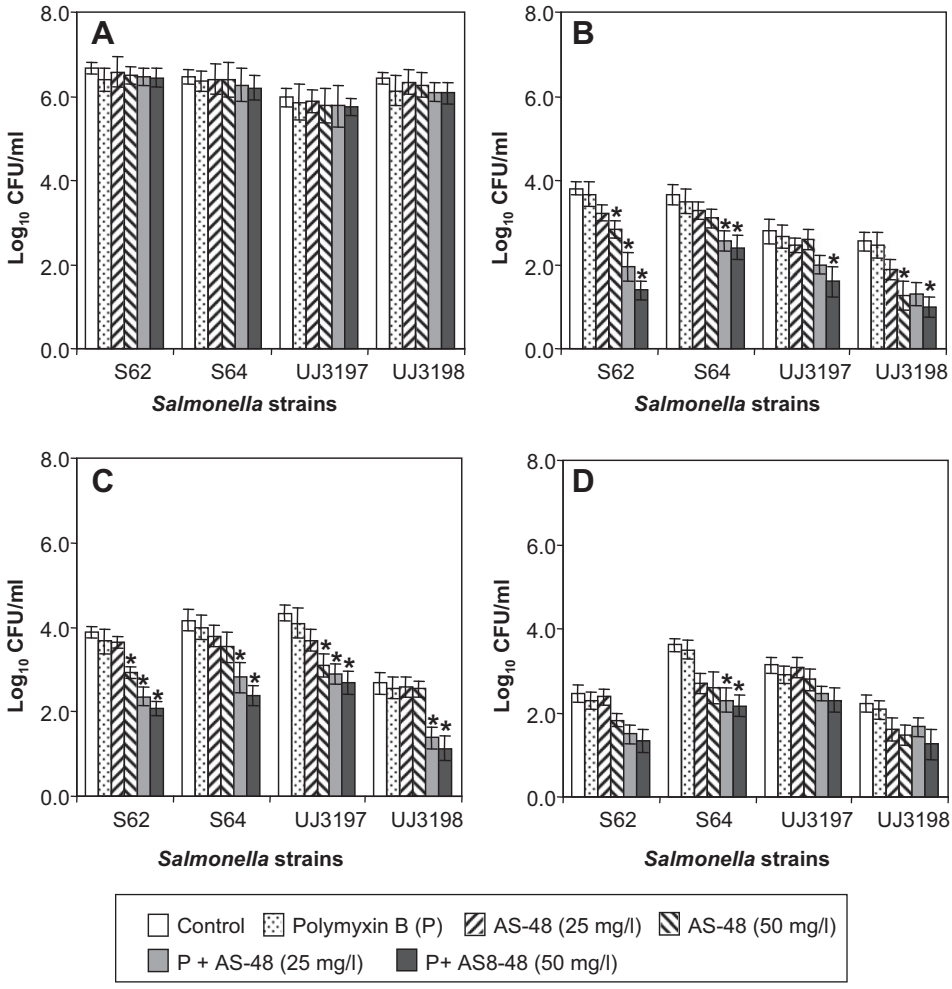

Fig. 3. Effect of polymyxin B (2 mg/l), enterocin AS-48 (25 or 50 mg/l) or both without biocides (A) or in combination with 10 g/l benzalkonium chloride (B), 20 g/l cetrimide (C) and 20 g/l hexadecylpyridinium chloride (D) against sessile *Salmonella enterica* strains S62, S64, UJ3197 and UJ3198. Asterisks denote statistically significant ($p < 0.05$) inactivation of salmonellae compared to the single biocide treatment.

