Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Investigation into the effect of detergents on disinfectant susceptibility of attached *Escherichia coli* and *Listeria monocytogenes*

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Keywords
benzalkonium chloride, cell surface hydrophobicity, detergent, flow cytometry, susceptibility.

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2007/1264: received 8 August 2007, revised 25 September 2007 and accepted 2 January 2008

doi:10.1111/j.1365-2672.2008.03805.x

Abstract

Aims: Investigate the effect of detergent treatment on susceptibility of attached *Escherichia coli* and *Listeria monocytogenes* to subsequent disinfectant treatment.
Methods and Results: Plate counts show that *E. coli* attached to stainless steel surfaces became significantly more susceptible to benzalkonium chloride (BAC) after treatment with sodium alkyl sulfate (SAS) and fatty alcohol ethoxylate (FAE). No change in susceptibility was observed with Sodium dodecyl sulfate (SDS). *L. monocytogenes* became significantly less susceptible to BAC after treatment with SAS and SDS yet no change in susceptibility was observed with FAE. Flow cytometry using the fluoresceine propidium iodide revealed significant increases in cell membrane permeability of both organisms by SAS and FAE, although the effect was much greater in *E. coli*. No change was observed with SDS. Hydrophobic interaction chromatography showed that both organisms became less hydrophobic following treatment with SAS and SDS but FAE had no effect.
Conclusions: In *E. coli*, detergents that increase susceptibility to BAC increase membrane permeability. In *L. monocytogenes*, detergents that reduce susceptibility to BAC lower cell surface hydrophobicity.
Significance and Impact of the Study: Detergents can influence the sensitivity of pathogenic food borne micro-organisms to BAC.

Introduction

In the food industry, regular cleaning and disinfection procedures are the most effective way of controlling levels of pathogenic micro-organisms such as *Escherichia coli* and *Listeria monocytogenes* that are prevalent in foods and the environment and have the potential to cause serious illness (Wilks *et al.* 2006; Chang and Fang 2007).

The cleaning procedures involve the use of detergents, which are not designed as antimicrobial agents but to break down food soils and remove surface contamination, followed by applications of disinfectants that reduce the viability of the remaining organisms (Gibson *et al.* 1999).

Detergents are molecules that are amphiphilic in nature with a hydrophobic hydrocarbon tail and a hydrophilic head group. It is the hydrophile on the head group that defines the detergent as anionic, cationic, nonionic or amphoteric, all of which may be used in detergent formulations. A limited range of disinfectants are used in the food industry including sodium hypochlorite, peracetic acid, alcohol based products and quaternary ammonium compounds (QACs) such as benzalkonium chloride (BAC). QACs which are predominantly used (Holah *et al.* 2002), are cationic in characteristic and cause damage to the outer membrane and promote their own intracellular uptake and entry (Russell 2002). The use of detergents is an important procedure before applying disinfectant as the presence of organic and inorganic soil can inactivate a disinfectant.

Although previous investigations have shown organisms in food processing environments to adapt to disinfectants through repeated exposure (Aase *et al.* 2000; To *et al.*

2002), few studies have yet investigated the possibility that pre-exposure to detergent during normal cleaning procedures may affect the susceptibility of the organisms to the subsequent disinfectant treatment.

Early studies by Brown and Richards (1964) observed an enhanced effect of BAC on *Pseudomonas aeruginosa* after treatment with Tween 80 and they suggested that the effect may be because of its effects on cell membrane permeability. These results are supported by Hugo and Russell (2004), who reported that anionic and nonionic surface active agents can render certain bacterial species more sensitivity to some antimicrobial agents possibly by altering the permeability of the outer envelope. Disorganization of this layer would render the outer membrane permeable to antimicrobial agents that would otherwise be unable to enter the cell.

Changes in bacterial susceptibility have been attributed to several cellular mechanisms and alterations in envelope composition. These include energy dependent efflux pumps, production of stress proteins, changes in cell membrane permeability, surface hydrophobicity and outer membrane ultra structure (Aase *et al.* 2000; Poole 2002; Russell 2002).

The aims of this work were to investigate the influence of detergents commonly used in the food industry on the susceptibility of *E. coli* and *L. monocytogenes* to BAC and to assess the relationship with membrane permeability and cellular hydrophobicity.

