COMPARISON OF DETECTION METHODS USED TO DETERMINE ESCHERICHIA COLI AND MEAT EXUDATE REMOVAL FROM STAINLESS STEEL SURFACES FOLLOWING DIFFERENT PHYSICAL CLEANING METHODS

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ABSTRACT
Food products can be contaminated by residual organic materials and food-borne pathogenic microorganisms through contact with biofouling present on surfaces. Efficient cleaning is needed to maintain hygienic requirements and for quality assurance of food contact surfaces. To evaluate the efficacy of cleaning procedures, it is essential to use reliable detection and quantification methods that can detect both organic material and microorganisms retained on surfaces.
Repeated fouling with both organic material (meat exudate) and microorganisms (Escherichia coli) on 304 2B finish stainless steel surfaces was carried out. The surfaces were then cleaned using either a soak, spray or wipe method (in water) in order compare various biofouling detection methods.
Following enumeration of the microorganisms by plate counting, the results demonstrated that the soak and spray washes were the best cleaning methods, whereas the wipe clean produced the least hygienic surface. ATP bioluminescence further demonstrated that the spray cleaned surface was the most hygienic followed by the soaked cleaned surfaces. However, percentage coverage counts demonstrated that the number of retained cells on following the soak wash was the greatest (77.38 % after 30 washes) and the amount of organic material retained was greater than 50% on all the surfaces, and was not significantly different between the different types of cleans or number of washes. Visualisation of the surfaces using epifluorescence microscopy and scanning electron microscopy demonstrated that the soak clean was the least hygienic in terms of bacterial retention. This suggests that the biofouling on the surface was difficult to remove following the soak cleaning method. UV detection demonstrated that it was difficult to detect organic material, regardless of the cleaning method used. However, when using more intense UV at selected wavelengths, the 330 nm - 360 nm illuminated the retained biofouling on the surfaces with the greatest intensity.
The use of the different cleaning assays resulted in differences in cell and organic material distribution across the surfaces. The recorded level of contamination varied depending on the detection method used in this study. Our results highlighted that, in addition to the quantification method, visual images and quantification may help to better understand the fouling process on surfaces since certain cleaning methods may result in organic material being difficult to remove and detect.

INTRODUCTION

The contamination of food contact surfaces by residual organic materials and food-borne microorganisms in the food processing industries has been an ever-persistent issue (Whitehead and Verran, 2015). In the food industry, open food contact surfaces present a solid-air or solid-liquid-air interface with organic materials that may be favourable for microbial attachment, retention and subsequent biofilm formation (Verran et al., 2008). The presence of organic material on a surface will affect cell–substratum interactions (Verran and Whitehead, 2006).

Such biofouling may result in increasing or decreasing subsequent cell viability and surface cleanability. Some food-borne pathogens, such as *Escherichia coli,* can grow in biofilms and then contaminate food products in food industries (Bridier et al., 2015). Many strains of Shiga toxigenic *E. coli* are human pathogens that cause illness ranging in severity from mild diarrhoea to severe renal complications, which can result in death (Hunt, 2010).

In order to maintain hygienic requirements and quality assurance there is need for regular and efficient cleaning and disinfection regimes (Otto et al., 2011). However, prior to develop efficient cleaning and disinfection methods, it is essential to develop efficient methods to detect and/or enumerate organic materials and microorganisms on surfaces (Whitehead et al., 2015). The effectiveness of cell and organic material biofouling detection and quantification depends on the methods used to remove organisms from the surface, and the ability to recover and quantify them. A number of studies have compared the efficacy of different sampling methods for food contact surfaces (Gómez et al., 2012; Kovacevic et al., 2009; Nyachuba and Donnelly, 2007; Vorst et al., 2004). In addition, a number of methods have been suggested for both *in vitro* and *in situ* assessment of organic food material and cells on surfaces (Verran and Whitehead, 2006; Verran et al., 2002). Many researcher have evaluated the hygienic status of a surface in terms of retained bacteria and organic material using such methods as ATP bioluminescence (Davidson et al., 1999; Whitehead et al., 2008), UV illumination (Whitehead et al., 2008), scanning electron microscope (SEM) (Gounga et al., 2007) or epifluorescent microscopy (Whitehead et al., 2009).

ATP bioluminescence is a well-established and rapid method of detecting materials of cellular origin on solid surfaces (Amodio and Dino, 2014). Epifluorescence microscopy allows the detection of both microorganisms and organic materials on surfaces. This technique involves the use of differential stains which aids imaging of cell:substratum attachment, distribution and retention in the presence and absence of organic material (Whitehead et al., 2009). Scanning electron microscopy (SEM) has been widely employed in the study of bacterial interactions because it allows the detection and visualisation of bacteria at specific surfaces, including host cells and bacterial colonies (An and Friedman, 1997). However, a distinct disadvantage of SEM in the study of bacterial interactions is that it is usually qualitative (Knutton, 1995). UV illumination can be used for monitoring cells:soil interaction on industrial surfaces (Whitehead et al., 2008).

As far as the authors are aware, these methods have never been compare to evaluate the impact of different cleaning methods in a same study. Therefore, the aim of this work was to determine the efficacy of three physical cleaning methods in water by the detecting the residual meat organic material and bacteria (*E. coli*) on stainless steel surfaces using a range of industrial and laboratory detection methods.

EXPERIMENTAL

Meat exudates (method kindly provided by Brigitte Carpentier AFSSA, France)

One kilogram of fresh rolled beef brisket (CO-OP, UK) was cut into 10 mm x 10 mm pieces, put into a stainless steel tray and covered in foil. This was covered by another stainless steel tray of the same size and weighed down with 8.4 Kg of stainless steel sheets and frozen at -20 °C for 24 h. The meat was defrosted at room temperature, the meat exudates were poured off and the meat squeezed to recover surplus exudates. The meat exudates were stored in 20 mL aliquots at -20 °C until needed.

