Developing application and detection methods for *Listeria monocytogenes* and fish extract on open surfaces in order to optimize cleaning protocols

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Abstract

Surfaces in the food industry are often fouled with bacteria and organic materials. A range of fouling and testing methods using two Listeria monocytogenes *strains (Scott A and N53-1) and organic material (0.4 g/ml fish extract) were designed to determine the efficacy of two different cleaning methods (spray and wipe) in 1% sodium hypochlorite. The optimum method for applying the cells and organic material to substrata occurred when the cells and organic material were mixed together, dried onto the surface and stained. As the number of cleaning and re-foulings increased, cells were removed from the surfaces but the organic material remained. The pattern of organic material retention was different on the surfaces with the different cleaning protocols, but neither method was better at removing the retained organic material. More cells were removed from the surfaces by the spray than the spray with wipe clean. There was no difference in cell number retention for either of the* Listeria monocytogenes *strains. These findings are valid for a 'dirty material' as classified in BS EN1276. To determine cleaning method efficacy, the application of cells and organic material to a surface is important, as is the detection methods used.*

Keywords: *Listeria monocytogenes*; fish extract; organic material; conditioning film; biofouling; cleaning

1.0 Introduction

The human pathogenic bacterium *Listeria monocytogenes* is of major concern in the food processing industry and is known to readily attach to stainless steel surfaces (Gram et al., 2007). *Listeria* spp. are known to be extremely resistant and may persist in a given premises, for example the food industry for many years (Bagge-Ravn et al., 2003; da Silva and De Martinis, 2013). One of the key processing areas where *L. monocytogenes* is problematic is the fish processing industry. The mortality rate of listeriosis is considerably higher than for other food borne pathogens, and listeriosis causes 20-30% of the annual deaths attributable to these microorganisms (Mead et al., 1999; Gomez et al., 2012).

The presence of organic material is a known factor in fouling in the food processing environment (Whitehead et al., 2008). This initial fouling layer, known as the conditioning film, may affect the hygienic status of materials (Herrera et al., 2007). If further organic material, cells, minerals, cleaning deposits and other debris are deposited onto a surface, this may be known as organic fouling. In food processing, there is constant fouling of surfaces by the food components being processed and this fouling forms the layer onto which microorganisms subsequently adhere (Whitehead et al., 2009a). This organic material may also potentially provide nutrients for the residual microorganisms, enabling multiplication and an increase in contamination of the surface, providing that other conditions conductive to growth are available (Whitehead et al., 2009b). The transfer of cells and organic material to the surface, (biofouling), may cause a gradual build-up of material unless it is removed using a cleaning process. If the presence of viable microorganisms on a surface presents a bio-transfer potential then it is important to also know if the presence of a conditioning film or organic fouling reduces or increases microbial attachment/retention to a surfaces, since this may enhance the potential for microbial contamination further downstream. Although it has been suggested that the presence of a conditioning film may increase the number of

microorganisms attached to a surface (Speers and Gilmour, 1985), it has also been shown that conditioning films such as aqueous cod extract significantly decreased bacterial attachment by a factor of 10–100 (Bernbom et al., 2006; Pillai et al., 2009). In order to maintain hygienic standards in the food industry, regular cleaning of equipment must be undertaken. However, it has been shown that the susceptibility of cells to biocides may be reduced by the presence of organic material on the surface (Aarnisalo et al., 2000; Bagge-Ravn et al., 2003 Gram et al., 2007). Retained organic material on a surface may also impede the recovery of cells and methods of detection (Whitehead et al., 2009a). One of the most common ways to evaluate cell viability on surfaces is to use a swabbing and serial dilution method. It has been shown that methods such as swabbing result in poor cell recoveries (Gomez et al., 2012). However, if only a few bacteria are left on the surface it may be impossible to detect them (Gram et al., 2007), thus a sufficiently high cell load needs to be initially added to the surface in laboratory conditions in order to be able to demonstrate cleaning protocol efficacy. If organic material is also present on the surface, and assays such as epifluorescence microscopy are to be used in cell evaluation, then too high a cell/organic material load will result in difficulty in enumeration of cell numbers and organic material retention. One way to overcome such difficulties is to use a range of methods and also to develop current methodologies.

The food industry (and others) relies on cleaning and disinfection to remove organic material and kill bacteria and it is well-known that the type of organic material present affects the effectiveness of cleaning and sanitizing (Whitehead et al., 2009a). Chlorine is known to work primarily against proteins (Schwach and Zottola, 1984) and also depolymerizes exopolymeric substances (Kumar and Arand, 1998). Sodium hypochlorite is a chlorine compound used as a disinfectant and its bactericidal effect is based on the penetration of the chemical and its strong oxidative action on essential enzymes in the cell (Lomander et al., 2004).

The aim of this work was to determine the effect of cells and fish extract on the method of application of cells (*L. monocytogenes*) onto a stainless steel surface so that both cells and organic material could be easily visualised and quantified. This was to enable a comparison of the efficacy of different types of cleaning assays using sodium hypochlorite (spray clean or a spray with wipe clean).

2.0 Materials and methods

2.1.1 Stainless steel preparation and cleaning

In order to examine the condition of the surfaces to be used in the cleaning assays, unused and surfaces that were continually reused with cleaning, were examined to determine if there was a change in the surface topography or wettability. Five by four centimeter pieces of 304 2B finish stainless steel (Outokumpu, UK) were used. In order to ensure a robust clean, the cleaning method consisted of autoclaving the stainless steel for 15 min at 121 ˚C, and then soaking for 30 – 60 min in an alkaline, phosphate free glass cleaner (Dri-decon, East Sussex, UK). The steel was then autoclaved for 15 min at 121 ˚C in the cleaner, cooled and rinsed in water. The steel was then soaked for 30 – 60 min in > 99% concentration acetone, rinsed again in water and autoclaved again. Samples were air dried before each use for a minimum of two hours and were stored in a sterile beaker covered with aluminum foil between uses.

2.1.2 Atomic Force Microscopy (AFM) of autoclave cleaned stainless steel

R values were obtained using an AFM (Quesant Instruments CA, US) operated in contact mode using silicon nitride tips with a force constant of 0.12 N/m.

2.1.3 Surface wettability of autoclave cleaned stainless steel

The wettability of the surfaces was measured using a Kruss goniometer (Kruss, France) using 5 µl diameter droplets of HiPerSolv HPLC grade H_2O (BDH, UK). Contact angle measurements of the substrata were taken using immediately following application of the water droplet.