Materials and methods

Strains and cultivation

Food industry isolates *E. coli* CRA400 and *L. monocytogenes* CRA359 were provided by Campden and Chorleywood Food Research Association (CCFRA, Gloucestershire, UK). Cell cultures were grown in Tryptone Soya Broth (TSB) at 37°C in an orbital shaker at 150 rpm for 24 h.

Detergents and disinfectant

Holchem Laboratories Ltd, (Lancashire, UK) provided the anionic detergent, sodium alkyl sulfate (SAS) and the nonionic detergent, fatty alcohol ethoxylate (FAE). Sodium dodecyl sulfate (SDS) was obtained from Sigma-Aldrich. The detergents were prepared to working concentrations of 0·2% (v/v), 0·1% (v/v) and 0·2% (w/v) by dilution with sterile distilled water, immediately prior to use. BAC, provided by Holchem, was used at a concentration of 0·05% (v/v) for *E. coli* and of 0·005% (v/v) for *L. monocytogenes*. This was lower than the recommended in use concentration but gave a measurable level of kill at which changes in susceptibility could be quantified. The disinfectant was diluted in water of standard hardness in accordance with the European Standard suspension test (Anon 1997). Fresh stocks were made on a daily basis.

Effect of detergent and disinfectant on *E. coli* and *L. monocytogenes*

Stainless steel coupons, type 316, measuring 0·8 × 0·8 × 0·1 mm were supplied by CCFRA. The coupons were prepared by washing in household detergent followed by thorough rinsing in tap water and distilled water and autoclaving for 20 min at 121°C.

Overnight cultures of cells for attachment procedures were harvested by centrifugation at 2000 ***g*** for 30 min, resuspended in Ringers solution and centrifuged again before being resuspended in half the volume of TSB. Prepared coupons were immersed in the cell suspension for 1 h, at room temperature, to allow attachment to take place and subsequently rinsed twice by swirling in Ringers solution to remove any unattached cells. Coupons with attached cells were then immersed in detergent for 20 min (± 10 s) at room temperature and twice rinsed in Ringers solution. The coupons were then immersed in BAC for 5 min (± 10 s) and twice rinsed again in Ringers solution prior to analysis. The exposure times were selected to simulate industry practice.

Control coupons were treated with only detergent, disinfectant or water and the attached cells were quantified using the method described by Gibson *et al.* (1999). The coupons were swabbed and the swabs were vortexed for 30 s in 5 ml Ringers solution, serially diluted in Ringers solution and plated on Tryptone soya agar (TSA). Plates were incubated overnight at 37°C.

Effects of detergents on membrane permeability

Membrane integrity of cells was determined by uptake of the fluorochrome propidium iodide (PI). 10 μl of PI (1 mg ml^{-1} in water) was added to 1ml of suspended cells and were analysed on a BD Facs Calibur Flow Cytometer, which measured forward scatter (FS), side scatter (SS) and red fluorescence (FL3) at 635 nm emitted by PI - stained cells (Ananta *et al.* 2004).

Effects of detergent on cell surface hydrophobicity

Cell surface hydrophobicity was determined by hydrophobic interaction chromatography (HIC), using the methods of Smyth *et al.* (1978), with Sepharose CL-4B (Sigma-Aldrich) as the nonhydrophobic control and Octyl Sepharose (Sigma-Aldrich) as the hydrophobic ligand. 0·1 ml of untreated or detergent treated cells was added to the

columns and washed through with 4·9 ml of 1 mol l^{-1} ammonium sulfate buffered with 10 mmol l^{-1} sodium phosphate buffer (pH 6·8). The eluate was serially diluted and plated on TSA plates, which were incubated at 37°C for 24 h. Changes in hydrophobicity were determined by calculating the $\log_{10}$ difference in total viable count (TVC) of untreated and treated cells eluted from the sepharose and octyl sepharose columns.

Gas chromatography-mass spectrometry

Detergents were analysed by gas chromatography – mass spectrometry (GC–MS) to determine carbon chain lengths of the hydrocarbon tails.

All GC–MS analyses were performed on an Agilent 6890GC with 5973 MSD using Chemstation version B01.00 software and Nbs75k.1 mass spectra library. The column used was HP – 5MS 5% phenyl methyl siloxane that was 30 m in length with a diameter of 250·0 μm and a nominal film thickness of 0·25 μm (Agilent Technologies). The initial temperature of the GC oven was maintained at 70°C for 2 min, and then increased by 10°C per minute to a final temperature of 280°C that was maintained for 15 min. 0·1 μl of detergent was injected with helium as the carrier gas and a split ratio of 1:150.