S. enterica shows an increased resistance to bacteriocins (Gálvez, Abriouel, Lucas-López, & Ben Omar, 2007). Enterocin AS-48 acts on the bacterial cytoplasmic membrane, and needs to overcome the outer membrane permeability barrier in order to reach its lethal target (Gálvez, Maqueda, Martínez-Bueno, & Valdivia, 1991). The natural resistance of *S. enterica* to enterocin AS-48 can be overcome by outer membrane-permeabilizing treatments (such as EDTA or heat) (Abriouel, Valdivia, Gálvez, & Maqueda, 1998) but also by treatment with other chemicals such as trisodium phosphate, lactic acid, peracetic acid, polyphosphoric acid, *p*-hydobenzoic acid or 2-nitro-propanol (Cobo Molinos et al., 2008, 2009) or by high-intensity pulsed-electric field treatments (Martínez Viedma et al., 2008). The present study showed that *S. enterica* treated with subinhibitory concentrations of polymyxin B also became sensitive to low concentrations of enterocin AS-48. This synergy could be explained by the detergent-like action of polymyxins on the bacterial outer membrane, and opens new possibilities for application of enterocin AS-48 in combination with polymyxins similar to previous reports on polymyxin E and nisin or pediocin PA-1 (Naghmouchi et al., 2011).

Since biocides are widely used in sanitation of food-related environments such as the surfaces of industrial equipments in contact with foods (Holah, 2000; Jones, Jampani, Newman, & Lee, 2000), it would be interesting to see if the activity of biocides on *S. enterica* could be potentiated by enterocin AS-48 in combination with polymyxin B. While a subinhibitory concentration of polymyxin B improved the inhibitory activity of only one biocide on planktonic salmonellae, a subinhibitory combination of polymyxin B (2 mg/l) and enterocin AS-48 (25 mg/l) improved remarkably the inhibitory effects of four of the biocides tested (cetrimide, hexadecylpyridinium chloride, chlorhexidine and poly-(hexamethylen guanidinium) hydrochloride) and it also caused a partial growth inhibition in combination with the rest of biocides. These results may be of interest in the development of biocide formulations with improved activity on salmonellae and reduced biocide content.

Biocides are important in sanitizing food-contact surfaces both in the home and in food-processing plants, where bacteria tend to persist embedded in biofilms (Kusumaningrum, Riboldi, Hazeleger, & Beumer, 2003; Kusumaningrum, Paltinaite, et al., 2003). Biofilms are surface-associated, three-dimensional multicellular structures whose integrity depends upon the extracellular matrix produced by their constituent bacterial cells (Branda, Vik, Friedman, & Kolter, 2005; Jefferson, 2004). Biofilm formation is one of the main problems for disinfection in the food industry and in other industrial and health sectors as well (Chia, Goulter, McMeekin, Dykes, & Fegan, 2009; Jun et al., 2010). Numerous studies have documented the ability of *Salmonella* spp. to adhere and form biofilms on surfaces such as plastic, cement, glass, and stainless steel (Dhir & Todd, 1995; Joseph et al., 2001) and on food surfaces as well (Lapidot et al., 2006; Iturriaga et al., 2007; Kroupitski, Pinto, Brandl,

