

Influence of Organic Material and Biofilms on Disinfectant Efficacy Against *Listeria monocytogenes*

HILDA NYATI^{a,b,*}, RIJKELT BEUMER^a, STIJN VAN DER VEEN^a, WILMA HAZELEGER^a, AND TJAKKO ABEE^a

^aLaboratory of Food Microbiology, Wageningen University, P.O. Box 8129, 6700 EV Wageningen, The Netherlands

^bDepartment of Applied Biology and Biochemistry, National University of Science and Technology, P.O. Box AC939, Ascot, Bulawayo, Zimbabwe

*hnyati@hotmail.com

TEL: +263 9 282842

FAX: +263 9 286 390

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Abstract

The effects of organic material and biofilm formation on the efficacy of Suma Tab D4 chlorine tablets and Suma Bac D10 quaternary ammonium compound (QAC) against *Listeria monocytogenes* was determined in suspension and on stainless steel and polystyrene surfaces according to standard disinfectant test methodology. Exposure to 200 and 740 mg L⁻¹ QAC and to 150 mg L⁻¹ active chlorine resulted in a > 5.0 log₁₀ CFU mL⁻¹ and > 5.0 log₁₀ CFU/coupon reduction of six *L. monocytogenes* strains within one minute, in suspension tests, and on stainless steel surfaces, respectively. Additionally, there was a reduction by as much as 5 log₁₀ CFU/coupon or 5 log₁₀ CFU/well of reference strains EGDe and Scott A biofilms within five minutes on stainless steel and polystyrene surfaces. Organic material, added as bovine serum albumin at 0.3% (w/v) completely prevented the inactivation of *L. monocytogenes* in 150 mg L⁻¹ chlorine, while reductions of only 0.6 ± 0.1 log₁₀ CFU mL⁻¹ were recorded in the presence of UHT milk at 3% (v/v). In contrast, reductions of ≥ 5 log₁₀ CFU mL⁻¹ were recorded within one minute on exposure to 740 mg L⁻¹ QAC in the presence of 0.3% (w/v) bovine serum albumin and within two minutes in the presence of 20 % (v/v) UHT milk. Although Suma D4 chlorine tablets and Suma Bac D10 QAC are effective listericidal agents at recommended concentrations, Suma Tab D4 chlorine efficacy against *L. monocytogenes* is impaired by the presence of low concentrations of organic material, while Suma Bac D10 QAC maintains its listericidal activity in high organic loads.

Keywords: *Listeria monocytogenes*, chlorine, quaternary ammonium compounds, organic residues, biofilms

1 Introduction

The presence of hazardous microorganisms such as *Listeria monocytogenes* on food-processing equipment emphasises the significance of proper cleaning and sanitizing practices as an aid in preventing contamination of processed foods. To be deemed effective, sanitizing agents must re-

duce a given test organism by at least 5 log₁₀ CFU mL⁻¹ or coupon within 5 minutes in suspension and carrier/surface tests (ECS, 2000; Fraise, 2008). However, efficacy is dependent on microbial species and practical conditions such as disinfectant concentration, contact time, organic load, water hardness, pH, presence of biofilms,

temperature, and presence of protective agents such as lipids (Gebel, Altena A. Vacata, & Exner, 2005).

The sanitizing agents that are active against a wide range of microorganisms include chlorine-based compounds, quaternary ammonium compounds (QACs), acid sanitizers, ozone, and iodophores (Bessems, 1998; Best, Kennedy, & Coates, 1990; Mustapha & Liewen, 1989). Of these, chlorine-based sanitizers and quaternary ammonium compounds are the most commonly used in the food industry. Chlorinated compounds are applied at 50 - 200 mg L⁻¹ available chlorine at 24°C or ambient temperature for at least 2 minutes. The bactericidal activity is attributed to undissociated hypochlorous acid, which diffuses into the bacterium inducing the formation of toxic oxidative species that combine with proteins, leading to the inhibition of oxidative phosphorylation, and damage to nucleic acids and enzymes (Lado & Yousef, 2007; McDonnell & Russell, 1999). The mode of action on the other hand, of QACs, which are applied at concentrations of 200 to 800 mg L⁻¹ for at least two minutes, is attributed to reaction with bacterial carboxylic groups resulting in the permeabilisation of the cytoplasmic membrane, denaturation of essential cell proteins and inactivation of cellular enzymes (Earnshaw & Lawrence, 1998; McDonnell & Russell, 1999).