2.2 Microbiology

2.2.1 Preparation of microorganisms for use in assays

Stock cultures of *L. monocytogenes* ScottA and N53-1 were inoculated on tryptone soya agar (Oxoid, UK). *L. monocytogenes* ScottA was chosen as a reference strain and *L. monocytogenes* N53-1 since it had previously been shown to persist in the fish processing environment (Vogel et al., 2001; Wulff et al., 2006). Cells were grown for 24 h at 30 ºC. A single colony of the microorganism was inoculated into 100 ml tryptone soya broth (Oxoid, UK), and incubated for 18 h with shaking at 30 ºC. An optical density (OD) of 1.0 (540 nm), equating to 10^8 colony forming units per ml (cfu/ml) were used to inoculate substrata.

2.2.2. Fish extract

Fresh fish fillets (cod) (CO-OP, UK) were cut into cubes and 500 ml of tap water was added per kg fish. The fish was boiled from cold (4 ºC) for 5 min. The juice was pressed from the fish flesh and the resulting solution was drained off, boiled for 5 min without stirring, then left for 5 min until flocs were seen in the liquid. The liquid was then filtered through doubled wood pulp (size 4) coffee filters (CO-OP, UK), then 0.1 M phosphate buffer was added (7.62 g $KH_2(PO)_4$ (BDH, UK) and 7.66 g $K_2H(PO)_4$/L (BDH, UK)). The pH was adjusted to 6.6. The optical density (OD) (600 nm) of the fish extract was measured and adjusted to 0.21. The extract was sterilized by autoclaving at 100 °C for 30 min and cooled to 0 °C – 5 °C, which resulted in clearing of the liquid. The liquid was aliquoted and stored at -20°C until use, whereby it was defrosted and stored at 4 °C, and was discarded if not used within 2 days (Whitehead et al., 2008).

2.2.3 Concentration of fish extract needed for detection by epifluorescence microscopy.

One hundred microlitres of fish extract was pipetted onto the stainless steel surface. Samples were air dried in a class 2 flow hood. Samples were stained with 0.03 % w/v acridine orange (Sigma, UK) in 2 % v/v glacial acetic acid (BDH, UK) for 2 min, (whilst being left in the dark

to avoid bleaching of the fluorescent dye), rinsed once, gently with 5 cm^3 distilled H_2O, with the distilled water bottle at a 45 ^{o} angle, with a 3 mm diameter nozzle opening. Samples were air dried for one hour in a class 2 hood and stored at 4 ^oC in the dark until visualised. A concentration of 0.03 % acridine orange was used since previous work in our laboratories had found that it was the optimum concentration for staining cells on stainless steel surfaces (Whitehead et al., 2007).

2.2.4 Determination of optimum cell and organic material concentrations to allow differentiation using epifluorescence microscopy.

Different strains of *L. monocytogenes* (Scott A and N53-1) were stained with 0.03 % w/v acridine orange demonstrating the presence of both potential viable (green) and non viable (red) cells in the presence of fish extract. One hundred microlitres organic material (0.04g/ml fish extract or 0.4g/ml fish extract), 100 µl cells and 40 µl 0.03 % w/v acridine orange stain were mixed together in an eppendorf tube. One hundred microlitres of the mixture was pipetted onto the substratum and air dried in a class 2 flow hood.

2.2.5 Optimization of application method

For assay 1, 100 µl of 0.4g/ml fish extract, 100 µl cells and 40 µl 0.03 % w/v acridine orange stain were mixed together in an Eppendorf tube. One hundred microlitres was pipetted onto the substratum and air dried for 30 min in a class 2 flow hood. For assay 2, the fish extract was applied to the surface and dried for 30 min, then the cells were added and air dried for 30 min, then the sample was stained. Assay 3 was as assay 2 except the cells were applied to the surface first and assay 4 was as assay 1 except the stain was added after the mixture of fish extract and cells had been dried for 30 min on the surface.

2.2.6 Cleaning methods

Substrata were inoculated with cells and fish extract as described in assay 4 and were used in subsequent cleaning assays.To inoculate the surfaces, 400 µl of *L. monocytogenes Scott A* or

L. monocytogenes N53-1 were mixed with 400 µl 0.4g/ml fish extract. From this stock solution, 40 µl of cells mixed with fish extract was applied to the stainless steel surface. The mixture was spread across 4 cm x 4 cm of the test piece using a glass spreader and air dried in a class 2 microbiological hood for 30 min. Inoculated coupons were then attached onto a stainless steel tray using blue tac (Bostik, UK) perpendicular to the surface. For the spray clean, the stainless steel tray with the attached fouled substrata was placed at the back of a Class 2 microbiology hood. The spray nozzle was set 20 cm away, perpendicularly to the test pieces. Before spraying began, pressure was attained. The test piece was sprayed with 1 % sodium hypochlorite (BDH, UK) for 5 sec at 9000 kPa. Immediately after spraying the test piece was either soaked for 2 min in Leethen broth (Lab M, UK), prior to microbial enumeration or was laid horizontally to air dry for 30 min prior to their next clean. The substrata were then either stained using acridine orange or were re-fouled and re-cleaned up to 15 times. The test pieces were stored at 4 °C in the dark until visualisation using epifluorescent microscopy. For the spray with wipe cleaning, following spraying, a sterile swab was dipped in 9.9 ml Leethen broth and used to swab the test piece to simulate a wipe clean. The swab was rotated as it was used across the surface and was used 10 x left to right, 10 x top to bottom. The swab was returned to the Leethen broth and vortexed for 20 s. Serial dilutions were made and plated out onto Leethen agar (Lab M, UK). Leethen broth and agar was used in these assays in order to neutralize the sodium hypochlorite so as to maximize recovery and assessment of the CFU/ml. Plates were incubated at 30 °C for 48 hours (n = 12). Replicate surfaces were dried and were stained using acridine orange. All acridine orange solutions were stored at 4 °C in the dark until use.

2.2.7 Visualisation of fouled and cleaned surfaces using epifluorescence microscopy

Substrata plus adherent organic material and/or microorganisms were visualised using epifluorescence microscopy (Nikon Eclipse E600, UK). The microscope was mounted with a Hitachi HV-D37P colour camera (Nikon, UK). This system used a Lucia Image Analysis

package (Nikon, UK). The percentage coverage area of the stained components was measured to determine the retained organic material and cells. Throughout this work cells were enumerated as a total percentage coverage and not according to viability. To obtain data for cells and organic material separately, an area was selected and an image captured. The threshold for the cells or conditioning film was set and the percentage coverage individually recorded. The UV wavelength band used was 510–560 nm (Whitehead et al., 2008:2009b).

2.3 Statistics

Replicate samples were tested in triplicate, and the experiments were repeated and the mean values plotted. Error bars on the graphs represent the standard error. Statistical differences were determined using Excel in order to calculate ANOVA and t-tests values. The statistical confidence interval was considered significant when $p < 0.05$.

