Microbiology

http://www.aimspress.com/journal/microbiology

AIMS Microbiology, 2(3): 340-358.
DOI: 10.3934/microbiol.2016.3.340
Received: 19 May 2016
Accepted: 14 August 2016
Published: 18 August 2016

Research article

Transfer of bacteria between stainless steel and chicken meat: A CLSM and DGGE study of biofilms

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Abstract: This study aimed to assess the interaction between bacteria and food processing surfaces using novel methods. Microbial cross contamination between stainless steel, a common food processing material, and raw chicken was studied using microbiological culture, specialized microscope and molecular techniques. Confocal laser scanning microscopy (CLSM) allowed the visualization of biofilms containing single or dual species of *Escherichia coli* O157:H7, *Salmonella typhimurium*, *Bacillus cereus*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, formed after 6 days' incubation on stainless steel or 4h on raw chicken. The results provided information on intra-biofilm location and stratification of species within dual species biofilms. Top-to-bottom Z-stack images revealed that, on both materials, *S. typhimurium* and *E. coli* attached concurrently, the former in greater numbers. *E. coli* and *B. cereus* segregated on steel, *E. coli* more frequent near the metal surface, *B. cereus* almost the only species in outer layers. Few cells of *S. aureus*, found at all depths, were seen in the 2.9 µm thick biofilm on steel with *E. coli*. Greatest attachment was shown by *P. aeruginosa*, followed by *S. typhimurium*, *E. coli* and finally Gram positive species. Large amounts of EPS in *P. aeruginosa* biofilms made visualization difficult on both materials, but especially on chicken meat, a limitation of this technique. Nevertheless, CLSM was useful for determining time sequence of adhesion and species makeup of thin biofilms. The technique showed that five min contact between bacterially-contaminated chicken and sterile steel resulted in greatest transfer of

P. aeruginosa, followed by *S. typhimurium*. This was confirmed using DGGE. Gram positive bacteria transferred poorly. A biofilm containing 2.3 × 10^5 cfu·cm^{-2} *B. cereus* on steel transferred an undetectable number of cells to chicken after 5 min contact. This species was unable to form biofilm on chicken when incubated for 4 h in growth medium. *S. typhimurium* and *P.aeruginosas* were most efficiently transferred from contaminated steel to raw chicken within 5 min contact, with 20–30% transfer from single species biofilms. All other species, and all cells in dual species biofilms, showed less than 2% transfer. CLSM and DGGE were shown to be useful techniques for the study of bacterial adhesion to stainless steel.

Keywords: biofilms; CLSM; cross-contamination; food-processing surfaces; stainless steel

Abbreviations

AISI	American Iron and Steel Institute;	*AO*	acridine orange;
BSA	bovine serum albumin;	*CFU*	colony-forming units;
DAPI	4,6-diamidino-2- phenylindole;	*DNA*	desoxyribonucleic acid;
OCT	optimal cutting temperature compound;	*OD*	optical density;
LB	Lysogeny Broth (or Luria-Bertani medium);	*PTFE*	polytetrafluoroethylene;
CLSM	confocal laser scanning microscopy;		
DGGE	denaturing gradient gel electrophoresis;		
DABCO	1,4-diazabicyclo[2.2.2]octane;		
EPS	extracellular polymeric substances;		
FITC	fluorescein isothiocyanate;		
PBS	phosphate-buffered saline;		
HDPE	high density polyethylene;		
PCR	polymerase chain reaction;		

1. Introduction

Microbial contamination of food and food contact surfaces is the most common cause of compromised food product safety. Bacteria, including foodborne pathogens, grow predominantly as biofilms in both natural and industrial environments and biofilm development on food processing equipment is a source of contamination that may lead to transmission of disease or spoilage organisms [1]. Cells attached in biofilms can have considerably increased persistence in the factory environment [2]. Such biofilms can thus lead to serious hygienic problems and economic losses due to food spoilage [3,4]. In the modern world, many foods are extensively processed in industrial settings. The mass production of foodstuffs, lengthy production cycles, and large surface areas available for biofilm development select for biofilm forming bacteria on food-contact surfaces.

Methods used for the study of bacteria in biofilms range from traditional techniques involving removal, suspension and plating, to direct microscopy in situ; researchers have begun to identify the genes involved in the formation of bacterial biofilms, generally using single bacterial cultures [5,6,7].

Dual species inocula have rarely been used in basic laboratory studies of biofilm formation. There is some information available in the literature about mixed species biofilms in the food processing industry [8–11], but this is generally orientated to studies of specific pathogenic bacterial species.