Lopes (1986), Brakett (1987), Mustapha and Liewen (1989), Best et al. (1990) and Aarnisalo et al. (2000) have demonstrated the effectiveness of sodium hypochlorite against *L. monocytogenes*. A 4 log₁₀ CFU mL⁻¹ reduction in planktonic cells by 200 mg L⁻¹ sodium hypochlorite within one minute was reported by Mustapha and Liewen (1989), while 4 log₁₀ CFU/coupon reductions were recorded after 5 minutes on smooth stainless steel surfaces. In the same study, 50 mg L⁻¹ of a QAC (n-alkyl dimethyl dichlorobenzyl ammonium chloride), resulted in > 4 log₁₀ reductions in vitro and on smooth and pitted stainless steel surfaces within 5 minutes. Similarly, Luppens (2002) reported a 3.9 log₁₀ CFU mL⁻¹ reduction of planktonic exponential phase cells of *L. monocytogenes* after a five minute exposure to 25 mg L⁻¹ of the QAC benzalkonium chloride.

Presence of organic material is one of the most

significant factors influencing the efficacy of disinfectants against microorganisms. Organic material may interfere with the efficacy of sanitization procedures by either inactivating the disinfectant or blocking it from surface contact. Generally, quaternary ammonium compounds perform better than chlorine sanitizers in the presence of organic matter, although their bactericidal efficacy is reduced to a variable degree depending on the type of organic matter, the kind of quaternary ammonium compound involved, as well as the type of microorganism (Bessems, 1998; Gonzalez-Fandos, Sanz, Garcia-Fernandez, & Garcia-Arias, 2005; Kawamura-Sato, Wachino, Kondo, Ito, & Arakawa, 2008).

Biofilm formation also compromises the sanitation of food processing surfaces due to increased microbial resistance, and the subsequent spreading of detached cells to other areas of processing plants. Proposed explanations for the observed resistance in biofilms include low diffusion through the cellular matrix and exopolysaccharides, chemical or enzymatic modification of the antibacterial agent, physiological changes due to slow growth rate and starvation responses, and the induction of attachment specific physiologies (Blackman & Frank, 1996; Lado & Yousef, 2007; Moretro & Langsrud, 2004; Pan, Breidt, & Kathariou, 2006; Trachoo, 2003). The attachment of *L. monocytogenes* to stainless steel, glass, rubber and polystyrene surfaces has been described by Kalmokoff et al. (2001).

In view of the significance of *L. monocytogenes* as a food-borne pathogen, and the dependence of effective sanitation on practical conditions and the selection of appropriate sanitizers, the objectives of this study were to determine the efficacy of recently introduced QAC and chlorine formulations against *L. monocytogenes* in suspension, on stainless steel surfaces, in the presence of organic material, and in biofilms.

2 Experimental

2.1 *L. monocytogenes* strains and culture conditions

A total of six *L. monocytogenes* strains (reference strains EGDe and Scott A, and four food

isolates Li0010, Li0013, Li0019, Li0025) were selected from the culture collection of the Laboratory of Food Microbiology, Wageningen University, for use in disinfectant tests. Stock cultures for each strain were prepared by mixing 700 μL of a 24h Brain Heart Infusion (BHI, BDH) broth culture with 300 μL of 87% glycerol (Sigma), and maintained at -20°C in screw-cap cryovials (Nalge NUNC International, Denmark) for the duration of the study. The cultures were activated before use by transfer to BHI agar (BDH), and incubated at 30°C for 48h. Single colonies were subsequently used for inoculation into BHI broth and incubated at 37°C for 24h.