Escherichia coli

A non pathogenic strain of *E. coli* 0157:H7 was a kind gift from Dr Brigitte Carpentier (Agence Française de Sécurité Sanitaire des Aliments (AFSSA), Maisons-Alfort, France), selected since it is a non-pathogenic *E. coli* O157:H7 wild type strain that does not carry stx1 and stx2 genes. This strain was recovered from heifers faecal samples by the laboratory of Dr C. Vernozy-Rozand (Vernozy-Rozand et al., 2000).

In preparation for retention assays, stock cultures of *E. coli* were defrosted at room temperature and a loop of liquid was taken and streaked out onto brain heart infusion agar (BHIA) (Lab M UK). The *E. coli* was grown at 37 °C for 24 h and then cultures were stored at 4 °C. One hundred microliters of BHIB were inoculated with a single colony of *E. coli* and incubated at 37 °C overnight. Following incubation, cells were harvested by centrifugation at 716 x *g* for 10 min and were washed once, by re-suspension in sterile distilled water, vortexing for 1 min, and then the cells were collected by centrifugation at 716 x *g* for 10 min. Cells were re-suspended to an OD of 1.0 at 540 nm in sterile distilled water. Colony forming units mL^{-1} (cfu mL^{-1}) were determined by serial dilution and were $1.07 \pm 0.84 \times 10^8$ cfu mL^{-1}.

Application of meat exudate and bacteria to surfaces
Meat exudates and *E. coli* cells were mixed in a 1:1 ratio in an eppendorf tube by vortexing for 5s. Twenty five microliters of preparation was pipetted on to a 20 mm x 20 mm unused 304 2B finish stainless steel plate, and spread across the surface with a sterile plastic spreader.

Cleaning assays
Soak clean
Samples were soaked in 1 L of room temperature sterile distilled water for 2 min, followed by immersion for 10 min at 60 °C in 1 L tap water and then were soaked in a third water bath in 1 L of room temperature sterile distilled water for 2 min. Following cleaning, samples were re-fouled and dried in a class 2 flow hood. They were put through the cleaning system again for 30 soak cleans.

Wipe clean
A piece of cloth (WYPALL® x80, Hydroknit®, cleaning clothes - folded blue, Kimberley-Clark UK) was cut into 45 mm x 45 mm pieces. One piece of 45 mm x 45 mm cut cloth was attached to the 16 mm diameter test finger of the crockometer (AATCC Crockometer, ALPLAS Technology, UK) and 1 ml of water was applied. Each sample was wiped 10 times, with 2 passes of the crockometer counting as 1 time. After four samples another 1 ml of sterile distilled water was added to the cloth. The cloth was changed every 10 samples. Following each clean the sample was re-fouled, and dried before being re-cleaned.

Spray clean
Three replicate substrata were fixed onto a stainless steel tray using adhesive gum (Impega, Malaysia). The tray and attached substrata were placed vertically into a microbiological class 2 hood. Two hundred milliliters of sterile distilled water was placed into the spray reservoir of a spray gun (Clarke Indy, UK). The spray gun was set to the 30 psi and at a distance of 10 cm the spray was used to clean the sample by spraying each one for 5 s. Immediately following spraying, samples were re- fouled and dried.

Detection methods
Recovery of bacteria using plate counting
Following cleaning the samples were swabbed using a sterile swab that had been moistened in a fresh blank of 10mL diluent (sterile distilled water). The 20 mm × 20 mm area of stainless

steel was swabbed using the swab in unidirectional strokes (10 vertically and 10 horizontally). The head of the swab was snapped off into the 10 mL diluent and was vortexed for 10 s and following serial dilutions were incubated for 24 h at 37 °C on BIHB where after the number of colony forming units per milliliter (CFU mL^{-1}) were calculated.

ATP bioluminescence

ATP bioluminescence (Hygiena, Herts, UK) measurements were carried out as per manufacturer's instructions (Hygiena, 2007), with the exception that in this study, coupons of 20 mm × 20 mm area were used and the cells and organic soil were applied to this area, rather than the manufacturer recommendation of a swab area of 100 mm × 100 mm. The 20 mm × 20 mm area of stainless steel was swabbed using the Ultrasnap™ ATP surface test swabs (Herts, UK) in unidirectional strokes (10 vertically and 10 horizontally). The swab was activated by snapping the valve and expelling the liquid by squeezing the bulb twice and shaking the device for 10 seconds. The Ultrasnap™ swab were then placed in the SystemSURE II luminometers device (Herts, UK) according to the manufacturer's instructions and the results were recorded.

Differential staining of meat exudate and bacteria and epifluorescence microscopy

4,6-Diamidino-2 Phenylindole (DAPI) (Sigma, UK) was made into a stock solution in water at a concentration of 0.3 g mL^{-1}. It was diluted for use to 0.1 g mL^{-1}. Rhodamine B (Molecular Probes, UK) was made into a stock solution by dissolving in 70 % ethanol at a concentration of 1 g mL^{-1} and was used at a concentration of 0.1 mg mL^{-1}. Solutions were stored at 4 ºC in the dark. Ten microliters of the 0.1 g mL^{-1} DAPI solution was pipetted onto the substrata with following cleaning and spread across the surface using a plastic L shaped spreader, then left to dry in the dark in a class 2 microbiological cabinet. After 10 minutes, the surface was gently washed for 5 s with sterile organic filtered purified water with a bottle at a 45° angle, with a 3 mm nozzle. Ten microlitres of 0.1 mg mL^{-1} rhodamine B solution was immediately applied to the surface and spread washed and dried as previously (Whitehead et al., 2009). The samples were then kept at 4 °C in the dark until analysed (n = 40). Substrata plus adherent organic material and/or microorganisms were visualized using epifluorescence microscopy (NikonEclipse E600, UK). The microscope was mounted with a HitachiHV-D37P colour camera (Nikon, UK) and using CellF software (Olympus, UK). The percentage coverage area of the stained components was measured to determine the retained organic material and cells. The UV wavelength band used was 330–380 nm (DAPI) and 590–650 nm (Rhodamine) (Whitehead et al., 2009).