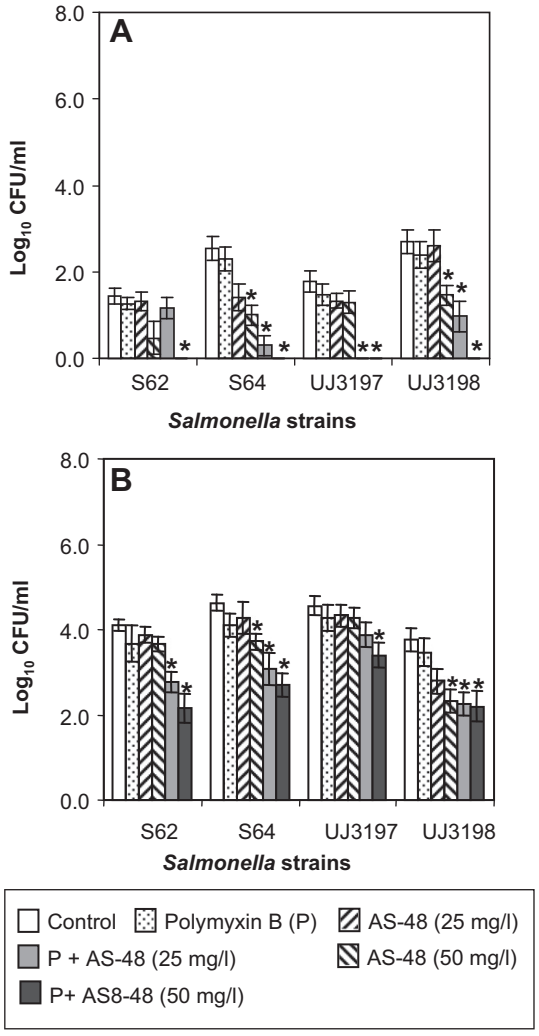

Fig. 4. Effect of polymyxin B (2 mg/l), enterocin AS-48 (25 or 50 mg/l) or both in combination with 5 g/l triclosan (A) or 20 g/l hexachlorophene (B) against sessile *Salmonella enterica* strains S62, S64, UJ3197 and UJ3198. Asterisks denote statistically significant ($p < 0.05$) inactivation of salmonellae compared to the single biocide treatment.

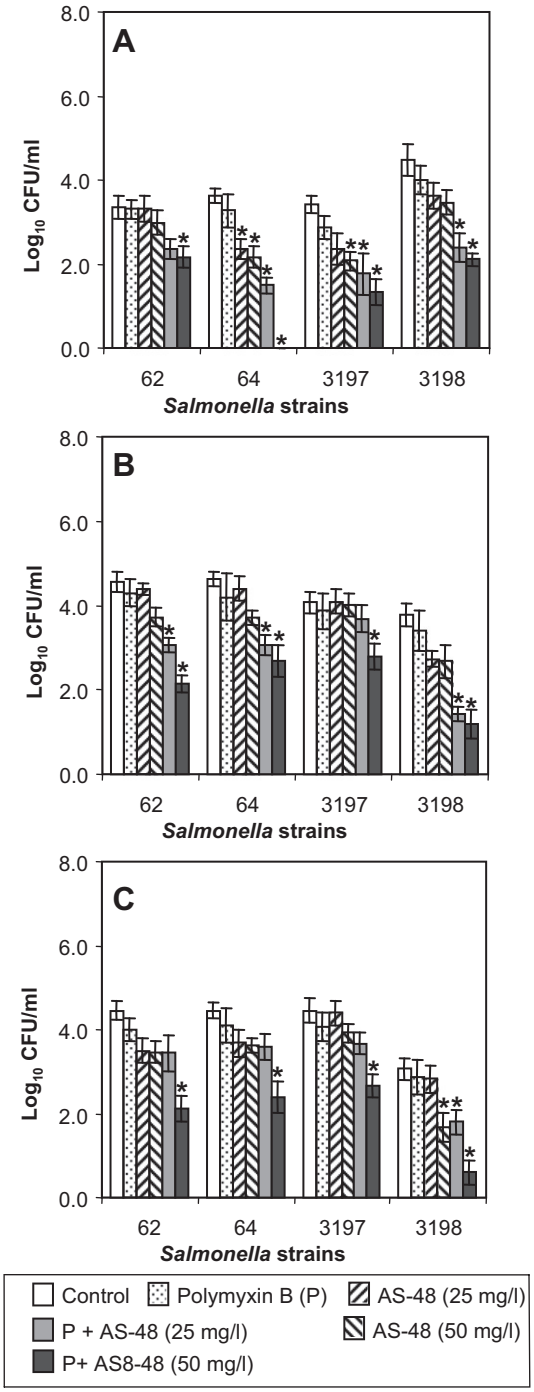

Fig. 5. Effect of polymyxin B (2 mg/l), enterocin AS-48 (25 or 50 mg/l) or both in combination with 20 g/l chlorhexidine (A), 16 g/l poly-(hexamethylen guanidinium) hydrochloride (B) or with 2.0% (v/v) of the sanitizer P3-oxonia (C) against sessile *Salmonella enterica* strains S62, S64, UJ3197 and UJ3198. Asterisks denote statistically significant ($p < 0.05$) inactivation of salmonellae compared to the single biocide treatment.