3.0 Results

3.1 The condition of the surfaces following 10x, 20x and 30x autoclave cleaning assays

When surfaces had been autoclave cleaned 10 or 30 times, it was demonstrated that the surfaces became more wettable with use (clean surface, 91.18º; 30 x clean, 19.07º) (Fig. 1). AFM examination of the surfaces did not reveal any obvious changes with repeated fouling and cleaning, although the 10 x cleaned surface had an elevated R_a value (Arithmetic average height from a mean centre line (Anon 2010) (237.65 nm) when compared to the used (157.98 nm) and 30 cleaned surfaces (104.75 nm) (Fig. 1), and there was a significant difference ($p < 0.05$) between the 10x cleaned surfaces when compared to the unused and 30x cleaned surface. The R_p values (the measure of the height of the highest point of the profile from the mean line) (Anon, 2010) demonstrated similar trends to the R_a values, however in this case there was a significant difference between the 30x cleaned surfaces and the unused and 10x cleaned surfaces. The R_t (total height of the profile of the evaluation length) (Anon 2010) and R_v values (the largest profile valley depth) (Anon, 2010) revealed a decrease in values with

increased cleans, whilst the R_z (the sum of R_p and R_v within a sampling length) (Anon, 2010) values of the three different surfaces demonstrated no differences. There was no significant difference in the R_p, R_v or R_z values for the three surfaces. Acridine orange staining of the surfaces and efluorescence microscopy revealed a gradual build-up of residual material (Fig. 2). The organic material was most prominent on the 10x cleaned surface which was reflected in the R_a values.

3.2 Determination of optimum cell and organic material concentrations

In order to carry out cleaning efficacy assays, it was necessary to find an optimum method of cell and organic material application that would allow a large enough number of cells on the surface, so the efficacy of cleaners could be tested, but with low enough numbers of cells and organic matter to enable cell and organic material visualisation, differentiation and quantification. It was demonstrated that a cell loading of 10^7 cfu/ml, and staining with 0.03 % w/v acridine orange gave a good visualisation of cells (Fig. 3).

A range of concentrations of fish extract was added to the stainless steel surface. The fish extract is a chemically and physically heterogeneous mixture obtained from boiling cod and extracting the juices, thus it was unknown how the organic material would be distributed on the surfaces. The results demonstrated that the optimum concentrations of fish extract to use in order to visualise and quantify the organic material would be either 0.4 g/ml and 0.04 g/ml fish extract. When 10^7cells were mixed with either 0.4 g/ml (Fig. 4a) or 0.04 g/ml (Fig. 4b) fish extract, the 0.4g/ml fish extract gave the optimum visualisation of both cells and organic material.

3.3 ***Optimization of application method***

Work was carried out to determine the best method of application of cells and organic material to the surface with an effective staining method in order to ensure optimization of the visualisation of cells and organic material (Fig. 5). Four different methods of cell and organic

material applications were tested; cells, organic material and stain were mixed together before applying them to the substrata and air drying (Assay 1); Organic material was added to the surface and air dried, then the cells were pipetted cells on top of the organic material air dried, stained, washed, air dried again (Assay 2). Assay 3 was as assay 2, except the cells were added to substratum before the organic material and finally in assay 4 the organic material and cells were mixed together and air dried onto the surface, stained, washed and air dried again. The retention of cells and organic material in assay 4 was the most likely situation to occur *in situ.* The optimum application and staining method was assay 1 (mixed the cells, organic material and stain together before applying them to the substrata and air drying) or 4 (the organic material and cells were mixed together and air dried onto the surface, stained, washed and air dried again) (Fig. 5 a and d). Both the fish extract and the cells could be quantified using these methods of application. Work in our laboratories using BSA and *L. monocytogenes* (data not shown) had also demonstrated that assay 4 gave the best differentiation between the cells and the organic material. Thus, this method was used in subsequent fouling and cleaning assays.

3.4 Cleaning methods

Fish extract and *L. monocytogenes* Scott A and fish extract and *L. monocytogenes* N53-1 were applied to a stainless steel surface, and cleaned using a spray method (Fig. 6a-d) or a spray method followed by wiping (Fig 7a-d). In the presence of the sodium hypochlorite cleaner, differential staining of cells and organic material using acridine orange was difficult, as both cells and organic materials became orange under the microscope. However, cells could be enumerated using this method as they were bright (Fig. 6a), whereas the organic material was dull in colour (Fig. 6c). The results found that although spraying (Fig. 6) or spraying followed by wiping (Fig. 7) removed cells from the surfaces, organic material remained. The pattern of the organic material retained on the surfaces was different following the different cleaning methods. Following the spray clean, the organic material was spread across the surfaces (Fig.

6) whereas the mechanical action of the wipe pushed the organic material into the grain boundaries of the stainless steel (Fig. 7).

Following percentage coverage results of the retained material, it was found that in the presence of the two different strains, similar trends in organic material retention occurred following the increased spray or the spray with wipe cleaning assays. Following the spray clean, the 5x clean retained the most organic material followed by the 15x > 10x > 0x cleans. For the spray with wipe clean, the retention of organic material was also greatest on the 5x, followed by 10 x > 15x > then the 0x clean. The different cleans produced different organic material patterns. For *L. monocytogenes* ScottA the only significant difference observed (p <0.05) in conditioning film retention between the spray or spray and wipe clean was for the 15 x clean (Fig. 8). For *L. monocytogenes* N53-1 there was a significant difference observed (p <0.05) in the retained conditioning film for the 0x and 10 x cleans.

Viable cells were only recovered prior to exposure to the cleaning agent (0 cleans), but given that 10^7 cells were added to the surface, only between 3.2 x 10^1 (*L. monocytogenes* Scott A) and 7.0 x 10^1 (*L. monocytogenes* N53-1) were recovered from the surfaces following the spray clean and 3.2 x 10^2 (*L. monocytogenes* Scott A) and 3.1 x 10^2 (*L. monocytogenes* N53-1) following the spray and wipe clean. When comparing the two *L. monocytogenes* strains, there was no significant difference in the cell numbers removed from the surfaces (p > 0.05) (data not shown).

4.0 Discussion

L. monocytogenes is a known potential pathogen that has been shown to persist in the fish industry. Since surfaces in the food industry will be fouled with both microbial cells and organic material, fish extract was used in these assays.

4.1 The condition of the surfaces following 10x, 20x and 30x autoclave cleaning assays

The surface properties of the stainless steel changed immediately after one clean, whereby the unused surfaces became more wettable. This is important since work by others has shown that results in relation to adhesion force tend to suggest that at least for hydrophobic bacteria, adhesion is greater on hydrophobic materials (Midlet and Carpentier, 2002; Boulange-Petermann et al., 2006; Jullien et al., 2008). Thus, it might be suggested that a change in surface wettability due to organic material retention may affect trends in initial cell attachment to a surface.