Scanning Electron Microscopy (SEM)

For the preparation of test pieces for SEM, samples were immersed in 4 % v/v gluteraldehyde (Agar, UK) for 24 h at 4 °C, washed in sterile distilled water, dried and stored at room temperature in phosphorous pentoxide (Sigma, UK) desiccators. The samples were fixed to stubs for gold sputter coating (Polaron E5100 UK). Samples were sputter coated at a vacuum of 0.0921 mbar, for 3 min, at 2500 V, in argon gas at a power of 18 - 20 mA. Images of substrata were obtained using a JEOL JSM 5600LV SEM (Jeol Ltd, UK). Replicates were carried out in triplicate.

UV detection of cells and organic material

A standard UV lamp (15 W bench lamp, 365 nm, 1680 µW cm^{-2} at 305 mm, 115 VAC. 60 Hz, Cole-Parmer, UK) with an optimum wavelength of 350 nm was used for analysis of surfaces inoculated with cells ± soil and retained surface soil on the stainless steel coupon. The UV bench lamp was placed on top of an exposure stand and the lamp was switched on for 5 min prior to images being taken to allow the lamp to warm up. The sample coupons were attached

to the bottom of the UV exposure stand using adhesive tack (Blu tack, UK). The coupons were placed at a 45° angle to the base of the exposure stand and images were taken using a Nikon coolpix 4500 (4.0 mega pixels with a 4 x optical and 4 x digital zoom) digital camera (Nikon UK Ltd., UK)

Detection of material using intense UV and different wavelength filters
In order to determine the optimum wavelengths of the UV to illuminate the organic material, fouled samples were visualised using an epifluorescence microscope described earlier. Different filters, 330 – 380 nm, 510 – 560 nm and 590 – 650 nm were used to select specific wavelength bands of UV. Dried but unstained samples were visualised under the differently filtered UV wavelength bands and imaged.

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 t-tests values. The statistical confidence interval was considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

In order to compare the detection methods to determine the retention of *E. coli* and meat exudate on the stainless steel substrata following the cleaning assays, surfaces were analysed using a range of techniques.

Recovery of bacteria using plate counting
Cells were recovered from the inoculated but un-cleaned surfaces using sterile swabs. The numbers recovered were low from all the 0 washed surfaces, (around 10^3 CFU mL^{-1} for soak and spray washes, and around 10^4 CFU mL^{-1} for wipe wash) even though 10^7 - 10^8 CFU mL^{-1} bacteria had been inoculated onto the stainless steel (Figure 1). Following the 10, 20 and 30 soak or spray washes, no cells were recovered from the surfaces. Between the 20 and 30 wipe wash there was a slight increase in the numbers of the cells recovered from the surfaces, approximately 10^3 CFU mL^{-1}. The results demonstrated the different cleaning assays resulted in differences in cell recovery from the surface in water. The soak and spray washes were the best cleaning methods, whereas the wipe clean produced the least hygienic surface. However, this detection method had two main limitations: the results were obtained only after one or two days, and the bacteria numbers may be lower since some cell remained trapped on the surface of possibly in the swab tip, resulting in surfaces seeming cleaner than they actually were.

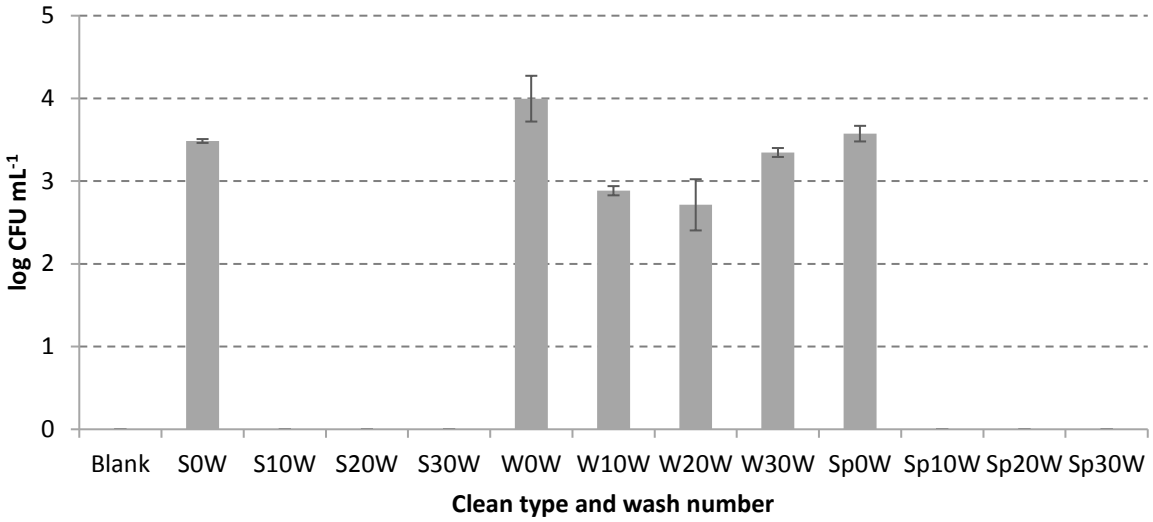

Figure 1. Number of CFU mL^{-1} following swabbing and serial dilution recovered from surfaces after soak, wipe or spray clean 0 – 30 washes (n = 8). S0W = Soak clean 0 wash, S10W = Soak clean 10 washes, S20W = Soak clean 20 washes, S30W = Soak clean 30 washes, W0W = Wipe clean 0 wash, W10W = Wipe clean 10 washes, W20W = Wipe clean 20 washes, W30W = Wipe clean 30 washes, Sp0W = Spray clean 0 wash, Sp10W = Spray clean 10 washes, Sp20W = Spray clean 20 washes and Sp30W = Spray clean 30 washes.