2.2 Preparation of disinfectants

Suma Bac D10 QAC disinfectant (Johnson Diversey, Utrecht, Netherlands) was used at the recommended concentration of 740 mg L^{-1} of the active compound alkyl dimethyl benzyl ammonium chloride by diluting 10 mL of disinfectant in 1 L of sterile demineralised water. Solutions containing 200 mg L^{-1} QAC were prepared by diluting 2.7 mL of Suma Bac D10 or 0.4 mL of a 50% benzalkonium chloride stock solution (Acros organics, New Jersey) in 1L of sterile demineralized water. Chlorine solutions (150 mg L^{-1}) were prepared in compliance with product usage instructions by dissolving one sodium dichloroisocyanurate tablet (Suma Tab D4, Johnson Diversey) in 10 L of sterile demineralised water. Solutions containing 50 mg L^{-1} chlorine were prepared by further dilution of 150 mg L^{-1} solutions.

2.3 Suspension tests

Initial counts of the test cultures were determined from 1 mL tryptone soya agar (TSA, Oxoid) pour-plates after incubation at 30°C for 48h. Suspension tests were carried out according to the standard EN 13727 disinfectant test protocol (ECS, 2000; Fraise, 2008). For each disinfectant, efficacy against *L. monocytogenes* was determined by adding 0.1 mL of a 24h BHI broth culture to 9.9 mL of a disinfectant solution at ambient temperature, giving an initial range of 10^7 - 10^8 CFU mL^{-1} , while in control samples the disinfectant was replaced by sterile de-mineralized

water. At the end of the required contact time, the disinfectant was inactivated by transferring 1 mL of the culture suspension to 9 mL of neutralizer composed of 0.3% lecithin, 3% Tween 80, 0.5% sodium thiosulphate, 0.1% L-histidine and phosphate buffer (ECS, 2000). Survivors were enumerated from TSA pour plates incubated at 30°C for 48h. All tests were carried out in triplicate.

2.4 Surface tests

For surface tests, 10 μL of a 24h BHI broth culture was placed on each 1.9 cm^2 stainless steel coupon (Type 304, Wageningen University, Netherlands), giving an initial range of 10^6 - 10^7 CFU/coupon, and allowed to air-dry for 1h in a class II biological safety cabinet (Bloomfield et al., 1994). The coupons were then placed in culture plate wells (Greiner bio-one) and covered with 1 mL of disinfectant. After the required contact time, each coupon was transferred aseptically to 10 mL of neutralizer containing about 4.5 g of 1 mm diameter glass beads (Emergo, Landsmeer, Netherlands). Cells were subsequently dislodged from stainless steel surfaces by vortexing for 30 seconds, and survivors enumerated using TSA pour plates. Initial counts on coupons after drying were determined by dislodging cells into 10 mL of neutralized bacteriological peptone (Oxoid) through bead vortexing, and subsequent enumeration in TSA pour plates.

2.5 Bovine serum albumin tests

The bovine serum albumin (BSA) solution used to simulate clean conditions was prepared by dissolving 0.3 g of BSA in 100 mL of demineralised water and sterilized by membrane filtration. A further ten-fold dilution gave a final concentration of 0.03% in disinfectant solutions. In tests that simulated dirty conditions the final concentration of BSA and washed sheep erythrocytes in disinfectants were 0.3% (w/v) and 0.3% (v/v), respectively (ECS, 2000).

2.6 Disinfectant tests in the presence of UHT milk

Whole UHT milk (3% fat content) was added to 50 mg L⁻¹ and 150 mg L⁻¹ Suma Tab D4 chlorine, and to 200 mg L⁻¹ and 740 mg L⁻¹ Suma Bac D10 QAC to give a final concentration of 1 - 50% UHT milk (v/v) in disinfectant solutions before use in suspension and surface tests.