The rate at which biofilm microorganisms pass from one surface to another in contact with it depends on the type of microorganism, the nature of the surface and the physicochemical conditions. For example, stainless steel has been shown to contaminate foods with *Listeria monocytogenes* more readily than does high density polyethylene [12] and both stainless steel and Formica transferred *Salmonella typhimurium* to cucumber slices more readily than did polypropylene or wood [13]. Conversely, *S. aureus* was found to transfer from contaminated chicken meat to food-processing surfaces at a similar rate, independent of the nature of the material [14] and thus exact factors affecting transfer may not always be identical. For example, the presence of calcium can increase adhesion of *Staphylococcus sciuri* and *Pseudomonas fluorescens* to stainless steel [15]; when biofilms are allowed to dry onto a food processing surface for just one hour the subsequent transfer rates to a food are substantially reduced [16].

In this paper, we report the use of confocal laser scanning microscopy (CLSM) in conjunction with specific fluorescent dyes, denaturing gel electrophoresis (DGGE) and traditional microbiological culture techniques to study the dynamics of dual species biofilm formation on stainless steel and raw chicken meat, and the efficiency of transfer between the two surfaces.

2. Materials and Methods

The methodology used is shown schematically in Figure 1.

2.1. Bacterial strains

Strains of typical food-poisoning bacteria, *Escherichia coli* O157:H7 (NCIMB 50139), *Bacillus cereus* (NCIMB 8012), *Staphylococcus aureus* (NCIMB 9518), *Pseudomonas aeruginosa* (NCIB 8295) and *Salmonella typhimurium* (NCIMB 13284), were used for the development of single or dual species biofilms with *E. coli* as the common component. Strains were obtained from the University of Portsmouth, School of Pharmacy and Biomedical Sciences bacterial collection. Stock cultures were grown for 24 h at 37 ^{o}C on slopes of appropriate selective agars, MacConkey agar, MacConkey sorbitol agar, *Bacillus cereus* agar base, *Staphylococcus* medium N110, *Shigella-Salmonella* agar (SS) and *Pseudomonas* Agar Base, all from Oxoid, Basingstoke, and were stored at 4 ^{o}C for up to 8 weeks. Cultures were routinely checked for purity by plating and incubating plates aerobically and anaerobically at 37 ^{o}C for up to 5 days.

2.2. Stainless steel coupons

Coupons (1 cm × 1 cm × 0.16 cm), cut from AISI 316 stainless steel in the University of Portsmouth workshop, were used as substratum for the development of biofilms. Polytetrafluoroethylene (PTFE) stubs (1 cm length × 1 cm diameter), cut from a 2 m long PTFE rod, served as support. Two separate grooves (0.2 cm width and 0.2 cm depth) were cut on one of the flat

sides of each stub. The distance between the grooves was 0.5 cm. Stubs were sterilised by autoclaving in watertight glass containers. Stainless steel coupons were immersed in 70% (v/v) aqueous methanol and flamed. Two of the coupons were fitted, using flame-sterilized forceps, into a sterile PTFE stub and the two-coupon assemblies were placed vertically inside glass universal bottles containing 20 ml of sterile LB medium. All procedures were performed aseptically in a laminar flow cabinet.