ATP Bioluminescence

ATP bioluminescence operates by measuring the amount of ATP removed on a surface by calculating the amount of light generated based on a luciferine/luciferase reaction. The ATP bioluminescence method relies on the swab efficiently removing the bacteria and assumes that the tested surface is representative of a much larger, possibly inaccessible, area of manufacturing plant (Brooks and Flint, 2008).

In the present study, ATP bioluminescence demonstrated that on the control surfaces, there was an ATP bioluminescence reading produced higher than 30 (Blank = 47.00 ± 8.53 relative light units), which according to the manufacturer's instructions deemed the surfaces needed cleaning (Figure 2). With the increase in soak washes, the ATP values gradually increased, although this was not reflected by the CFU mL^{-1} results. For the wipe cleans, there was no significant difference between the ATP bioluminescence values for the different number of wipe cleans carried out. Following the spray clean a different trend was again determined whereby all following the 10, 20 or 30 spray clean, the surfaces recorded relatively low ATP bioluminescence values. According to this method, in agreement with the swabbing and colony forming units method, the wipe cleaned surface was the least hygienic followed by the soaked cleaned surfaces. The spray method gave the best cleaned surfaces.

All three different cleaning methods gave a different pattern of ATP bioluminescence results. Further, the ATP bioluminescence replicates following the different cleaning methods demonstrated high variability of the results (49 to 674 RLU). This is interesting because if only one ATP measurement is carried out, the surfaces might be perceived to be relatively clean. Indeed, if organic material is retained in surface features and is not removed during swabbing prior to (indirect) assessment, this method may result in a hygiene pass level despite the presence of unwanted organic material (Whitehead et al., 2008). However, direct assessment

from the surface has been suggested as a solution for this inaccuracy caused by swabbing (Storgårds, 2000).

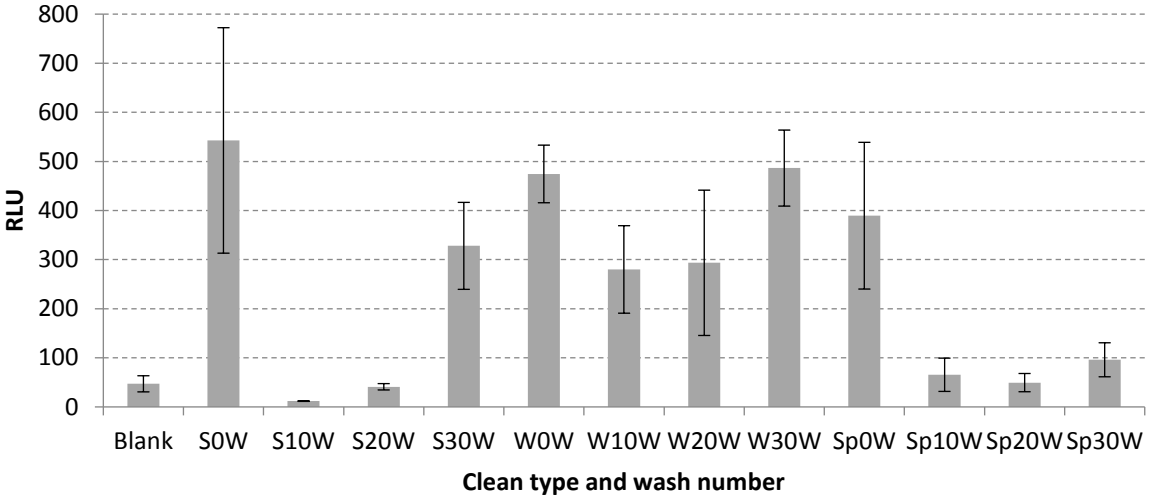

Figure 2. ATP bioluminescence results of surfaces after soak, wipe or spray clean 0 – 30 washes (n = 8). S0W = Soak clean 0 wash, S10W = Soak clean 10 washes, S20W = Soak clean 20 washes, S30W = Soak clean 30 washes, W0W = Wipe clean 0 wash, W10W = Wipe clean 10 washes, W20W = Wipe clean 20 washes, W30W = Wipe clean 30 washes, Sp0W = Spray clean 0 wash, Sp10W = Spray clean 10 washes, Sp20W = Spray clean 20 washes and Sp30W = Spray clean 30 washes.

Epifluorescence microscopy

Epifluorescence microscopy relies on differential staining of particular organic material (Verran and Whitehead, 2006), and of microorganisms (Declerck et al., 2003). Unlike SEM, this *in situ* staining technique permits to visualise and quantify the amount of cells and organic material that was retained on the surfaces rather than what was removed from them (Whitehead et al., 2009). Moreover, epifluorescence microscopy has the significant advantage that the results are immediate, ensuring that a true representation of numbers of cells present is obtained within the limits of detection of the method. However, staining methods can sometime be difficult to optimise.