2.7 Disinfectant tests on stainless steel biofilms

L. monocytogenes biofilms on stainless steel surfaces (Figure 1a) were prepared by placing sterile stainless steel coupons (1.9 cm²) in 50 mL centrifuge tubes containing 3 mL of sterile BHI broth. Each tube was inoculated with 30 µL of a 24h BHI broth culture and incubated at 20°C for 48 or 72h. Prior to disinfectant tests, the stainless steel coupons were rinsed three times with sterile demineralized water for the removal of unattached cells. Each coupon was then placed in a culture plate well and covered with 1 mL of disinfectant for 1 to 5 minutes. After the required contact time coupons were transferred to 10 mL of neutralizer containing 4.5 g of 1 mm diameter beads, and the attached cells dislodged by vortexing for 1 minute. Viable cells were enumerated using TSA pour plates.

2.8 Disinfectant tests on polystyrene biofilms

Polystyrene 12-well culture plates (Greiner bio-one, Netherlands) containing 1 mL of BHI broth in each well were inoculated with 10 µL of a 24h BHI broth culture and incubated at 20°C for 48h. Growth medium was discarded from culture wells by inverting the plates, and non-adherent cells removed by washing three times with sterile demineralized water. Disinfectant (1 mL) was added to each well and 3 mL of neutralizer added after 1 or 5 minutes of exposure to the disinfectant. Biofilms (Figure 1b) were then detached from culture plates by pipetting the medium up and down several times before transferring the entire contents of the well to an additional 6 mL

of neutralizer. Viable cells were enumerated after serial dilution using 1 mL TSA pour-plates. The absence of clumps in biofilm suspensions was confirmed by microscopic examination.

3 Results and Discussion

3.1 Effectiveness of chlorine and QACs against *L. monocytogenes* in suspension tests

For all six *L. monocytogenes* strains under study, > 5 log₁₀ CFU mg L⁻¹ reductions in suspended cells were recorded within one minute in 150 mg L⁻¹ chlorine. A 5 log₁₀ reduction was also achieved within one minute in 50 mg L⁻¹ chlorine for five strains, while 4.3 ± 0.2 log₁₀ reductions were recorded for strain Li0019 (Table 1). Similarly, five log reductions in cell suspensions were achieved within one minute in 200 mg L⁻¹ or 740 mg L⁻¹ Suma-Bac D10 QAC and 200 mg L⁻¹ benzalkonium chloride (Table 2). The observations are consistent with reports by Best et al. (1990) and Gonzalez-Fandos et al. (2005) where > 5 log₁₀ CFU mg L⁻¹ reductions in suspension tests were observed within 1-5 minutes at the lowest recommended QAC concentrations.

3.2 Efficacy of chlorine and QACs against *L. monocytogenes* on stainless steel surfaces

Suma Bac D10 QAC was equally effective against *L. monocytogenes* on stainless steel surfaces, resulting in five log₁₀ CFU/coupon reductions within one minute of exposure to 200 mg L⁻¹ and 740 mg L⁻¹ solutions. For Suma Tab D4, although five log₁₀ reductions were recorded within one minute of exposure to 150 mg L⁻¹ chlorine, five minutes was required for a five log kill in 50 mg L⁻¹ chlorine on stainless steel surfaces. The higher resistance to chlorine disinfectants observed in surface tests may be due to the protection of cells in inner layers by those on surface layers or the initiation of stress responses as suggested by Hill, Cotter, Sleator, and Gahan