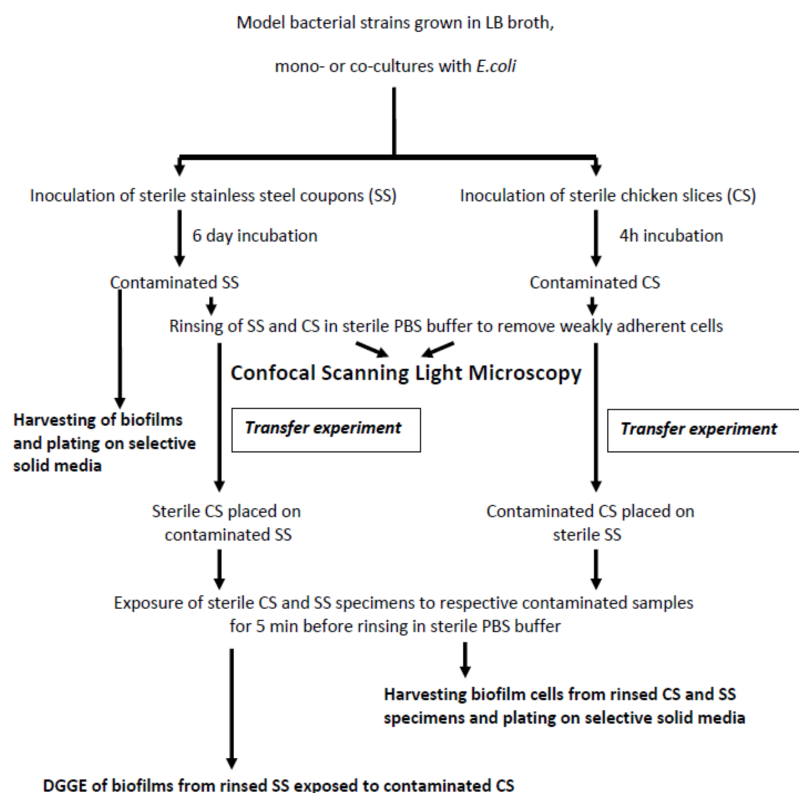

Figure 1. Flow chart of experimental design

2.3. Biofilm production on stainless steel in bacterial cultures

Bacteria were sub-cultured from 1-day old agar plates into 20 ml of Lysogeny Broth (Luria-Bertani medium) and incubated for 24 h at 37 ^{o}C on a rotary shaker at 200 rpm. Cell suspensions were adjusted with LB medium, using OD measurements at 600 nm followed by total cell counting in an improved Neubauer haemocytometer, to give a final concentration of 10^5 cell·ml^{-1} for each species in coupon-containing bottles. These were left for single and dual species biofilms to form on the coupons in LB broth for 6 days at 37 ^{o}C. Controls consisted of coupons incubated in sterile LB medium. Triplicate coupons were used in each run and all biofilm formation experiments were repeated at least 5 times.

2.4. Production of biofilms on raw chicken slices

Raw chicken drumsticks were purchased from a local grocery store in Portsmouth. Skin and fat were removed and longitudinal meat sections (1 cm × 1 cm × 0.3 cm) were cut using a sterile scalpel. They were disinfected by treating with 3% hydrogen peroxide for 1h at room temperature and washed 3 times in sterile distilled water.

Overnight bacterial cultures grown as single and dual species in LB medium (as described above) were used as inocula for contaminating chicken samples. Slices of chicken were placed separately into universal bottles containing 20 ml of sterile LB medium and inoculated with 1ml of either pure or dual species bacterial cultures (final concentration 10^5 cells·ml^{-1}). Controls contained only sterile medium. All assays were incubated at 37 ^{o}C for 4 h.

2.5. Transfer between chicken and stainless steel surfaces

Following incubation, the contaminated chicken specimens were carefully removed from the bottles using heat sterilised metal forceps. They were rinsed by dipping into 200 ml sterile 0.9% PBS to remove loosely attached cells. Clean, heat-sterilised AISI 316 stainless steel (SS) coupons were placed in sterile Petri dishes and a segment of contaminated chicken placed on top of each and left for 5 min., this being deemed representative of the time for which the chicken might remain on the surface during processing.

Hydrogen peroxide treated and rinsed chicken slices were exposed for 5 min to control (sterile) steel coupons or to the various types of biofilm on coupon surfaces by placing one slice on each carefully rinsed steel surface.

Cells transferred from contaminated chicken to sterile SS and from contaminated SS to sterile chicken were determined as described in the next section. Triplicates were used in all cases.

2.6. Analysis of biofilm

2.6.1. Removal and culture

After rinsing SS coupons in 0.9% PBS to remove loosely attached bacterial cells, remaining cells were collected from the surfaces by scraping with a scalpel blade and washing with 1 ml 0.9% PBS. The resuspended cells were transferred to sterile 1ml Eppendorf tubes and the number of viable cells was estimated by preparing 10-fold serial dilutions and plating on appropriate selective solid medium, using drops of 100 µl of each dilution. Contaminated chicken slices were placed directly on selective solid media and left for 1 min. After incubation at 37 ^{o}C for 48 h, bacterial colonies were identified using fluorescent and specific immunostains as detailed in the following section. In the case of chicken slices, no quantitative measurements were made; the tests were intended merely to demonstrate the production of biofilms on chicken.

2.6.2. Epifluorescence microscopy on stainless steel surfaces

After rinsing in 0.9% PBS, coupons were fixed with 4% formaldehyde (Sigma Chemical Ltd, UK) for one hour at room temperature and gently washed three times with 0.9% PBS. They were then immersed in 1% PBS/bovine serum albumin (BSA) for 30 min at 37 ^{o}C. Cells were stained with non-specific fluorescent stains, 3,6-bis dimethylaminoacridium chloride (acridine orange, AO, 0.025%), which stains DNA green and RNA red, rhodamine, a viable cell stain, or 4,6-diamidino-2-phenylindole (DAPI; 1mg.ml^{-1} in 0.9% NaCl), which stains DNA blue, for 20 min in a light-tight box, followed by 3 rinses in PBS. Various stains were used to ensure that results were reproducible.

Specific stains were also used to enable more ready differentiation of species with similar morphology. Coupons with biofilms comprising *E. coli* and *B. cereus* were first stained with biotinylated α-E. coli antibody (Europa Bioproducts Ltd, Cambridge, UK) 1:50 (v:v) in 1% PBS/BSA at 37 ^{o}C for 30min and then with Streptavidin Texas Red (Vector Laboratories Inc. USA) 1:25 (v:v) in 100mM NaHCO3 buffer with 150mM NaCl (pH 8.5) at 37 ^{o}C for an additional 30 min. The coupons were then washed three times with 0.9% PBS and counterstained with nuclear counter stain, 1:50 (v:v) DAPI in 0.9% NaCl (Sigma Chemical Ltd, UK). After additional washing with 0.9% PBS (×3) the coupons were rinsed with sterile distilled water and prepared for viewing.