Most cleaning procedures remove gross organic material, but there is concern regarding organic material that is retained on surfaces, especially in surface features (Briandet et al., 2001; Whitehead and Verran, 2007). The condition of the surfaces following 10x, 20x and 30x autoclave cleaning assays demonstrated that there was a build-up of organic material on the surfaces with increased cleans even when none was added. In-use surfaces subject to wear may well present surface features that will hamper the removal of cells by swabbing and cleaning and thence confound interpretation of data (Whitehead and Verran, 2006).

4.2 Determination of optimum cell and organic material concentrations

Different strains of *L. monocytogenes* (Scott A and N53-1) were stained with acridine orange demonstrating the staining, presence and distribution of cells on the surface. A cell loading of 10^7 cfu/ml, and staining with 0.03 % w/v acridine orange gave a good visualisation of both live and dead cells.

A range of concentrations of fish extract was added to the stainless steel surface in order to enable visualisation of both the cells and the organic materials before the cleaning assays. The results from this work demonstrated that the optimum concentration fish extract for use in these assays was 0.4 g/ml.

4.3 Optimization of application method

The optimum method of cell and organic material application to the substratum that gave the best visualisation of cells and organic material for subsequent cleaning assays was obtained when the cells and organic material were mixed together, dried onto the surface and then stained. Others have also demonstrated the importance of correct setting up of microbiological assays (Gram et al., 2007).

4.4 Cleaning methods

Viable cells were only recovered prior to exposure to NaOCl in low numbers. More cells were recovered from the surfaces following the spray and wipe clean than the spray clean alone. The results demonstrated that in the presence of the sodium hypochlorite cleaner, differential staining of both the organic material and cells using acridine orange was more difficult and the viability of the cells could not be determined.

The results demonstrated that the methods of cleaning resulted in cell removal, and in differences in organic material distribution on the surfaces. Following the spray clean, organic material was distributed across the surfaces. The mechanical action of the wipe clean pushed the organic material into the grain boundaries where it was retained. Following percentage coverage results of the retained material neither cleaning method was better at removing the retained material. There was no difference in the numbers of cells removed for the different *L. monocytogenes* strains.

4.5 Difficulties in selecting methodologies

With high numbers of microorganisms potentially present on surfaces, accurate methods for enumeration are essential. It is highly likely that in the food engineering plant, microorganisms will be present on the surface alongside organic material. There are a number of methods used to evaluate the number of cells retained on a surface following surface fouling, or cleaning and disinfection procedures. However, traditional methods for the detection of cells on surfaces have indicated that they may not be reliable (Gomez et al.,

2012). Recommended standards such as EN1276 specifies a test method and the minimum requirements for bactericidal activity of chemical disinfectant and antiseptic products. The standard states that the product shall demonstrate at least a 5 decimal log reduction, and that simulated clean conditions require a 0.3 g/l bovine albumin solution, or simulated dirty conditions require 3 g/l .bovine albumin solution (Anon, 2009). Although the method advised in the BS EN 1276 standard where not followed in this work our method did demonstrate some similarities. This work neutralised the cleaning solution following cleaning in order to maximise cell recovery. Our work also used a greater loading of fish extract than was recommended in the standard, but this was necessary in order to visualise the material using epifluorescence microscopy. Thus, these findings are valid for a dirty material.

Direct epifluorescence microscopy enables visualisation of contamination on the surface, and estimation of cell coverage. Imaging cells *in situ* has a significant advantage over culture, since results are immediate, a true representation of numbers of cells present is obtained, and organic material can also be visualised, and quantitatively assessed (Whitehead et al., 2009b). However, the limitations of this method encompass accessibility of equipment and expertise to industry, and the microscopic scale at which data are acquired thus necessitating large numbers of replicate samples and a confidence that the entire surface of concern is adequately represented.

5.0 Conclusions

This paper describes the development of a method to ensure the optimum application of cells and organic material onto a surface, in order to determine cleaning efficacy assays and also produce quantitative analysis of the residual organic material (fish extract) and *L. monocytogenes* cells. The optimum method for applying the cells and organic material to substrata occurred when the cells and organic material were mixed together, dried onto the surface and stained. Cells were generally removed following cleaning but organic material was

not, and the pattern of organic material retained was dependent on the cleaning method used. Neither method was better at removing the retained organic material. In this work, the spray method was the most effective in terms of cell removal. The strain of *L.* monocytogenes used did not affect the results.

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References

Aarnisalo, K., Salo, S., Miettinen, H., Suihko, M.L., Wirtanen, G., Autio, T., Lunden, J., Korkeala, H., Sjoberg, A.M., 2000. Bactericidal efficiencies of commercial disinfectants against *Listeria monocytogenes* on surfaces. J. Food Safety 20, 237-250.

Anonomous, 2010. Assessment of surface texture – Guidance and general information, BS 1134. British Standards Institute, UK.

Anonomous, 2009. Chemical disinfectants and antiseptics — Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic andinstitutional areas — Test method and requirements (phase 2, step 1). BS EN 1276. British Standards Institute, UK.

Bagge-Ravn, D., Ng, Y., Hjelm, M., Christiansen, J.N., Johansen, C., Gram, L., 2003. The microbial ecology of processing equipment in different fish industries - analysis of the microflora during processing and following cleaning and disinfection. Inter. J. Food Microbiol. 87, 239-250.

Bernbom, N., Licht, T.R., Saadbye, P., Vogensen, F.K., Norrung, B., 2006. *Lactobacillus plantarum* inhibits growth of *Listeria monocytogenes* in an *in vitro* continuous flow gut model, but promotes invasion of *L. monocytogenes* in the gut of gnotobiotic rats. Inter. J. Food Microbiol. 108, 10-14.

Boulange-Petermann, L., Gabet, C., Baroux, B., 2006. Relation between the cleanability of bare or polysiloxane-coated stainless steels and their water contact angle hysteresis. J. Adh. Sci. Tech. 20, 1463-1474.

Briandet, R., Herry, J.M., Bellon-Fontaine, M.N., 2001. Determination of the van der Waals, electron donor and electron acceptor surface tension components of static Gram-positive microbial biofilms. Colloids Surf. B-Biointerfaces 21, 299-310.

da Silva, E.P., De Martinis, E.C., 2013. Current knowledge and perspectives on biofilm formation: the case of *Listeria monocytogenes*. Appl. Microbiol. Biotechnol. 97, 957-968.

Gomez, D., Arino, A., Carraminana, J.J., Rota, C., Yangueela, J., 2012. Comparison of sampling procedures for recovery of *Listeria monocytogenes* from stainless steel food contact surfaces. J. Food Protection 75, 1077-1082.

Gram, L., Bagge-Ravn, D., Ng, Y.Y., Gymoese, P., Vogel, B.F., 2007. Influence of food soiling matrix on cleaning and disinfection efficiency on surface attached *Listeria monocytogenes*. Food Control 18, 1165-1171.