Statistical analyses

Triplicate samples were repeated at least three times for all experiments. Statistical analysis was carried out using *t*-tests and two-way ANOVA (first factor being presence or absence of detergent, second factor being presence or absence of disinfectant) using the statistical software MINITAB (Version 14).

Results

The aim of the work was to assess the influence of detergents on the susceptibility of attached *E. coli* and *L. monocytogenes* to BAC. It can be seen that the TVC of control cells was 5–5·5 $\log_{10}$ (Fig. 1a–c) and that none of the detergents had an effect on the numbers of *E. coli* attached to surfaces. BAC reduced the TVC by approximately 1 $\log_{10}$ in each case giving an expected reduction of 1 $\log_{10}$ overall if detergent and subsequent disinfectant treatments were combined. However, combined treatments of SAS or FAE followed by BAC reduced the TVC by more than the 1 $\log_{10}$ expected (1·6 and 2·28 $\log_{10}$ respectively) and a divergence of the lines shows that the cells became significantly more susceptible to BAC after treatment with SAS and FAE ($P < 0·05$). No difference in susceptibility to BAC was observed after treatment with

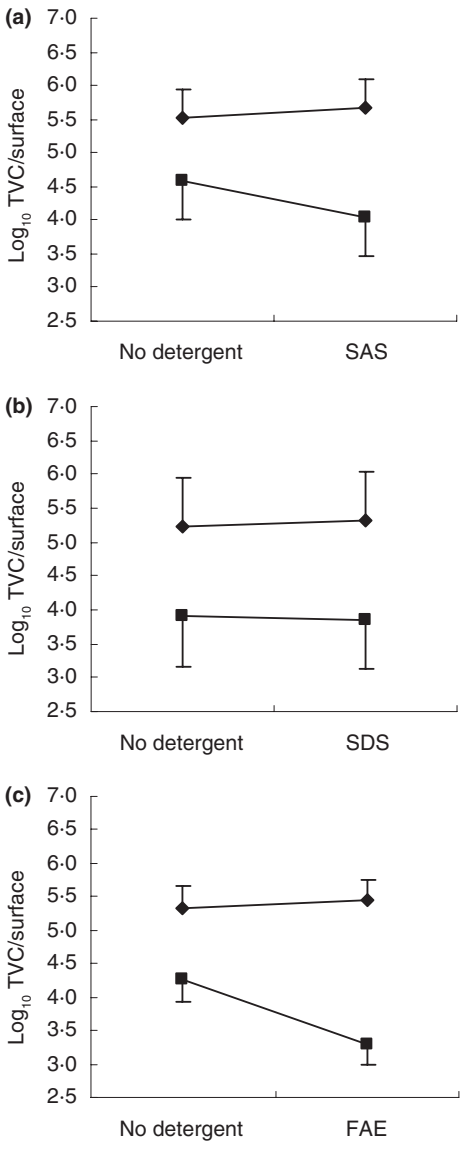

Figure 1 Interaction plots of effect of detergent, disinfectant and combined treatments on $\log_{10}$ viable count of attached *E. coli* on ◆ = No BAC, ■ = BAC. SD shown. (a) SAS: $n = 12$; (b) SDS: $n = 9$; (c) FAE: $n = 12$.

SDS. The TVC of control cells for attached *L. monocytogenes* was 6–6·6 $\log_{10}$, which was reduced by 4·19 and 2·06 $\log_{10}$ following treatment with SAS and SDS, while BAC reduced the population on each of the surfaces by an average of 3·22 $\log_{10}$ (Fig. 2a,b). The nonionic detergent, FAE, had no effect on TVC. If no interaction were to occur, it would be expected that the treatment with detergent followed with that of disinfectant would reduce the TVC by the sum of the individual treatments. However, combinations of SAS or SDS followed by BAC reduced the TVC to less than the expected 7·41 and 5·28 $\log_{10}$