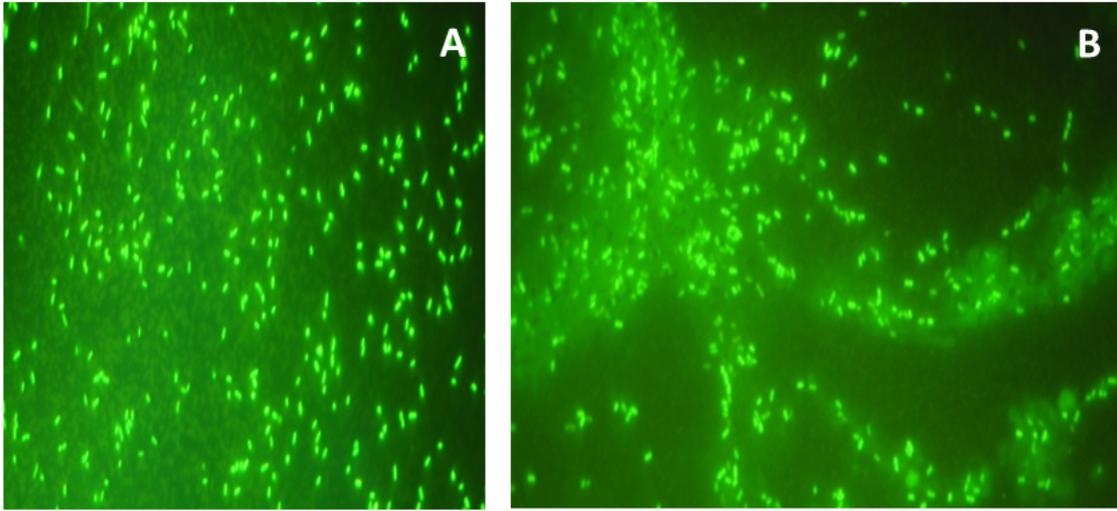


Figure 1: *L. monocytogenes* (EGDe) 48h biofilm on stainless steel (A) and on Polystyrene (B).

Table 1: Efficacy of Suma Tab D4 chlorine against *L. monocytogenes* in suspension tests.*

Strain	Log_{10} reduction (CFU mg L^{-1}) in 1 min	
	50 mg L^{-1} chlorine	150 mg L^{-1} chlorine
EGDe (serotype 1/2a)	>5.0	>5.0
Scott A (serotype 4b)	>6.4	>6.4
Li0010 (from goose liver pate)	>6.4	>6.4
Li0013 (nisin resistant)	5.4 ± 0.3	>6.4
Li0019 (nisin resistant)	4.3 ± 0.2	>6.1
Li0025 (nisin resistant)	>5.6	>5.6

*Results are mean of triplicate samples.

(2002) and Kastbjerg, Nielsen, Arneborg, and Gram (2009).

3.3 Effect of BSA on the efficacy of chlorine and QACs against *L. monocytogenes*

The antimicrobial activity of 150 mg L^{-1} Suma D4 chlorine was inhibited by the presence of 0.3% BSA, resulting in a 0-0.6 log_{10} CFU mg L^{-1} reduction of suspended cells after five minutes, compared to the 5 log reduction within 1 minute in the absence of organic material. This may be explained by the fact that chlorine bactericidal activity is based on its reaction with proteins

and enzymes (Lado & Yousef, 2007; McDonnell & Russell, 1999). It is conceivable that pre-exposure to bovine serum albumin results in chlorine reacting with extraneous protein instead of the microbial targets, thus preventing microbial inactivation. In contrast, the efficacy of 200 mg L^{-1} or 740 mg L^{-1} Suma Bac D10 QAC against *L. monocytogenes* was not impaired by the presence of 0.3% BSA, a concentration that is representative of high organic loads.

Table 2: Efficacy of quaternary ammonium compounds against *L. monocytogenes* in suspension tests.*

Strain	Log_{10} reduction (CFU mg L^{-1}) in 1 min		
	200 mg L^{-1}	740 mg L^{-1}	200 mg L^{-1}
	Suma Bac D10	Suma Bac D10	Benzalkonium chloride
EGDe	>5.0	>5.0	>5.1
Scott A	>6.4	>6.4	>6.6
Li0010	>6.3	>6.3	>6.5
Li0013	>6.5	>6.5	>6.6
Li0019	>6.2	>6.2	>6.5
Li0025	>5.7	>5.7	>6.0