In the present study, differential staining of the surfaces and quantification using percentage coverage of the cells and the organic material retained was carried out following the three cleaning methods. The results demonstrated that cell retention increased with the number of soak cleans, however most were removed from the surfaces following the wipe and the spray cleans (Figure 3a). There was no significant difference in the amount of organic material retained on the surfaces regardless of the type of clean used (Figure 3b). The percentage coverage of organic material retained was great in number and varied in distribution. This suggests that the mechanical action of the wipe and spray clean removes cells from the surface, but not organic material. Further, the amount of organic material did not alter with increased washes. It might be suggested that the organic material, once retained maintains a steady state of fouling, which is then difficult to remove.

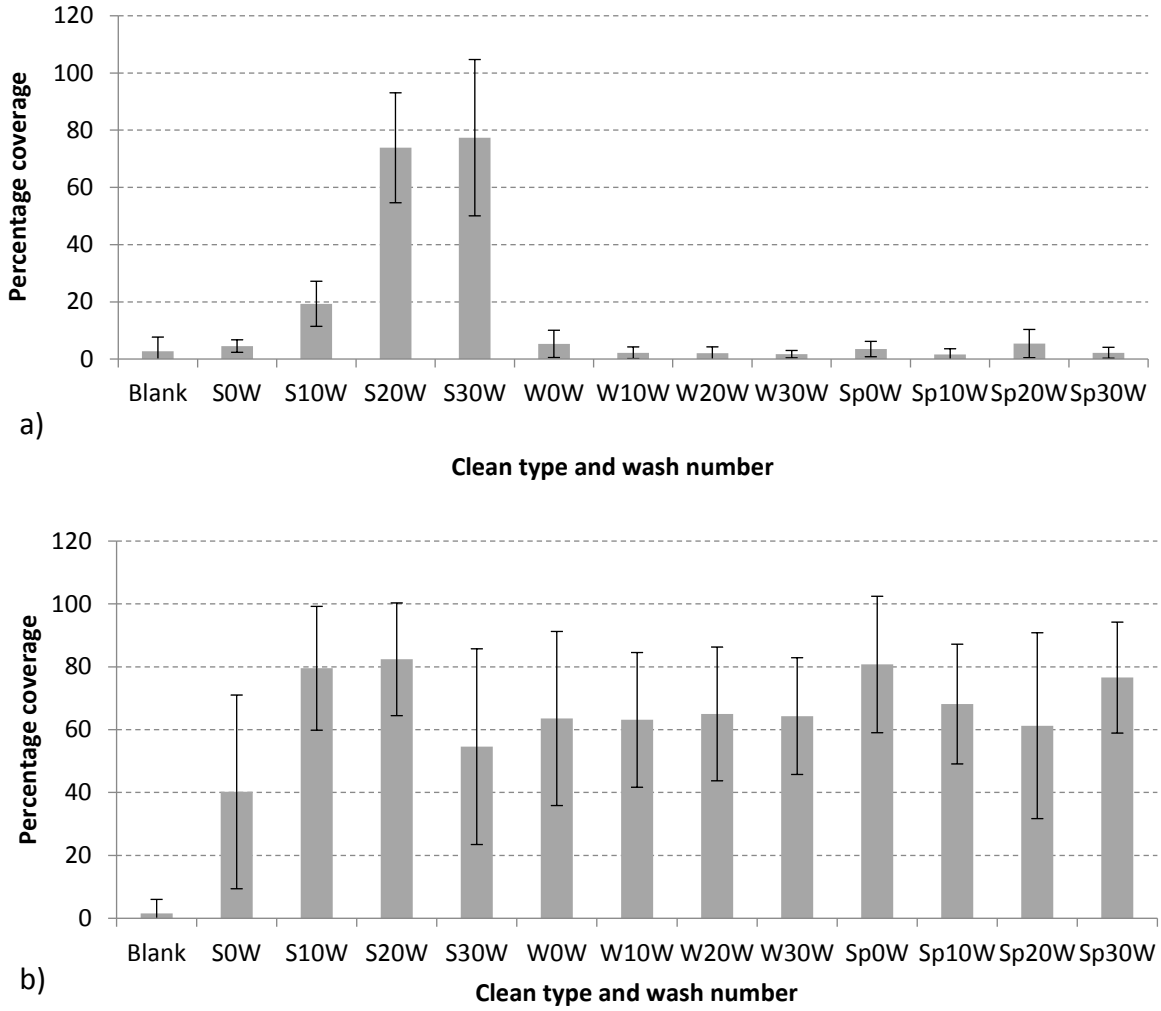

Figure 3. Percentage coverage for a) cells and b) meat extract using differential staining following 0 – 30 washes in water using a soak, wipe and spray clean (n = 40). S0W = Soak clean 0 wash, S10W = Soak clean 10 washes, S20W = Soak clean 20 washes, S30W = Soak clean 30 washes, W0W = Wipe clean 0 wash, W10W = Wipe clean 10 washes, W20W = Wipe clean 20 washes, W30W = Wipe clean 30 washes, Sp0W = Spray clean 0 wash, Sp10W = Spray clean 10 washes, Sp20W = Spray clean 20 washes and Sp30W = Spray clean 30 washes.

The results for the cells retained on the surfaces demonstrated a different trend to the results for the assays which are dependent on cell recovery from a surface. In these results, the soak clean would be determined to be the least hygienic in terms of bacterial retention, yet it is not known if the cells retained on the surfaces are viable. Imaging of the differential staining demonstrated that the distribution following the soak clean (Figure 4 a-d) of the organic material was more heterogeneous across the surfaces than was observed following the wipe (Figure 4 e-h) and spray clean (Figure 4 i-l), however this could only be evidenced from the images, and emphasizes the importance of visual images alongside numerical data. Following the soak cleans, the cells were heterogeneously distributed across the surface, whereas following the soak and the spray cleans, cells and organic material could be observed to be retained in the grain boundaries of the stainless steel. Thus, visualisation of the surfaces demonstrated that the type of cleaning method used influenced the pattern of both organic material and cell retention. The mechanical cleaning action of the wipe and spray cleans pushed material into surface

defects where it was retained, whereas the soak clean left cells and organic material in clumps on the surface.