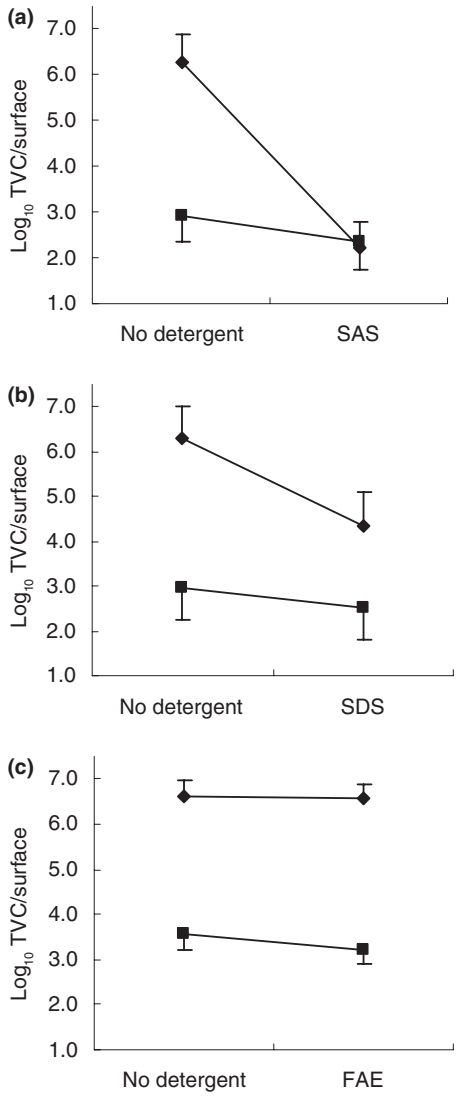

Figure 2 Interaction plots of effect of detergent, disinfectant and combined treatments on $\log_{10}$ viable count of attached *L. monocytogenes* ◆ = No BAC, ■ = BAC. SD shown. (a) SAS: $n = 9$; (b) SDS: $n = 8$; (c) FAE: $n = 6$.

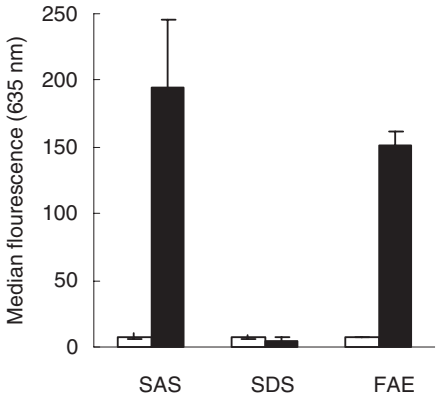

Figure 3 Flow cytometric analysis of PI uptake in *E. coli* treated with detergents. SAS: $n = 12$; SDS: $n = 9$; FAE: $n = 9$. SE shown. □ = Control, ■ = Detergent treated.

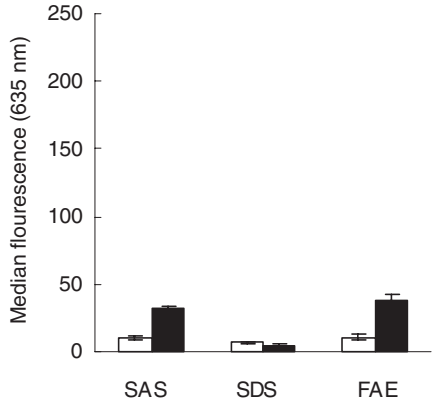

Figure 4 Flow cytometric analysis of PI uptake in *L. monocytogenes* treated with detergents. SAS: $n = 12$; SDS: $n = 9$; FAE: $n = 15$. SE shown. □ = Control, ■ = Detergent treated.

respectively and a convergence of the lines shows that the cells became significantly less susceptible to BAC after treatment with these anionic detergents ($P < 0{\cdot}05$). No difference in susceptibility to BAC was observed after treatment with FAE (Fig. 2c). These observations were not limited to attached cells because the same effects of detergent, disinfectant and combined treatments were also observed for both organisms in suspension (results not shown).

Membrane permeability changes as a result of detergent treatment were assessed using flow cytometry with PI, which diffuses across membranes that have become permeable and binds to nucleic acids, which increases its fluorescence. Analysis of median fluorescence readings (Figs 3 and 4) shows that SAS and FAE had significant effects on membrane permeability in *E. coli* and *L. monocytogenes*, although the extent of the effect was greater in *E. coli* ($P < 0{\cdot}05$). SDS had no effect on membrane permeability in either of the organisms.