Coupons that contained dual species biofilms of *E. coli* O157:H7 and *S. typhimurium* were stained with α-*Salmonella* antibody labelled with FITC (Europa Bioproducts Ltd, Cambridge, UK) 1:50 (v:v) in 1% PBS/BSA at 37 ^{o}C for 30 min and washed three times with 0.9% PBS. The coupons were then incubated with biotinylated α-*E. coli* antibody for an additional 30 min, stained with Streptavidin Texas Red for a further 30 min and subsequently washed with 0.9% PBS and with sterile distilled water, prior to viewing.

After washing, coupons were placed onto glass microscope slides. A drop of anti-fading agent DABCO/Glycerol (Sigma Chemical Ltd, UK) was deposited on the surface of each coupon and cover slips were positioned over them. Coupons were examined with a Nikon-Eclipse E600 epifluorescence microscope under ×100 oil immersion objective. Images were acquired using a Leica DC Viewer with a digital camera linked to a dedicated computer. Ten randomly selected areas within the field of view were observed on each coupon and representative biofilm images were recorded. At least 3 coupons were examined for each microorganism/stain group.

2.6.3. Confocal laser scanning microscopy

2.6.3.1. Stainless steel coupons

Coupons stained as described above were examined under a Zeiss LSM 510 CLSM with a 63× objective and images were acquired at HeNe 543 nm laser / filter LP560 for Streptavidin Texas Red, Ar 488 nm laser/filter BP 505-530 for FITC and Diode 405 nm for DAPI. For acridine orange, default channel 2 was used with filter BP505-530 or LP505.

2.6.3.2. Chicken slices

Gently rinsed slices of contaminated chicken were transferred into a capsular mold that had been covered with Tissue-Tek® OCT (Sakura, USA). The capsules were placed in liquid nitrogen for 20 min and transferred to −70 °C freezer where they stayed overnight. Chicken samples were removed from the capsules and placed onto supports sprayed with Tissue-Tek®O.C.T. (Sakura, USA). Cryo-sections (5–7µm) were cut in a cryostat set at −20 °C (Leica, Germany). Cut sections were mounted on poly-L-lysine-coated glass microscope slides and air dried for 30 min at room temperature. The sections were fixed in 2% paraformaldehyde in 0.9% PBS for 10 min at room temperature, removed from fixing solution and washed twice with 0.9% PBS. After fixing and blocking with BSA, specimens carrying *E. coli* O157:H7 and a second bacterial species other than *Salmonella spp*. were stained with biotinylated α-*E. coli* antibody 1:50 (v:v) in 1% PBS/BSA at 37 °C for 30 min and then with Streptavidin Texas Red 1:25 (v:v) in 100 mM $NaHCO_3$ buffer with 150 mM NaCl (pH 8.5) at 37 °C for an additional 30 min. The coupons were then washed three times with 0.9% PBS, counterstained with DAPI and prepared for epifluorescence microscopy.

Specimens that contained dual species biofilms of *E. coli* O157:H7 and *Salmonella typhimurium* were stained with α-*Salmonella* antibody labelled with FITC 1:50 (v:v) in 1% PBS/BSA at 37 °C for 30 min and washed three times with 0.9% PBS. The coupons were then incubated with biotinylated α-*E.coli* antibody for an additional 30 min, stained with Streptavidin Texas Red for further 30 min and subsequently washed with 0.9% PBS and with sterile distilled water and handled as described above. All procedures were carried out at room temperature (approximately 21 °C).

Specimens were examined under a Zeiss LSM 510 CSLM as described previously.

2.6.4. DGGE of biofilms transferred from chicken to steel

In order to confirm the presence of target microorganisms on stainless steel, we deliberately selected DGGE rather than new generation sequencing methods (pyrosequencing or Illumina), since the bacteria were already defined species. Cell suspensions (0.9 ml) of pure and dual species biofilms removed from stainless steel coupons as described previously were centrifuged at 10,000 g for 15 min. Supernatants were discarded and pellets were used for DNA extraction using the QIAGEN DNeasy kit (QIAGEN, Germany), following the manufacturer's instructions. DNA concentration was determined at 260 nm. In some cases, biofilm cells were submitted directly to the PCR without prior DNA extraction.