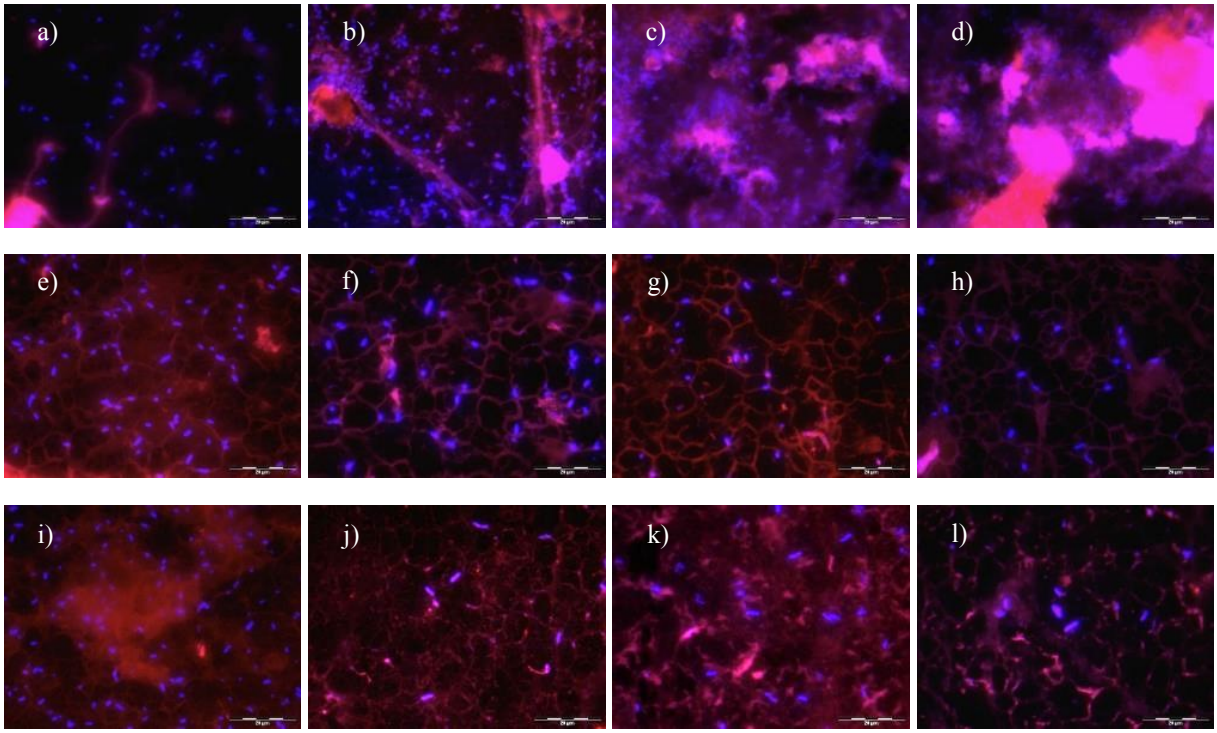

Figure 4. Differential staining of *E. coli* (blue) and meat extract (red) showing retained cells and organic material on stainless steel surfaces following soak clean a) 0, b) 10, c) 20 and d) 30 washes, wipe clean e) 0, f) 10, g) 20 and h) 30 washes and spray clean i) 0, j) 10, k) 20 and l) 30 washes.

Scanning Electron Microscopy of the substrata

SEM analysis of the surfaces (Figure 5) confirmed the observations seen following differential staining; however, results from the differential staining and image analysis gave quantitative and more informative qualitative data. It was sometimes difficult to detect the presence of residual organic material especially when it was retained in the grain boundaries on the less heavily fouled surfaces. Although SEM provide qualitative visualisation of the bacteria and conditioning films, the requirement for specialist equipment, training and extensive preparation of samples further limits its application due to its high financial demand and complexity in operation.