Belausov, & Sela, 2009; Patel & Sharma, 2010; Vestby et al., 2009). *S. enterica* forms biofilms that are relatively resistant to chemical sanitizing treatments. A previous study reported an MIC value of 0.5 mg/l for triclosan against planktonic *S. enterica* serovar Typhimurium (Tabak et al., 2007), which is slightly lower than the 2.5 mg/l value obtained in the present study. Nevertheless, at 1 g/l it only achieved a one-log reduction of viable counts when tested on sessile *Salmonella* (Tabak et al., 2007). The tolerance of *Salmonella* towards triclosan in the biofilm was attributed to low diffusion through the extracellular matrix, but also to the induced expression of genes involved in reduced influx of triclosan, increased efflux of the compound and enhanced exopolysaccharides production. In the present study, treatment with 5 g/l triclosan failed to completely inactivate sessile salmonellae, while the combinations with polymyxin B and enterocin AS-48 did. TC was also the biocide with greater bactericidal effects on sessile salmonellae when applied singly (5 g/l compared to 20 g/l for the rest of biocides). Triclosan blocks lipid biosynthesis by specifically inhibiting the enzyme enoyl-acyl carrier protein reductase (Levy et al., 1999), resulting in multiple secondary effects derived from alterations in lipid and phospholipid metabolism (Schweizer, 2001). A synergistic effect between triclosan and enterocin AS-48 would be expected from the mechanism of action of this bacteriocin, which interacts with the phospholipids in the bacterial cytoplasmic membrane (Gálvez et al., 1991).

Remarkably, results from the present study indicated that sessile *S. enterica* cells were much more tolerant to biocides than their planktonic cells, and, with the exception of triclosan, biocide concentrations up to 20 g/l failed to reduce viable populations

below 2.5–4.5 log. In addition, biocide tolerance in the sessile salmonellae was observed for all four strains tested, with slight differences in sensitivity between strains. The concentrations tested in our study are much higher than those commonly used in the industry. For example, benzalkonium chloride, chlorhexidine digluconate, and polyhexamethylenebiguanide formulations are used for sanitizing the surfaces of utensils and instruments in the food industry at concentrations of 0.05–0.2%, 0.01–0.02%, and 0.1–0.2% as the active ingredient, respectively, while hypochlorite solutions are usually used to a maximum of 200 mg/l chlorine (Ueda & Kuwabara, 2007). However, tolerance to commonly used biocide concentrations among sessile salmonellae has also been reported previously (Moretro et al., 2009; Ueda & Kuwabara, 2007). By using commercial disinfectant solutions, Moretro et al. (2009) reported that exposure to acidic peroxygen-based disinfectants resulted in complete reduction (>4 log) of the number of *Salmonella* on stainless steel coupons while in general, disinfectants based on cationic tensides, glutaraldehyde or hypochlorite did not show sufficient bactericidal effect on salmonellae. In another study, at the recommended user concentrations, only sodium hypochlorite showed 100% reduction in viable *Salmonella* cells in biofilms (Wong et al., 2010). Benzalkonium chloride and chlorhexidine gluconate were the least effective against biofilms, followed by quaternary ammonium compound which only showed 100% reduction in viable cells from 5-day-old biofilms. While many studies indicate that salmonellae from food environments are sensitive to disinfectants in their planktonic state (Davison et al., 2003; Gradel, Randall, Sayers, & Davies, 2005; Moretro, Midtgaard, Nesse, & Langsrud, 2003), more work is needed on biocide tolerance in the sessile state. Following disinfection, biofilms containing surviving salmonellae may act as reservoirs for contamination of foods and other items either by direct contact with the contaminated surfaces or by the release of planktonic bacteria that may spread away from the biofilm.