Herrera, J.J.R., Cabo, M.L., Gonzalez, A., Pazos, I., Pastoriza, L., 2007. Adhesion and detachment kinetics of several strains of *Staphylococcus aureus* subsp aureus under three different experimental conditions. Food Microbiol. 24, 585-591.

Jullien, C., Benezech, T., Le Gentil, C., Boulange-Petermann, L., Dubois P.E., Tissier, J.P., Traisnel, M., Faille, C., 2008: Physico-chemical and hygienic property modifications of

stainless steel surfaces induced by conditioning with food and detergent. Biofouling 24, 163-172.

Kumar, C.G., Arand, S.K., 1998. Significance of microbial biofilms in food industry: a review. Inter. J. Food Microbiol. 42, 9-27.

Lomander, A., Schreuders, P., Russek-Cohen, E., Ali, L., 2004. Evaluation of chlorines' impact on biofilms on scratched stainless steel surfaces. Bioresource Technol. 94, 275-283.

Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M., Tauxe, R.V., 1999. Food-related illness and death in the United States. Emerging Infectious Diseases 5, 607-625.

Midelet, G., Carpentier, B., 2002. Transfer of microorganisms, including *Listeria monocytogenes*, from various materials to beef. Appl. Environ. Microbiol. 68, 4015-4024.

Pillai, S., Arpanaei, A., Meyer, R.L., Birkedal, V., Gram, L., Besenbacher, F., P., K., 2009. Preventing protein adsorption from a range of surfaces using an aqueous fish protein extract. Biomacromolecules 10, 2759-2766.

Schwach, T.S., Zottola, E.A., 1984. Scanning Electron-Microscopic study on some effects of sodium-hypochlorite on attachment of bacteria to stainless steel. J. Food Protection 47, 756-759.

Speers, J.G.S., Gilmour, A., 1985. The influence of milk and milk components on the attachment of bacteria to farm dairy equipment surfaces. J. Appl. Bacteriol. 59, 325-332.

Vogel, B.F., Huss, H.H., Ojeniyi, B., Ahrens, P., Gram, L., 2001. Elucidation of *Listeria monocytogenes* contamination routes in cold-smoked salmon processing plants detected by DNA-based typing methods. Appl. Environ. Microbiol. 67, 2586-2595.

Whitehead, K.A., Verran, J., 2006. The effect of surface topography on the retention of microorganisms. Food Bioproducts Processing 84, 253-259.

Whitehead, K. A., Verran, J., 2007. The effect of application method on the retention of *Pseudomonas aeruginosa* on stainless steel, and titanium coated stainless steel of differing topographies. *Inter. Biodeterioration Biodegradation* 60, 74-80.

Whitehead, K.A., Smith, L.A., Verran, J., 2008. The detection of food soils and cells on stainless steel using industrial methods: UV illumination and ATP bioluminescence. Inter. J. Food Microbiol. 127, 121-128.

Whitehead, K.A., Benson, P., Smith, L.A., Verran, J., 2009a. The use of physicochemical methods to detect organic food soils on stainless steel surfaces. Biofouling 25, 749-756.

Whitehead, K.A., Benson, P., Verran, J., 2009b. Differential fluorescent staining of *Listeria monocytogenes* and a whey food soil for quantitative analysis of surface hygiene. Inter. J. Food Microbiol. 135**,** 75-80.

Wulff, G., Gram, L., Ahrens, P., Vogel, B.F., 2006. One group of genetically similar *Listeria monocytogenes* strains frequently dominates and persists in several fish slaughter- and smokehouses. Appl. Environ. Microbiol. 72, 4313-4322.

Figures

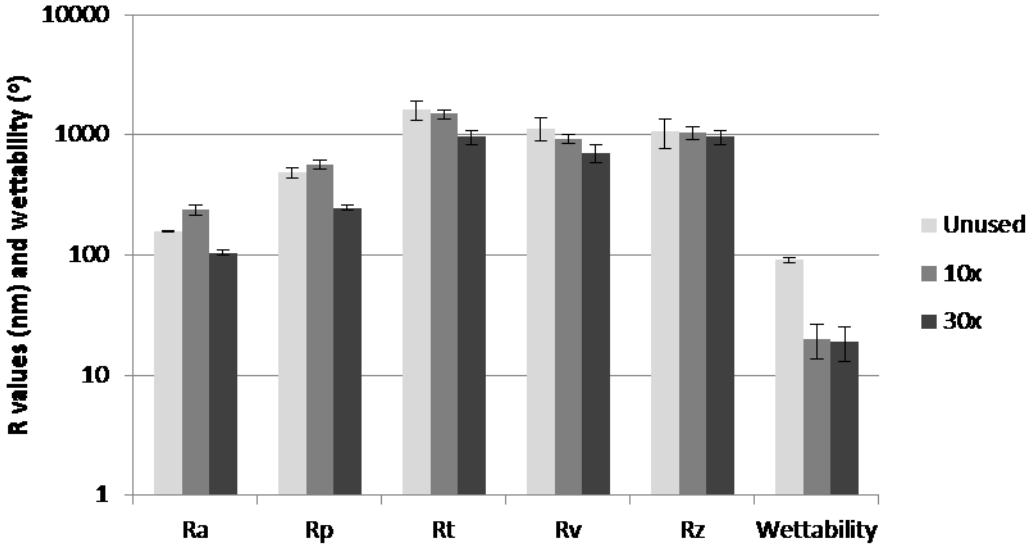

Fig.1 – *R* and wettability values of unused, 10x and 30x autoclaved cleaned 304B stainless steel surfaces demonstrating that there was a change in surface topography measurements, (with the exception of R_z) and a decrease in surface wettability with increased cleans