* Results indicated as a mean of triplicate samples

3.4 Effect of UHT milk on Suma Tab D4 and Suma Bac D10 QAC efficacy

The sensitivity of chlorine disinfectants to organic debris was confirmed by the inhibition of 150 mg L^{-1} Suma D4 chlorine in 3% UHT milk, resulting only in a $0.6 \pm 0.2 \log_{10}$ CFU mg L^{-1} reduction of *L. monocytogenes* (EGDe) after a five minute exposure in suspension (Figure 2). Suma Bac D10 QAC (740mg L^{-1}) was less sensitive to milk residues than Suma D4 chlorine, and achieved 5 log reductions in *L. monocytogenes* suspensions within two minutes in 20% UHT milk (Figure 3). The stability of the QAC in the presence of milk residues is consistent with observations from bovine serum albumin tests. However, in 30% UHT, 740 mg L^{-1} Suma Bac D10 QAC required a 10 minute exposure for a 5 log reduction in suspension tests.

Reduction of QAC concentration resulted in greater sensitivity to the presence of milk residues in suspension tests, as shown by the requirement for a ten-minute exposure for a 5 log kill in 10% UHT milk for 200 mg L^{-1} Suma Bac D10 compared to the one minute required in 10% UHT milk for 740 mg L^{-1} QAC. Use of the highest recommended disinfectant concentration is therefore critical for the maintenance of disinfectant efficacy in the presence of organic material.

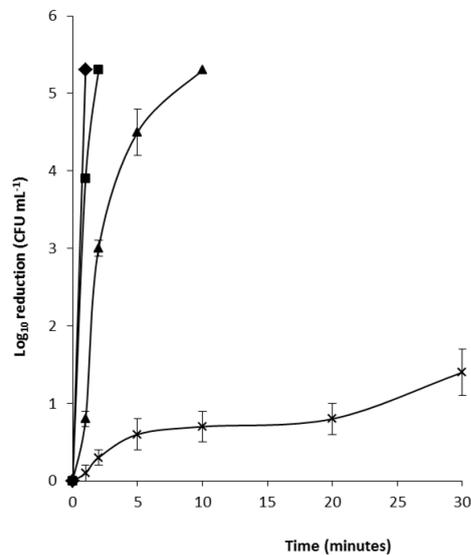


Figure 2: Effect of UHT milk (♦ 0%, ■ 1.5%, ▲ 2%, × 3%) on the efficacy of 150 mg L^{-1} Suma Tab D4 chlorine against *Listeria monocytogenes* (EGDe) in suspension tests.

3.5 Effectiveness of chlorine and QACs against *L. monocytogenes* biofilms

Five log reductions of 48h and 72h SS and PS EGDe and Scott A biofilms were recorded within one minute in 150 mg L^{-1} chlorine and 740 mg L^{-1} QAC (see Figures 4 and 5). In a similar study, Ronner and Wong (1993) reported 3-5 \log_{10} CFU/coupon reductions of *L. monocytogenes* biofilms on application of 200 mg L^{-1} QAC and 100 mg L^{-1} chlorine for 1 and 2 minutes,

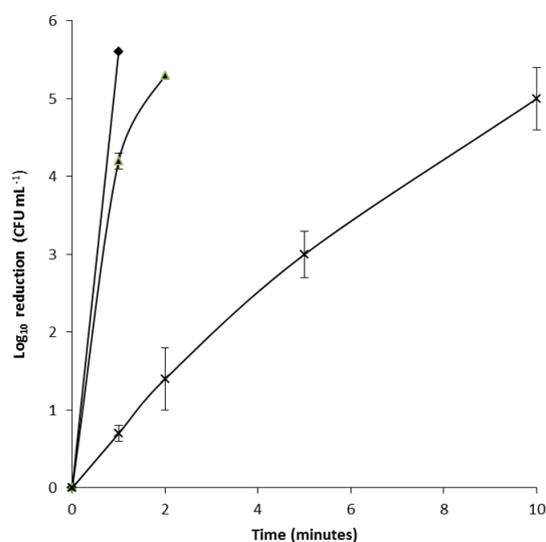


Figure 3: Effect of UHT milk (\blacklozenge 10%, \blacktriangle 20%, \times 30%) on the efficacy of 740 mgL^{-1} Suma Bac D10 QAC against *Listeria monocytogenes* (EGDe) in suspension tests.