There is a need to improve the efficacy of biocides in the food industry to completely solve the problems of biofilm formation. At the same time, there is a need to reduce the negative impact of biocides and detergents such as toxicity, corrosive effects, ease of removal and the subsequent sensory value effects on the final products (Moretro et al., 2009). The use of biosolutions containing enzymes, phages, interspecies competitions, microbially-derived antimicrobial compounds (Simões, Simões, & Vieira, 2010), essential oils (Valeriano et al., 2012) and natural plant molecules (Chorianopoulos, Giaouris, Skandamis, Haroutounian, & Nychas, 2008) has been proposed as an alternative to biocides in the food industry. Within this context, enterocin AS-48 could improve the efficacy of biocides either directly, as in the case of *L. monocytogenes* (Caballero Gómez et al., 2012) or together with polymyxin B or another outer membrane-permeabilizing agent for inactivation of Gram-negative bacteria.

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References

Abriouel, H., Lucas, R., Ben Omar, N., Valdivia, E., & Gálvez, A. (2010). Potential applications of the cyclic peptide enterocin AS-48 in the preservation of vegetable foods and beverages. *Probiotic and Antimicrobial Proteins, 2*, 77–89.

Abriouel, H., Valdivia, E., Gálvez, A., & Maqueda, M. (1998). Response of *Salmonella choleraesuis* LT2 spheroplasts and permeabilized cells to the action of the bacteriocin AS-48. *Applied and Environmental Microbiology, 64*, 4623–4626.

Abriouel, H., Valdivia, E., Martínez-Bueno, M., Maqueda, M., & Gálvez, A. (2003). Method for semi-preparative-scale production and recovery of enterocin AS-48 derived from *Enterococcus faecalis* subsp. *liquefaciens* A-48-32. *Journal of Microbiological Methods, 55*, 599–605.

Ammor, S., Tauveron, G., Dufour, E., & Chevallier, I. (2006). Antibacterial activity of lactic acid bacteria against spoilage and pathogenic bacteria isolated from the same meat small-scale facility: 2—behaviour of pathogenic and spoilage bacteria in dual species biofilms including a bacteriocin-like-producing lactic acid bacteria. *Food Control, 17*, 462–468.

Bower, C. K., McGuire, J., & Daeschel, M. A. (1995). Suppression of *Listeria monocytogenes* colonization following adsorption of nisin onto silica surfaces. *Applied and Environmental Microbiology, 61*, 992–997.

Branda, S. S., Vik, A., Friedman, L., & Kolter, R. (2005). Biofilms: the matrix revisited. *Trends in Microbiology, 13*, 20–26.

Caballero Gómez, N., Abriouel, H., Grande, M. A., Pérez Pulido, R., & Gálvez, A. (2012). Effect of enterocin AS-48 in combination with biocides on planktonic and sessile *Listeria monocytogenes*. *Food Microbiology, 30*, 51–58.

Chorianopoulos, N. G., Giaouris, E. D., Skandamis, P. N., Haroutounian, S. A., & Nychas, G. J. E. (2008). Disinfectant test against monoculture and mixed-culture biofilms composed of technological, spoilage and pathogenic bacteria: bactericidal effect of essential oil and hydrosol of *Satureja thymbra* and comparison with standard acid-base sanitizers. *Journal of Applied Microbiology, 104*, 1586–1596.

Chia, T. W., Goulter, R. M., McMeekin, T., Dykes, G. A., & Fegan, N. (2009). Attachment of different *Salmonella* serovars to materials commonly used in a poultry processing plant. *Food Microbiology, 26*, 853–859.

Cobo Molinos, A., Abriouel, H., Lucas, R., Valdivia, E., Ben Omar, N., & Gálvez, A. (2008). Combined physico-chemical treatments based on enterocin AS-48 for inactivation of Gram-negative bacteria in soybean sprouts. *Food and Chemical Toxicology, 46*, 2912–2921.