To aliquots of 10 µl or 20 µl of chromosomal DNA or biofilm cells (appro$\times 10^7$) were added 5 µl of 10× PCR reaction buffer (Sigma Chemical Ltd, UK), 1 µl each of 10 µM primers (F3 plus GC clamp [5'CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGC AGCAG3'] and Rev2 [5'ATTACCGCGGCTGCTGG3']), 1 µl of 10 mM deoxynucleotide triphosphates and 1 U of Red Taq ™ genomic DNA polymerase (Sigma Chemical Ltd, UK). The volume was made up to 50 µl with nuclease-free ddH_2O (Sigma Chemical Ltd, UK). Samples were subjected to amplification in a RoboCyler® (Stratagene). Initial denaturation was at 95 °C for 15 min, 30 cycles were then carried out with denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min, elongation at 70 °C for 1 min and a final elongation for 1 min at 72 °C. Products were analysed by

agarose gel electrophoresis. Correctly sized PCR products were then subjected to DGGE, using Ingeny apparatus (Ingeny International BV, The Netherlands).

Volumes of 10–25 µl of each PCR product and of the control DNA ladder were applied to 9% (wt/vol) polyacrylamide gels in 0.5× TAE buffer (20 mM Tris acetate, 10 mM sodium acetate, 0.5 mM Na_2-EDTA). Control consisted of mixed PCR products of DNA extracted from all the five species used in the experiments. Electrophoresis was performed at 60 °C using gel containing 40% to 90% urea-formamide denaturing gradient (100% corresponded to 7 M urea and 40% (v/v) formamide). Gels were run for 10 min at 200 V and for 20 h at 80 V and then stained with ethidium bromide (1 $\mu g.ml^{-1}$ in dd water) for at least 30 min, rinsed thoroughly with tap water and finally with distilled water. Gels were photographed under UV light using an Alpha Innotech Gel Documentation System (Alpha Innotech Corporation, USA) and images processed using free software ImageJ (rsb.info.nih.gov/ij/) to compare relative density of bands in PCR-DGGE profiles.

All experiments were repeated at least five times and the standard deviation was always within 5%.

3. **Results and Discussion**

3.1. Stainless steel biofilms produced in liquid cultures

CLSM showed that dual species biofilms formed on stainless steel after 6 days incubation in LB medium varied in thickness. Dual species *P. aeruginosa* and *E. coli* biofilms had an average thickness of 52.56 µm, whilst other dual species biofilms with *E. coli* were much thinner, 7.5 µm for *B. cereus*, 6.7 µm for *S. typhimurium* and only 2.9 µm for *S. aureus*. All biofilms apart from those containing *P. aeruginosa* were thinner than the *Salmonella* spp. biofilms that Niemira and Solomon [17] measured by CLSM on glass surfaces after 48 h incubation in tryptic soy broth (18–24 µm). Other workers have noted the lower adhesion of bacterial cells to stainless steel than to glass [18], but Kives et al. [19] reported that a biofilm of only 10 µm thickness was produced on glass by *P. fluorescens* in 24 h. In our case, large quantities of extracellular polymeric substances (EPS) were seen in the *P. aeruginosa*-containing biofilms and this added considerably to the thickness.

The “top-to-bottom Z-stack” CLSM images in Figures 2 and 3 cover the same area of each individual biofilm. The Z-coordinate in the upper left corner indicates the distance of the focal plane from the upper biofilm surface (i.e., the “depth” of the section below the surface). Figure 1 showed that many more cells of *S. typhimurium* (green) attached than of red *E. coli* O157:H7 when these two species were present together. Both species apparently attached concurrently, and dual species stacks (shown in yellow) were found in the middle of the biofilm (see circled areas).