Hydrophobic interaction chromatography was carried out to determine whether treatment with the detergents had an effect on cell surface hydrophobicity. Changes in hydrophobicity were determined by comparison of the difference in $\log_{10}$ numbers of detergent treated and of untreated cells eluted from the sepharose and octyl sepharose columns. The results show that both *E. coli* and *L. monocytogenes* became significantly less hydrophobic after treatment with SAS and SDS as more of the cells were eluted from the octyl sepharose column compared to that from the sepharose column (Figs 5 and 6). FAE

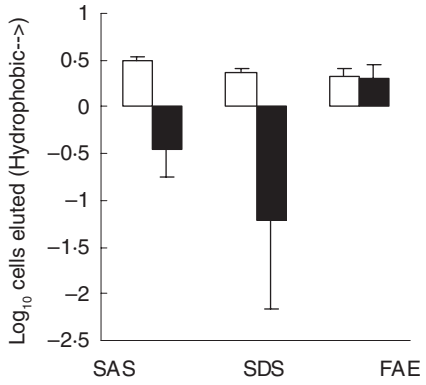

Figure 5 Changes in hydrophobicity as determined by $\log_{10}$ TVC of *E. coli* eluted from Sepharose and Octyl Sepharose columns. Control: $n = 16$; SAS: $n = 19$; SDS: $n = 16$; FAE: $n = 15$. SE shown. □ = Control, ■ = Treated.

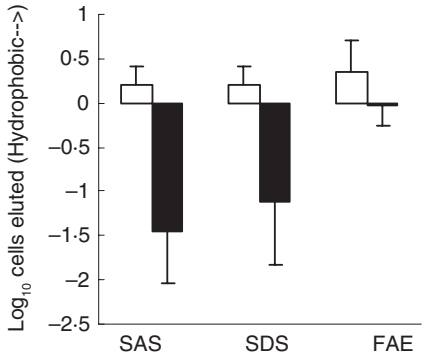

Figure 6 Changes in hydrophobicity as determined by $\log_{10}$ TVC of *L. monocytogenes* eluted from Sepharose and Octyl Sepharose columns. Control: $n = 5$; SAS: $n = 7$; SDS: $n = 5$; FAE: $n = 6$. SE shown. □ = Control, ■ = Treated.

had no effect on *E. coli* and a slight reduction in hydrophobicity was observed in *L. monocytogenes*.

Analysis of the detergents by GC-MS showed that alkyl chain lengths of C12 are predominant in all of the detergents but SAS and FAE also contain additional longer chain components of up to C17 that are yet to be identified (results not shown).

Discussion

No effect was observed on the total viable count (TVC) of attached *E. coli* after treatment with any of the detergents. However, a significant reduction in the TVC of *L. monocytogenes* was observed after treatment with SAS and SDS which would not be expected, at in use concentrations, as detergents are designed to remove organic soil and are not intended to be antimicrobial agents. Glover *et al.* (1999) investigated cationic, anionic and nonionic surfactant action on the membranes of *Proteus mirabilis* and *Staphylococcus aureus* and found that biocidal efficiency of detergents was organism dependent. As anionic detergents are recognized as having greater antimicrobial effects on Gram positive bacteria than on Gram negative bacteria (Gaibraith *et al.* 1971), and the same effect was observed in suspended cells, the reduction in the TVC of *L. monocytogenes* was attributed to bactericidal effects and not to the removal of cells from the surfaces.

Combined treatments of SAS or FAE followed by BAC showed a significant enhanced reduction in the TVC of *E. coli* suggesting increased susceptibility to the disinfectant, while no such change in susceptibility was observed following treatment with SDS. In contrast, *L. monocytogenes* cells that survived treatment with the SAS or SDS were observed to be less susceptible to subsequent treatment with BAC, while no such change in susceptibility was observed following treatment with FAE.

Gram negative bacteria are protected by the outer membrane (OM) (Sundheim *et al.* 1998) and the presence of lipopolysaccharide (LPS) is considered as a permeability barrier (Braoudaki and Hilton 2005). According to Vaara (1992), cation binding sites of the LPS are essential for the integrity of the OM of Gram negative bacteria that can resist uptake of biocides particularly in organisms, where high Mg^+ content produces strong LPS – LPS links (Russell 2001). However, the OM can be permeabilized by naturally occurring polycationic antibiotics and EDTA, which chelates Ca^+ and Mg^+ (Vaara 1992), and may allow for easier access to the cytoplasmic membrane by other agents. Glover *et al.* (1999) suggested that surfactant action may be similar to that of chaotropic anions that are able to break up the water structure near the polar head groups, which perturbs the lipid bilayer.