Cobo Molinos, A., Lucas, R., Abriouel, H., Ben Omar, N., Valdivia, E., & Gálvez, A. (2009). Inhibition of *Salmonella enterica* cells in deli-type salad by enterocin AS-48 in combination with other antimicrobials. *Probiotics and Antimicrobial Proteins, 1*, 85–90.

Collignon, S., & Korsten, L. (2010). Attachment and colonization by *Escherichia coli* O157:H7, *Listeria monocytogenes, Salmonella enterica* subsp. *enterica* serovar Typhimurium, and *Staphylococcus aureus* on stone fruit surfaces and survival through a simulated commercial export chain. *Journal of Food Protection, 73*, 1247–1256.

Davison, S., Benson, C. E., Munro, D. S., Rankin, S. C., Ziegler, A. E., & Eckroade, R. J. (2003). The role of disinfectant resistance of *Salmonella enterica* serotype enteritidis in recurring infections in Pennsylvania egg quality assurance program monitored flocks. *Avian Diseases, 47*, 143–148.

Dhir, V. K., & Todd, C. E. R. (1995). Susceptibility of suspended and surface-attached *Salmonella enteritidis* to biocides and elevated temperatures. *Applied and Environmental Microbiology, 61*, 1731–1738.

Djordjevic, D., Wiedmann, M., & McLandsborough, L. A. (2002). Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. *Applied and Environmental Microbiology, 68*, 2950–2958.

EFSA. (2010). The community summary report on trends and sources of zoonoses and zoonotic agents and food-borne outbreaks in the European Union in 2008. *The EFSA Journal*, 1946.

Gálvez, A., Abriouel, H., Lucas-López, R., & Ben Omar, N. (2007). Bacteriocin-based strategies for food biopreservation. *International Journal of Food Microbiology, 120*, 51–70.

Gálvez, A., Maqueda, M., Martínez-Bueno, M., & Valdivia, E. (1991). Permeation of bacterial cells, permeation of cytoplasmic and artificial membrane vesicles, and channel formation on bilayers by peptide antibiotic AS-48. *Journal of Bacteriology, 173*, 886–892.

Gilbert, P., Das, J. R., Jones, M. V., & Allison, D. G. (2001). Assessment of resistance towards biocides following the attachment of microorganisms to, and growth on, surfaces. *Journal of Applied Microbiology, 91*, 248–254.

Gilbert, P., McBain, A. J., & Rickard, A. H. (2003). Formation of microbial biofilm in hygienic situations: a problem of control. *International Biodeterioration and Biodegradation, 51*, 245–248.

Gradel, K. O., Randall, L., Sayers, A. R., & Davies, R. H. (2005). Possible associations between *Salmonella* persistence in poultry houses and resistance to commonly used disinfectants and a putative role of *mar*. *Veterinary Microbiology, 107*, 127–138.

Guerrieri, E., de Niederhäusern, S., Messi, P., Sabia, C., Iseppi, R., Anacarso, I., et al. (2009). Use of lactic acid bacteria (LAB) biofilms for the control of *Listeria monocytogenes* in a small-scale model. *Food Control, 20*, 861–865.

Hall-Stoodley, L., Costerton, J. W., & Stoodley, P. (2004). Bacterial biofilms: from the natural environment to infectious diseases. *Nature Reviews Microbiology, 2*, 95–108.

Holah, J. T. (2000). Cleaning and disinfection. In C. Dennis, & M. F. Stringer (Eds.), *Chilled foods: A comprehensive guide* (pp. 397–428). Cambridge: Woodhead Publishing.

Horton, J., & Pankey, M. D. (1982). Polymyxin B, colistin, and sodium colistimethate. *Medical Clinics of North America, 66*, 135–142.