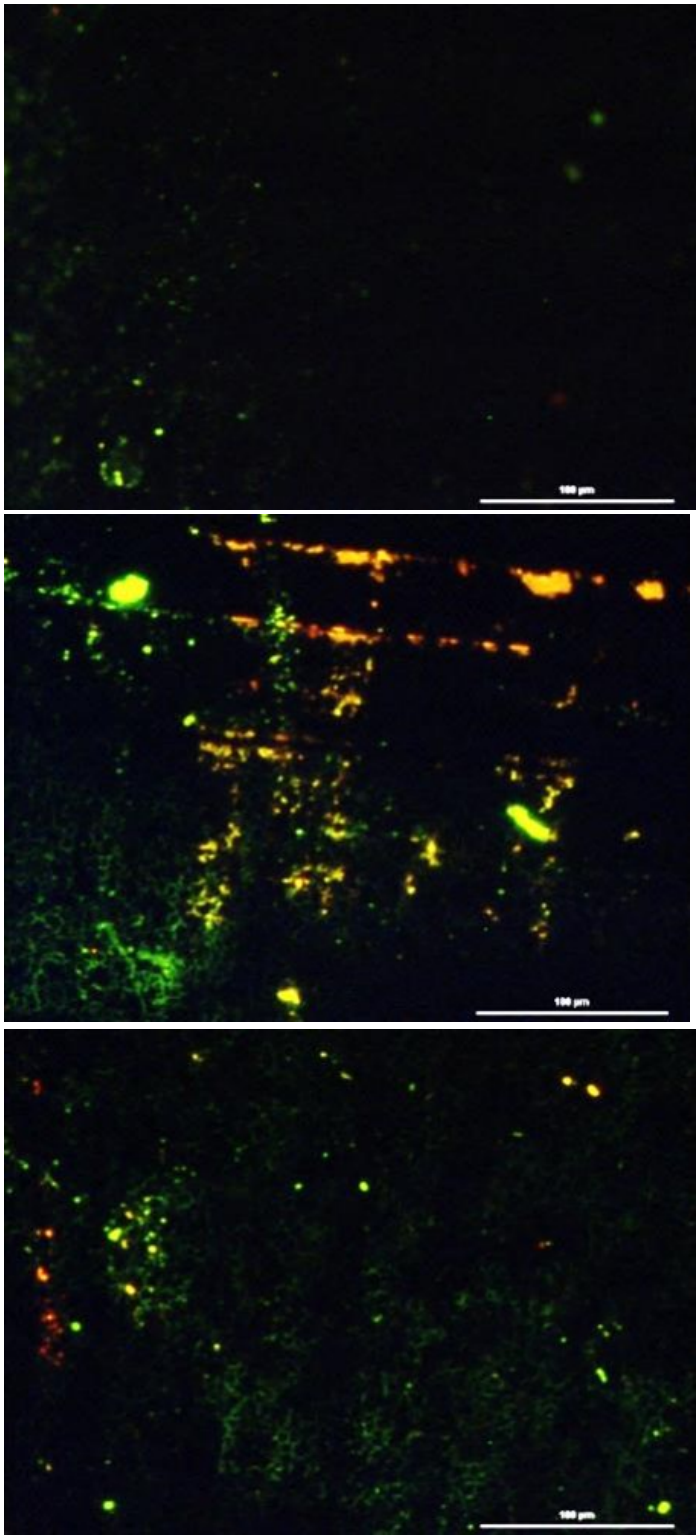

Fig. 2 - Organic material retained on surfaces following autoclave cleaning a) unused b) 10x and c) 30 x cleans demonstrating a change in organic material retention with increased cleans

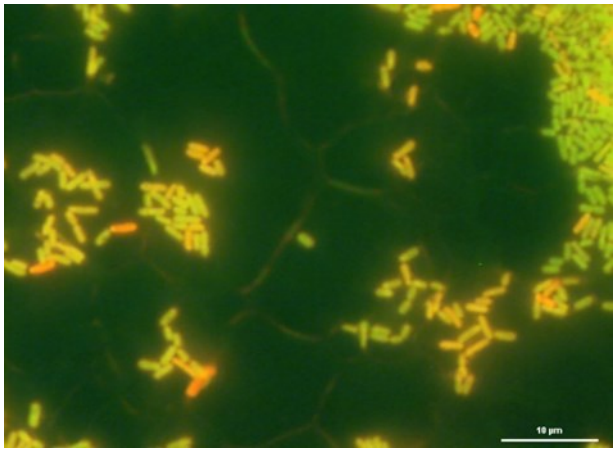

Fig. 3 - *L. monocytogenes* Scott A stained with 0.03 % w/v acridine orange demonstrating the presence of both viable (green) and non-viable (red) cells

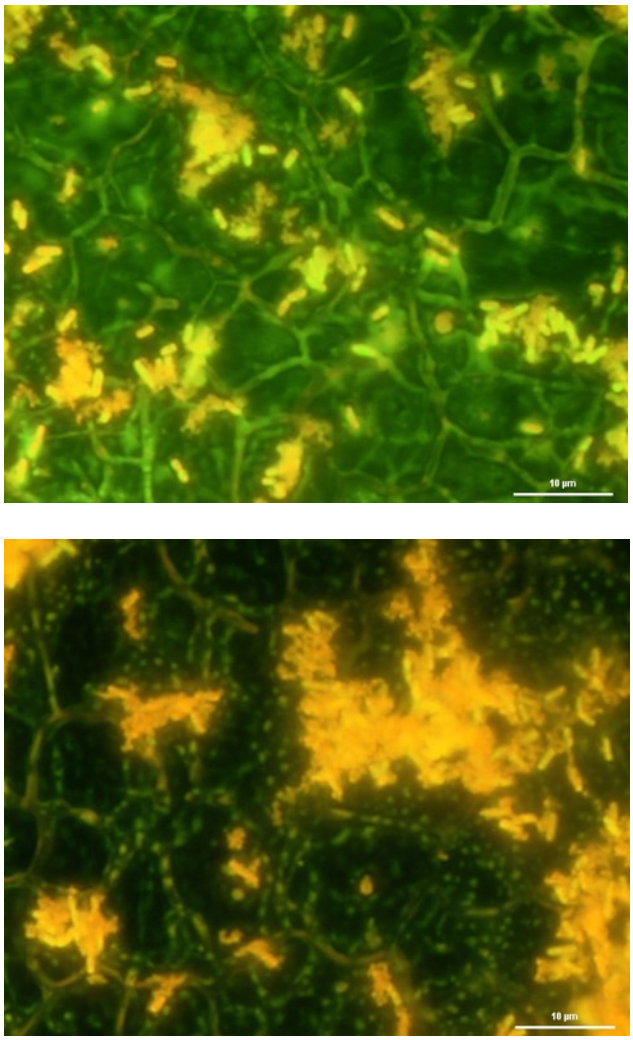

Fig. 4 – Visualisation of percentage coverage of a) 0.4 g ml^{-1} fish extract b) 0.04 g ml^{-1} fish extract