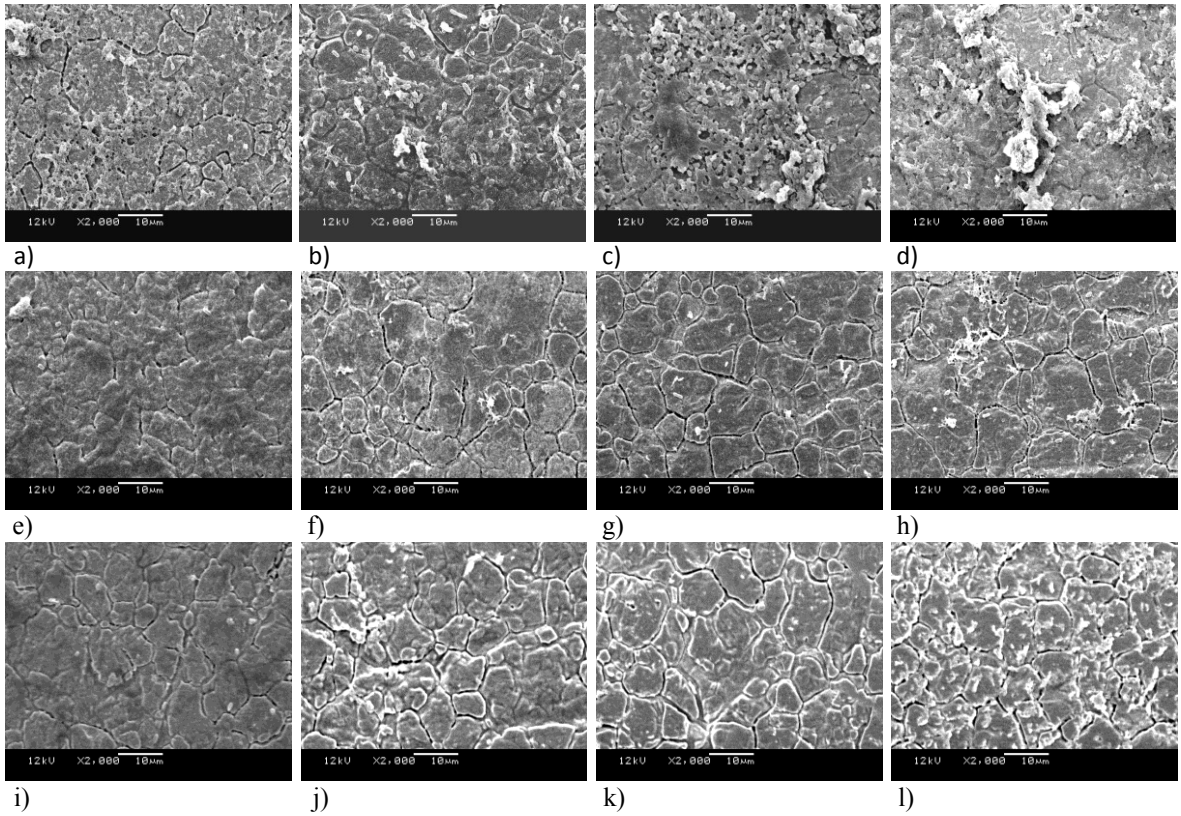

Figure 5. SEM images of surfaces following soak clean a) 0, b) 10, c) 20 and d) 30 washes, wipe clean e) 0, f) 10, g) 20 and h) 30 washes and spray clean i) 0, j) 10, k) 20 and l) 30 washes.

UV detection of cells and meat exudates retained on the substrata

The molecular configuration of organic material allows some organic residues to fluoresce when illuminated with UV at a wavelength of approximately 353 nm (Adhikari and Tappel, 1975). In the present study, the surfaces were also illuminated using UV light following the different cleans (Figure 6). Neither the cells nor organic materials were detected on the surfaces following the washes. It suggests that either the level of surface contamination is too low for detection using this method, or the UV wavelength should be optimized.

Indeed, a UV wavelength of between 330-380 nm were found to be ideal for detection of residual soils in meat and cheese industry while higher wavelengths were required for residual soils from fish industry (Whitehead et al., 2008). This highlights the need to optimise and standardize the wavelength used in the UV spectra for the assessment of different food soils (Whitehead et al., 2010). Similarly, it is difficult to make distinction between the cells and soil using the technique as also seen with ATP bioluminescence. UV illumination does not require direct contact with the surface as with ATP bioluminescence, which gives the technique the advantage of being non-invasive. UV may be used to detect residual soil on contact surfaces, highlighting areas in an industrial plant that need a more rigorous cleaning regime.