It is possible that the anionic SAS is acting like a chelating agent by permeating the membrane and binding the cations that maintain the structure of the LPS. This may result in increased permeability to BAC, although this would not account for the changes observed with FAE. The lack of effect of SDS on disinfectant susceptibility was unexpected as it is a similar anionic detergent to SAS.

It is hypothesized that the observed increase in susceptibility to BAC after treatment with SAS may because of longer chain components detected in SAS by GC-MS analysis. Properties of alkyl sulfates vary with the alkyl chain length distribution (Schmitt 2001) and longer chain lengths may be able to penetrate further into the LPS to bind with the cations and influence permeability. FAE is also a longer molecule because of the ethylene oxide units of the nonionic head group. Other studies by Moore *et al.* (2006) have shown that the chain length of

detergent influences membrane properties. although the effect of chain length on disinfectant susceptibility has yet to be established.

Alterations in the cell membrane brought about by the action of the detergents may explain the observed changes in susceptibility to BAC. Flow cytometric analysis revealed significant increases in cell membrane permeability in both the organisms after treatment with SAS or FAE, although the effect was much greater in *E. coli* and no change was observed after treatment with SDS. These results are supported by those of Glover *et al.* (1999) who observed that nonionic alcohol ethoxylate induced greater membrane fluidization in *Proteus mirabilis, Staphylococcus aureus* and *Saccharomyces cerevisiae* compared to that of SDS. Nonionic detergents are generally assumed to be inactive (Glover *et al.* 1999), yet Brown and Richards (1964) observed enhanced antibacterial activity of benzalkonium chloride on *Pseudomonas aeruginosa* after treatment with nonionic Polysorbate (Tween) 80, while the detergent on its own had no observable effect. They suggested interference by the surfactant with the packing or molecular organization of the cell membrane/envelope and using reconstituted lipid preparations they observed that nonionic agents reduced electrostatic resistance and increased cation permeability. Brown and Winsley (1971) later proposed that the outer lipid structure of the envelope of *P. aeruginosa* was altered by the nonionic detergent allowing easier access of cationic polymyxin to the underlying membrane.

This suggests that the increase in membrane permeability observed in *E. coli* following treatment with SAS or FAE may be the causative factor for the cells to become more permeable to the cationic BAC resulting in the increase in susceptibility. In contrast, the effect of SAS and SDS on *L. monocytogenes* resulted in an apparent reduction in susceptibility to BAC, while SAS and FAE produced small changes in membrane permeability suggesting that other mechanisms must be involved.

Cell surface hydrophobicity decreased in both organisms following treatment with SAS or SDS and may be a contributory factor in the decrease in susceptibility to BAC observed in *L.monocytogenes* after treatment with these detergents. No significant change was observed after treatment with FAE. This would be expected as the nonionic detergent carries no net charge, while the anionic detergents may attach to or insert into the surface of the organisms making them more polar and less hydrophobic. Braoudaki and Hilton (2005) observed in *Salmonella enterica* that changes in cell surface hydrophobicity associated with reduced susceptibility are strain specific.

Alternatively, the observed decrease in susceptibility in *L. monocytogenes* may be because of some other mechanism such as efflux pumps triggered by the action of the detergents. Russell (2002) investigating antibiotic and biocide resistance stated that bacteria may adopt strategies such as energy dependent efflux pumps that can remove low concentrations of biocides and Aase *et al.* (2000) found that BAC resistance was mediated by a proton motive force efflux pump in *L. monocytogenes.* As the nonionic detergent had no effect on susceptibility of the cells to BAC, it may be the charge on a detergent molecule that triggers efflux. It is likely that there will be more than one factor involved in the changes that have been observed in disinfectant susceptibility.

This work demonstrates that there is a significant difference in the effects of detergents on the susceptibility of gram positive and gram negative bacteria to disinfectant and further work is required to establish whether this is the same for other pathogenic organisms of interest in the food industry. An understanding of the effects of detergents on disinfectant susceptibility can be applied to the design of cleaning procedures for effective control of pathogens in food processing environments.

Acknowledgements

We would like to thank Holchem Laboratories and CCFRA for supplying materials and advice.

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