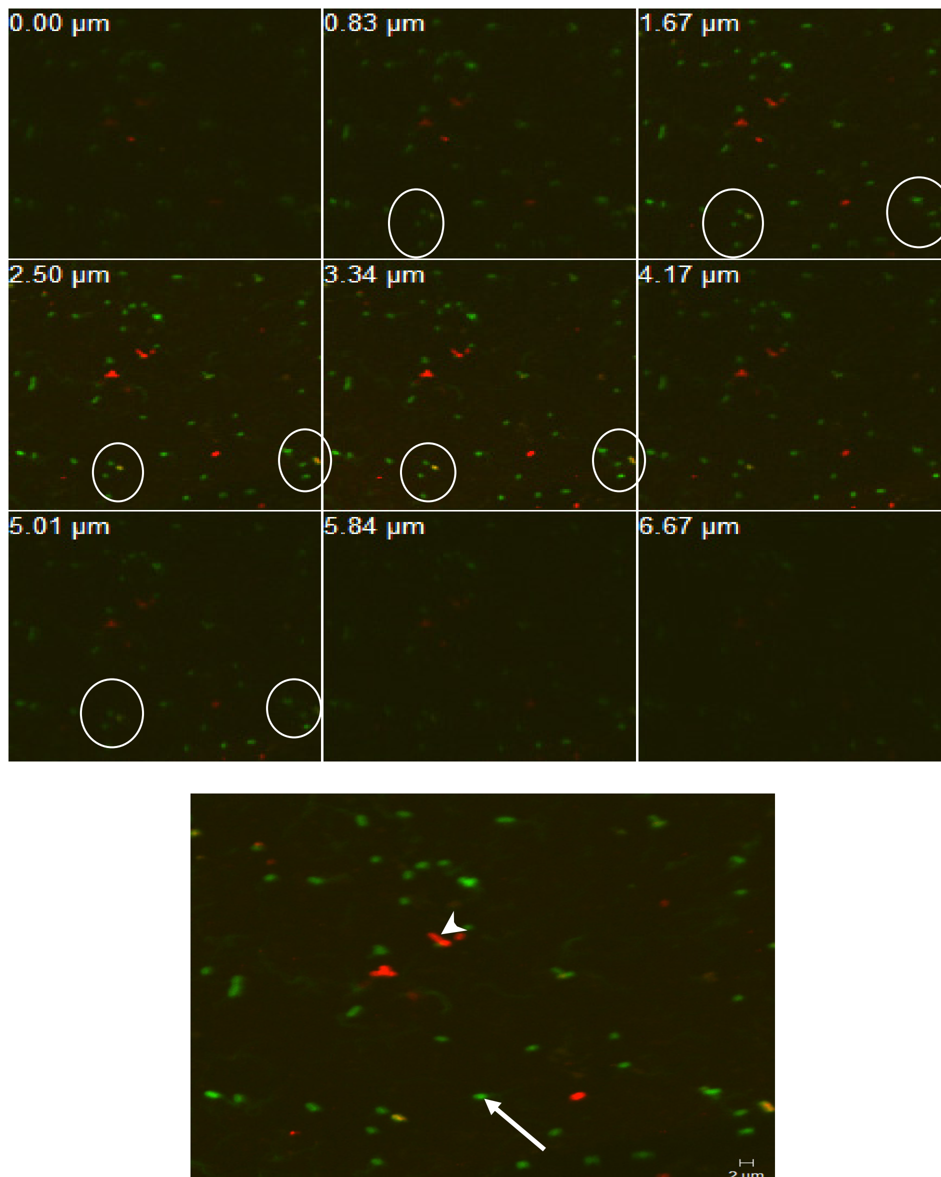

Figure 2. CSLM images of 6-day-old biofilms developed on surfaces of AISI 316 stainless steel in dual cultures of *E. coli* O157:H7 and *S. typhimurium* grown in LB medium at 37 °C. *E. coli* O157:H7 cells are stained red with *E. coli* α-biotin-labelled antibody and with Streptavidin Texas Red (groups of cells indicated by white arrow head); *S. typhimurium* cells are stained green with Acridine Orange (individual cells indicated by long white arrow). Sequence of images in upper panels shows the same area of the biofilm (60 µm × 60 µm) at different depths. The distances marked in the upper left corner represent the Z coordinate, i.e. the distance from the biofilm surface. The overall thickness of the imaged depth is 6.67 µm with 0.83 µm distance between successive images. Lower panel is a magnification of the image obtained for Z = 2.50 µm.

It has been reported that bacterial coaggregation in the planktonic phase plays a key role in the development of multi-species biofilms [20,21] and that dominance of some species can be attributed to the ability of the colonising bacteria to rapidly form microcolonies and to produce antibacterial compounds inhibiting other organisms [22]. In our single-species experiments, *S. typhimurium* was the least likely to form microcolonies on the steel surface after 6 days (Figure 2) and so its dominance over *E.coli* cannot be attributed to this characteristic.

Figure 3 shows a difference between the presence of *E. coli* (red) and *B. cereus* (blue) cells in these dual species biofilms. *E. coli* was seen mainly in the inner part of the biofilm, in close proximity to the metal surface, while *B. cereus* alone was seen in the outer parts (0.00–1.66 μm), suggesting that the Gram negative organism adhered first. In more central parts, mixed (purple-colored) regions showed that the organisms were able to co-exist within a single biofilm stack in these areas.