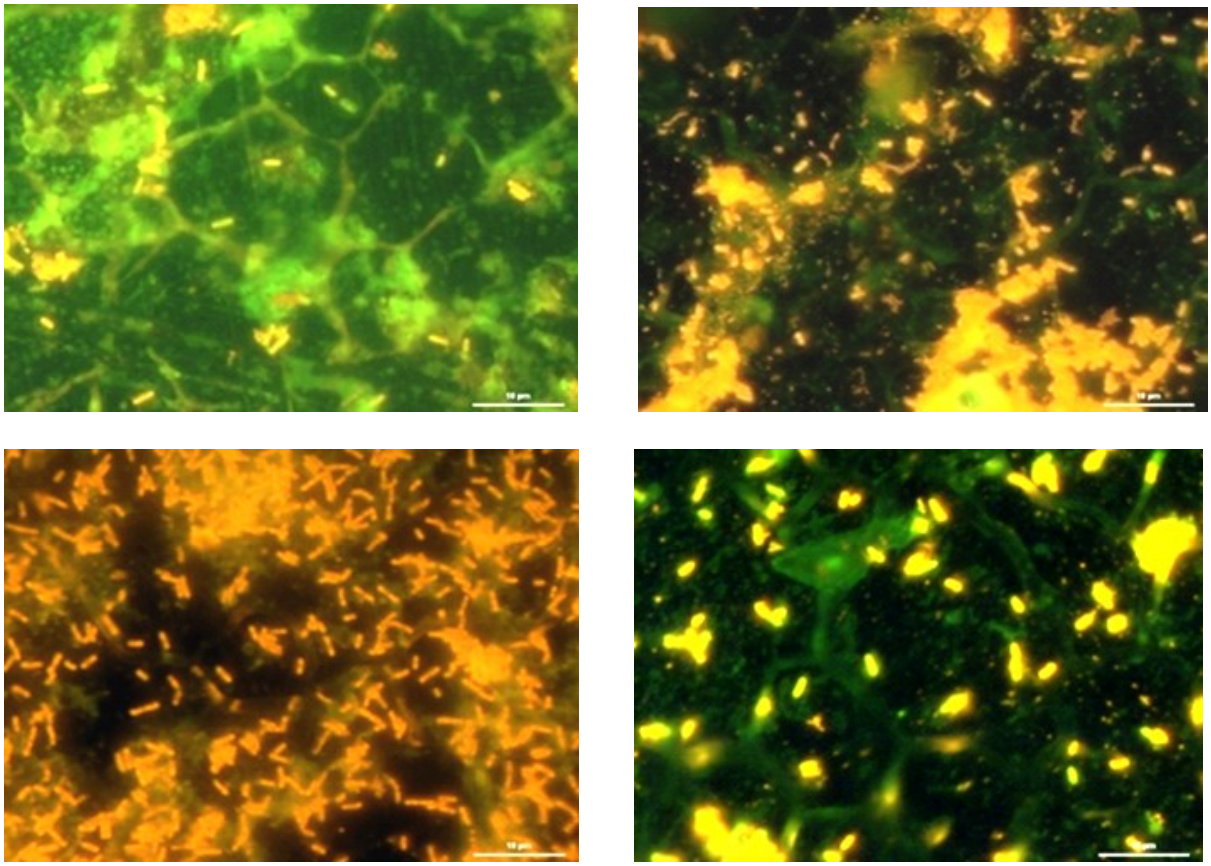

Fig. 5 - *L. monocytogenes* and 0.4g/ml fish extract stained as a) assay 1 b) assay 2 c) assay 3 and d) assay 4 demonstrating that assay 1 (mixed the cells, organic material and stain together before applying them to the substrata and air drying) and assay 4 (the organic material and cells were mixed together and air dried onto the surface, stained washed and air dried again) provided the best application combinations for cell and organic material visualisation

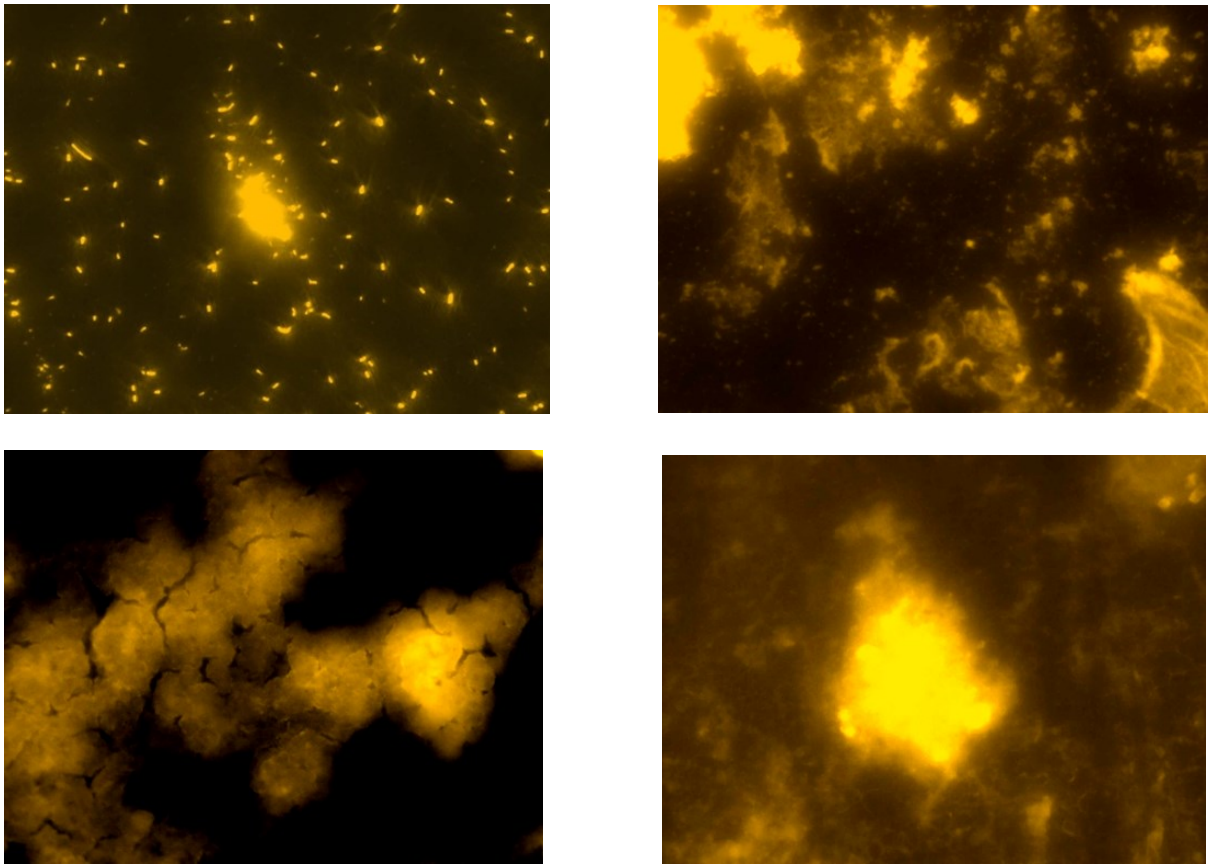

Fig. 6 – Epifluorescence images of *L. monocytogenes* ScottA and fish extract following a spray clean a) 0x, b) 5x, c) 10x and d) 15x demonstrating that although spraying removed cells the organic material was spread across the surfaces