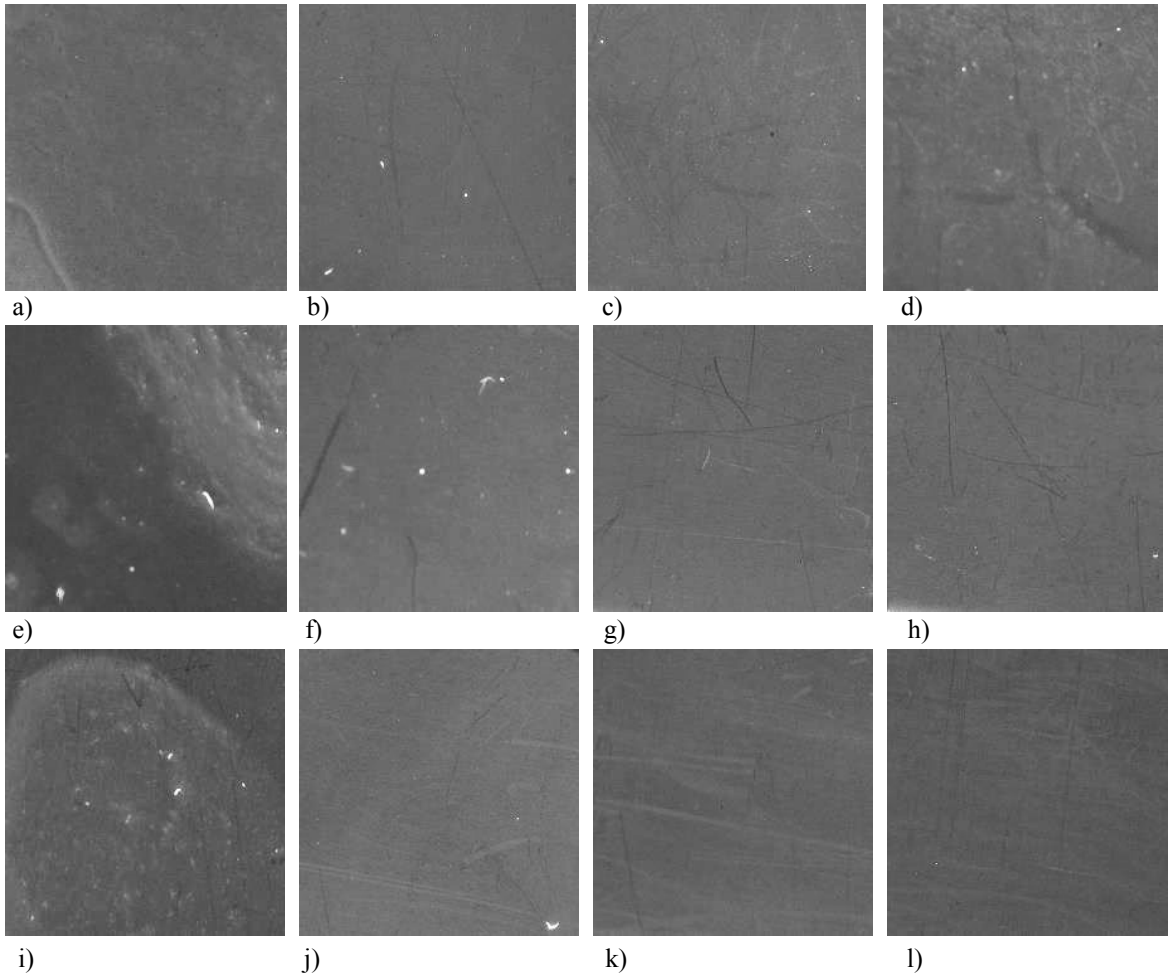

Figure 6. Images of surfaces following UV detection (350 nm) of residual material soak clean a) 0, b) 10, c) 20 and d) 30 washes, wipe clean e) 0, f) 10, g) 20 and h) 30 washes and spray clean i) 0, j) 10, k) 20 and l) 30 washes.

Defined UV detection of cells and meat exudates

The surfaces were visualised under different wavelengths of UV in order to determine which gave the best detection of residual material (Figure 7). It was demonstrated that following 10 cleans, regardless of the cleaning method used, the surfaces demonstrated the most fluorescent visualisation of retained material. In all cases, the UV wavelength of 330 nm – 380 nm gave the best results.

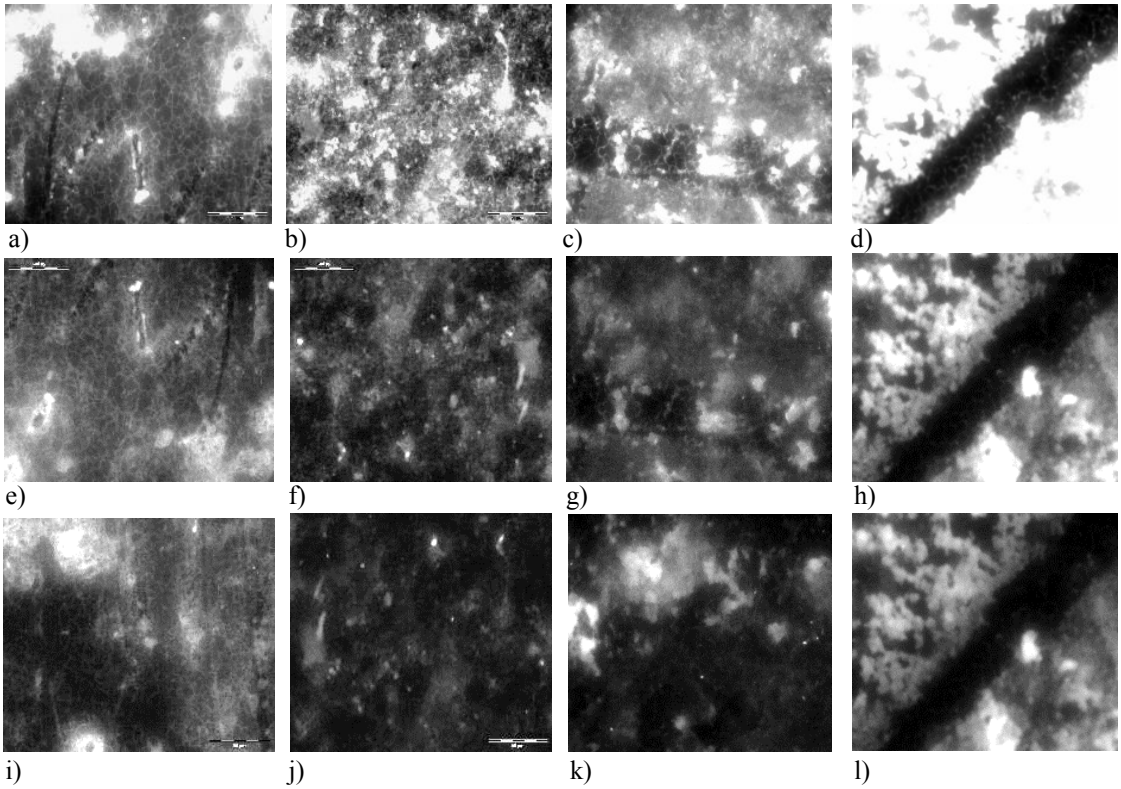

Figure 7. Images of surfaces following different wavelengths of UV after cleaning using the soak method a) 0, b) 10, c) 20 and d) 30 washes, 330– 380 nm e) 0, f) 10, g) 20 and h) 30 510–560 nm and i) 0, j) 10, k) 20 and l) 30 washes 590–650 nm.

CONCLUSIONS

These results demonstrate the different cleaning assays resulted in differences in cell and organic material distribution across the surface. When using conditioning films assays, careful consideration needs to be given to the methodologies used; visual images may help elucidate and understand the fouling process. Furthermore, these visual detection techniques would be time effective when employed in large scale for the assessment of cleaning regime used in food industry as compared to the traditional plate count method.

Although the impact of organic materials in bacterial retention cannot be overlooked, there is need to devise a quantitative detection method that would account for cell viability following different cleaning regimes since it is mainly the activities of these viable cells that plays a vital role in the various stages of biofilm formation.

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