Iturriaga, M. H., Tamplin, M. L., & Escartín, E. F. (2007). Colonization of tomatoes by *Salmonella* Montevideo is affected by relative humidity and storage temperature. *Journal of Food Protection, 70*, 30–34.

Jefferson, K. K. (2004). What drives bacteria to produce a biofilm? *FEMS Microbiology Letters, 236*, 163–173.
Jessen, B., & Lammert, L. (2003). Biofilm and disinfection in meat processing plants. *International Biodeterioration and Biodegradation, 51*, 265–269.
Jones, R. D., Jampani, H. B., Newman, J. L., & Lee, A. S. (2000). Triclosan: a review of effectiveness and safety in health care settings. *American Journal of Infection Control, 28*, 184–196.
Joseph, B., Otta, S. K., Karunasagar, I., & Karunasagar, I. (2001). Biofilm formation by *Salmonella* spp. on food contact surfaces and their sensitivity to sanitizers. *International Journal of Food Microbiology, 64*, 367–372.
Jun, W., Kim, M. S., Cho, B., Millner, P. D., Chao, K., & Chan, D. E. (2010). Microbial biofilm detection on food contact surfaces by macro-scale fluorescence imaging. *Journal of Food Engineering, 99*, 314–322.
Kroupitski, Y., Pinto, R., Brandl, M. T., Belausov, E., & Sela, S. (2009). Interactions of *Salmonella enterica* with lettuce leaves. *Journal of Applied Microbiology, 106*, 1876–1885.
Kumar, S., Parvathi, A., George, J., Krohne, G., & Karunasagar, I. (2009). A study on the effects of some laboratory-derived genetic mutations on biofilm formation by *Listeria monocytogenes*. *World Journal of Microbiology and Biotechnology, 25*, 527–531.
Kusumaningrum, H. D., Paltinaite, R., Koomen, A. J., Hazeleger, W. C., Rombouts, F. M., & Beumer, R. R. (2003). Tolerance of *Salmonella* Enteritidis and *Staphylococcus aureus* to surface cleaning and household bleach. *Journal of Food Protection, 66*, 2289–2295.
Kusumaningrum, H. D., Riboldi, G., Hazeleger, W. C., & Beumer, R. R. (2003). Survival of foodborne pathogens on stainless steel surfaces and cross-contamination to foods. *International Journal of Food Microbiology, 85*, 227–236.
Lapidot, A., Romling, U., & Yaron, S. (2006). Biofilm formation and the survival of *Salmonella* Typhimurium on parsley. *International Journal of Food Microbiology, 109*, 229–233.
Leriche, V., Chassaing, D., & Carpentier, B. (1999). Behaviour of *Listeria monocytogenes* in an artificially made biofilm of a nisin-producing strain of *Lactococcus lactis*. *International Journal of Food Microbiology, 51*, 169–182.
Levy, C. W., Roujeinikova, A., Sedelnikova, S., Baker, J. B., Stuitje, A. R., Slabas, A. R., et al. (1999). Molecular basis for triclosan activity. *Nature, 398*, 383–384.
Lobos, O., Padilla, A., & Padilla, C. (2009). In vitro antimicrobial effect of bacteriocin PsVP-10 in combination with chlorhexidine and triclosan against *Streptococcus mutans* and *Streptococcus sobrinus* strains. *Archives of Oral Biology, 54*, 230–234.
Maqueda, M., Gálvez, A., Martínez-Bueno, M., Sánchez-Barrena, J., González, C., Albert, A., et al. (2004). Peptide AS-48: prototype of a new class of cyclic bacteriocins. *Current Protein and Peptide Science, 5*, 399–416.
Martínez Viedma, P., Sobrino, A., Ben Omar, N., Abriouel, H., Lucas López, R., Valdivia, E., et al. (2008). Enhanced bactericidal effect of enterocin AS-48 in combination with high-intensity pulsed-electric field treatment against *Salmonella enterica* in apple juice. *International Journal of Food Microbiology, 128*, 244–249.