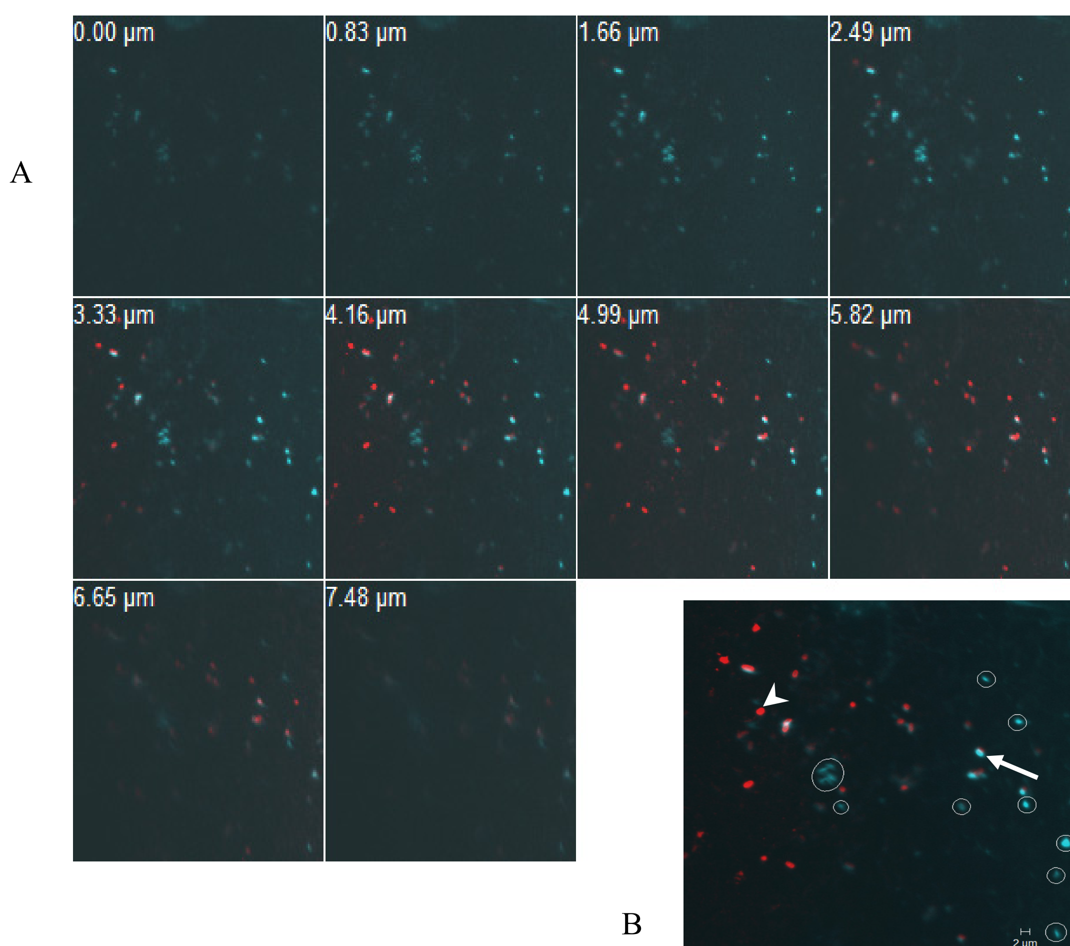

Figure 3. CSLM images of 6-day-old biofilm of *E. coli* O157:H7 and *B. cereus* dual culture on the surface of AISI 316 stainless steel grown in LB medium at 37 °C. Cells of *E. coli* O157:H7 are stained red with *E. coli* α-biotin-labelled antibody and with Streptavidin Texas Red (arrowhead in B), while *B. cereus* cells are stained blue with DAPI (long arrow in B). **(A)** Top-to-bottom Z-stack images of a total thickness of 7.48 μm and 0.83 μm spacing between images. **(B)** Z-slice at 4.16 μm. Selected *B. cereus* cells are surrounded with white circles.

In the 6-day-old *E. coli* O157:H7 and *S. aureus* biofilm, which was only 2.9 µm thick on average, very few red/orange-stained cells of *S. aureus* were seen, although they were apparent in low proportions at all levels (not shown). These observations were supported by removal, suspension and plating of biofilm organisms (Table 1). *E. coli* O157:H7 was the dominant species when grown as a biofilm with *S. aureus*. This confirmed the results reported by Rossoni and Gaylarde [23], who showed that *S. aureus* adheres much more sparsely to stainless steel than *E. coli*. Once again, this suggested that cell clumping has little effect on biofilm-forming ability as *Staphylococcus* cells typically occur in clumps in the planktonic phase.

Table 1. Viable counts of sessile populations on stainless steel stubs (cfu·cm^{-2}) incubated for 6 days with single or dual species (*E. coli* plus one other) bacterial species. Numbers are means of 3 replicates.

Microorganism(s)	Single cultures	St + Ec	Sa + Ec	Bc + Ec	Pa + Ec
E. coli O157:H7 (Ec)	1.5×10^5	0.8×10^5	2.4×10^5	1.2×10^6	0.5×10^5
S. typhimurium (St)	3.1×10^6	0.9×10^6			
S. aureus (Sa)	3.1×10^4		1.8×10^2		
B. cereus (Bc)	2.3×10^5			1.7×10^3	
P. aeruginosa (Pa)	2.0×10^8				3.3×10^7

The dominance of *P. aeruginosa* over *E. coli* O157:H7 numbers was very clear in biofilms formed in the presence of these two species (Figure 4) and this was confirmed by the viable counts (Table 1).