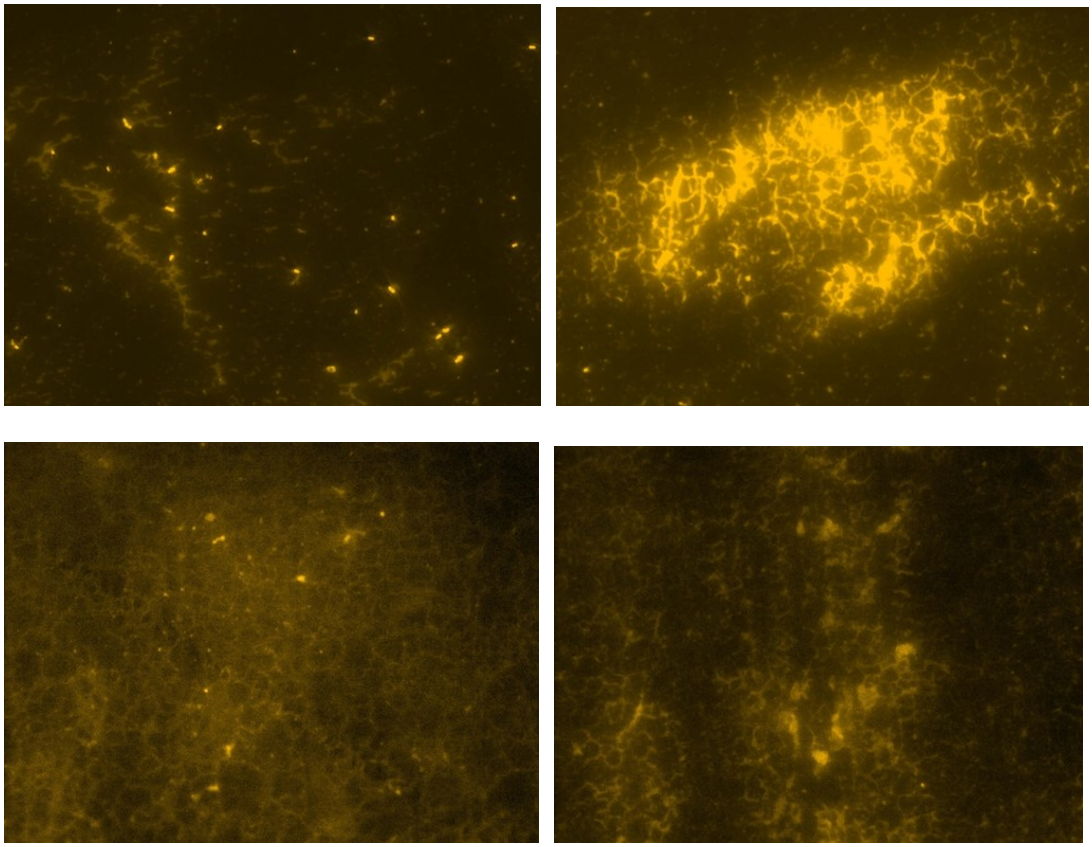

Fig. 7 – Epifluorescence images of *L. monocytogenes* ScottA and fish extract following a spray with wipe clean a) 0x, b) 5x, c) 10x and d) 15x demonstrating that although cells were removed the mechanical action of the wipe pushed the organic material into the grain boundaries of the stainless steel

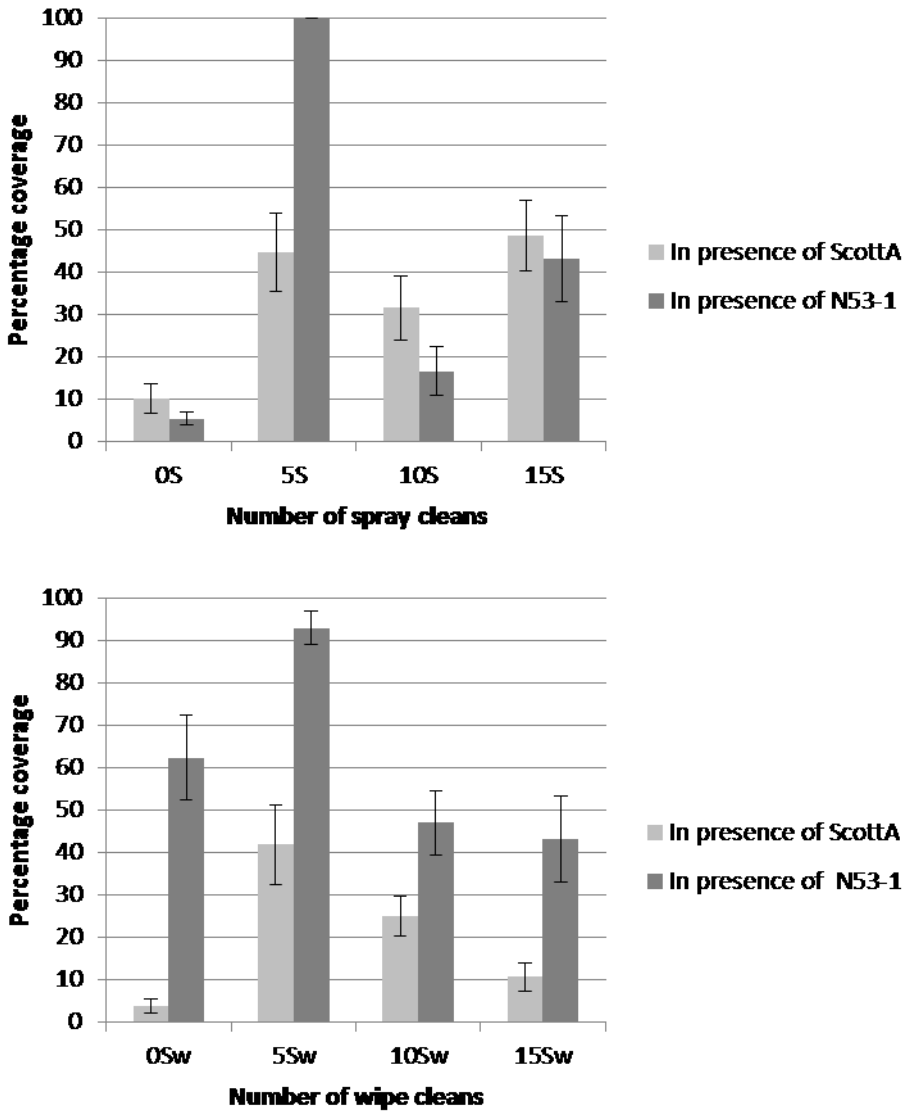

Fig. 8. Percentage coverage of organic material retained on the surfaces following spray (S) and spray with wipe (Sw) assays a) fish extract (in the presence of *L. monocytogenes* Scott A) and b) fish extract (in the presence of *L. monocytogenes* N53-1) demonstrating different trends of organic material retention on the surfaces following the different cleaning methods