Minei, C. C., Gomes, B. C., Ratti, R. P., D'Angelis, C. E. M., & De Martinis, E. C. P. (2008). Influence of peroxyacetic acid and nisin and coculture with *Enterococcus faecium* on *Listeria monocytogenes* biofilm formation. *Journal of Food Protection, 71*, 634–638.
Moretro, T., Midtgaard, E. S., Nesse, L. L., & Langsrud, S. (2003). Susceptibility of *Salmonella* isolated from fish feed factories to disinfectants and air-drying at surfaces. *Veterinary Microbiology, 94*, 207–217.
Moretro, T., Vestby, L. K., Nesse, L. L., Storheim, S. E., Kotlarz, K., & Langsrud, S. (2009). Evaluation of efficacy of disinfectants against *Salmonella* from the feed industry. *Journal of Applied Microbiology, 106*, 1005–1012.
Morton, L. H. G., Greenway, D. L. A., Gaylarde, C. C., & Surman, S. B. (1998). Consideration of some implications of the resistance of biofilms to biocides. *International Biodeterioration and Biodegradation, 41*, 247–259.
Naghmouchi, K., Belguesmia, Y., Baah, J., Teather, R., & Drider, D. (2011). Antibacterial activity of class I and IIa bacteriocins combined with polymyxin E against resistant variants of *Listeria monocytogenes* and *Escherichia coli*. *Research in Microbiology, 162*, 99–107.
Patel, J., & Sharma, M. (2010). Differences in attachment of *Salmonella enterica* serovars to cabbage and lettuce leaves. *International Journal of Food Microbiology, 139*, 41–47.
Schweizer, H. P. (2001). Triclosan: a widely used biocide and its link to antibiotics. *FEMS Microbiology Letters, 202*, 1–7.
Simões, M., Simões, L. C., & Vieira, M. J. (2010). A review of current and emergent biofilm control strategies. *LWT – Food Science and Technology, 43*, 573–583.
Tabak, M., Scher, K., Hartog, E., Romling, U., Matthews, K. R., Chikindas, M. L., et al. (2007). Effect of triclosan on *Salmonella typhimurium* at different growth stages and in biofilms. *FEMS Microbiology Letters, 267*, 200–206.
Ueda, S., & Kuwabara, Y. (2007). Susceptibility of biofilm *Escherichia coli, Salmonella* Enteritidis and *Staphylococcus aureus* to detergents and sanitizers. *Biocontrol Science, 12*, 149–153.
Valeriano, C., Oliveira, T. L. C., Carvalho, S. M., Cardoso, M. G., Alves, E., & Piccoli, R. H. (2012). The sanitizing action of essential oil-based solutions against *Salmonella enterica* serotype Enteritidis S64 biofilm formation on AISI 304 stainless steel. *Food Control, 25*, 673–677.
Vestby, L. K., Moretro, T., Langsrud, S., Heir, E., & Nesse, L. L. (2009). Biofilm forming abilities of *Salmonella* are correlated with persistence in fish meal- and feed factories. *BMC Veterinary Research, 27*, 5–20.
Winkelströter, L. K., Gomes, B. C., Thomaz, M. R. S., Souza, V. M., & De Martinis, E. C. P. (2011). *Lactobacillus sakei* 1 and its bacteriocin influence adhesion of *Listeria monocytogenes* on stainless steel surface. *Food Control, 22*, 1404–1407.
Wong, H. S., Townsend, K. M., Fenwick, S. G., Maker, G., Trengove, R. D., & O'Handley, R. M. (2010). Comparative susceptibility of *Salmonella* Typhimurium biofilms of different ages to disinfectants. *Biofouling, 26*, 859–864.
Zhao, T., Doyle, M. P., & Zhao, P. (2004). Control of *Listeria monocytogenes* in a biofilm by competitive-exclusion microorganisms. *Applied and Environmental Microbiology, 70*, 3996–4003.