respectively. The susceptibility of *L. monocytogenes* biofilms to disinfectants may be explained by its attachment as single cell layers on stainless steel surfaces and the production of loosely attached biofilms on polystyrene, which result in the exposure of cell surfaces to antimicrobials.

Although a five log reduction in 48h biofilms was achieved within five minutes on application of 200 mg L^{-1} QAC, only $3.5 \pm 0.1 \log_{10}$ reductions were recorded for 72h Scott A biofilms, suggesting an increase in resistance with aging. Increased biofilm resistance by up to two logs on aging has also been reported by Sommer, Martin-Rouas, and Mettler (1999).

4 Conclusion

Suma Tab D4 chlorine tablets and Suma Bac D10 QAC are effective listericidal agents when applied at the recommended concentrations, achieving the required five log reduction within 1-5 minutes in planktonic cells, on stainless steel surfaces, and in biofilms. However, Suma Tab D4 is inactivated in the presence of low concentrations of organic material, while Suma Bac D10

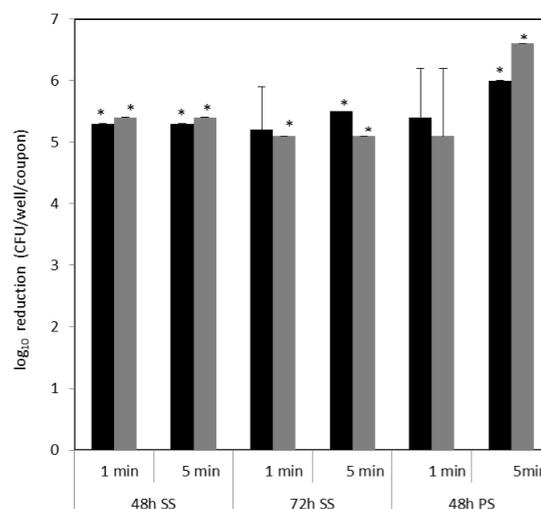


Figure 4: Susceptibility of *L. monocytogenes* (EGDe \blacksquare , Scott A \blacksquare) biofilms to 150 mgL^{-1} Suma TabD4 chlorine. * residual counts below detection levels. SS (stainless steel surfaces), PS (Polystyrene surfaces).

QAC maintains its anti-microbial activity in the presence of high levels of organic residues.

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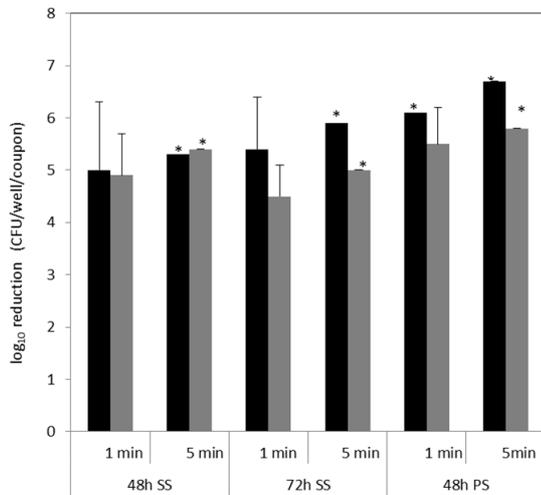


Figure 5: Susceptibility of *L. monocytogenes* (EGDe ■, Scott A ■) biofilms to 740 mgL⁻¹ Suma Bac D10 QAC. * residual counts below detection levels. SS (stainless steel surfaces), PS (Polystyrene surfaces).

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