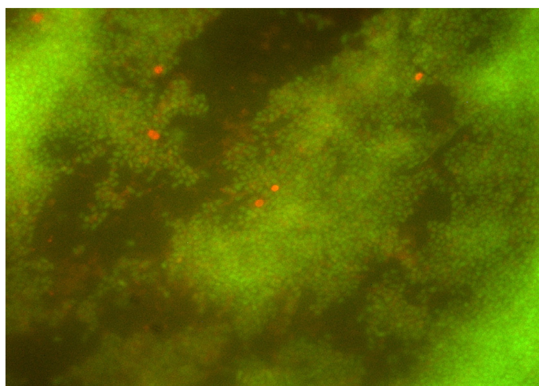

Figure 4. Epifluorescence microscopy image of 6-day-old, dual species biofilm of *E. coli* O157:H7 and *P. aeruginosa* grown in LB medium on the surface of AISI 316 stainless steel. Immunofluorescence staining with *E. coli* α-biotin-labelled antibody and Streptavidine Texas Red shows cells of *E. coli* O157:H7 in red, while *P. aeruginosa* cells are stained green with Acridine Orange.

Large amounts of EPS, almost certainly produced by the former species, a recognized producer of EPS [19 and references therein], were seen on plates and under the microscope and doubtless

explain the much greater thickness of these biofilms. In the CLSM images the EPS component rendered cell visualization problematic; *E. coli* cells were extremely difficult to see and suitable photographs could not be obtained. However, microscope observations clearly indicated that *P. aeruginosa* attached in much greater numbers near the metal surface and thus may be the first colonizer of the stainless steel surface.

The biofilm populations after 6 days` incubation, determined by removal and plating, are shown in Table 1. Including *E.coli* in the inoculum with either of the Gram positive bacteria (*S. aureus* and *B. cereus*) reduced the concentration of the latter cells in the biofilms. This inhibitory effect of Gram negative bacteria on Gram positive biofilm formation on stainless steel was also shown by Alavi and Hansen [8], who used *Listeria monocytogenes* as the Gram positive and *P. fluorescens* and *Shewanella baltica* as the Gram negative species. Gram positive bacteria have previously been shown to be less able than Gram negative to form biofilms on stainless steel [22], as well as other materials [10]. This has been attributed to the higher hydrophobicity, surface charge, and greater propensity to form EPS of Gram negative bacteria [7].

3.2. Biofilms on contaminated chicken slices

CLSM examination of chicken meat samples proved difficult because of interference by the chicken tissue. A more detailed investigation of the use of different types of stain, more specific to the bacterial cells, should resolve this issue. Mixed *S. typhimurium* and *E. coli* contamination did, however, produce results. Analysis of the Z-stack images of contaminated chicken specimens revealed that *S. typhimurium* was the more aggressive coloniser (Figure 5); much higher numbers of *S. typhimurium* than *E. coli* were present on slices incubated with this dual species culture.

There was no obvious aggregation of cells and cells from each strain were interdispersed over the tissue. However, *S. typhimurium* cells were found in every imaged area of the chicken, whereas *E. coli* O157:H7 cells were detected only in certain locations. What could be considered EPS was seen associated with colonies of *S. typhimurium*.

For *E. coli* and *S. aureus* co-cultures, very few coccus-shaped cells were detected in the biofilms, which were mainly composed of rod-shaped *E. coli*, while in co-cultures of DAPI-stained *P. aeruginosa* and FITC-labelled *E. coli* O157:H7 strong background fluorescence from DNA in chicken tissues stained with DAPI prevented visualisation of *P. aeruginosa* cells and only *E. coli* was distinguishable. These micrographs are not shown, as the quality could not be improved sufficiently for publication. A much more careful selection of stains must be used for fluorescence/adherence studies on tissues.

3.3. Transfer from chicken to stainless steel

The results of transfer experiments from chicken to stainless steel using the viable count method are shown in Table 2.

Preliminary experiments revealed that *B. cereus* failed to colonise chicken specimens, irrespective of the concentration in the inoculum (10^3, 10^5 and 10^9 cells·ml^{-1} were investigated), or of the length of incubation period (up to 48 h). The detection limit for cells adhering to chicken is

obviously a single cell, as seen under the fluorescence microscope, and so this data is highly meaningful. This strain was, therefore. excluded from the food to steel contamination study. When the *B. cereus* biofilms on steel were used to contaminate disinfected chicken slices left in contact with the dry coupons for 5 min (not described in methods), once again, no cells were detected on the chicken. Since the number of cells of *B. cereus* adhering to steel surfaces was 2.3×10^5 cfu·cm^{-2} for single and 1.7×10^3 cfu·cm^{-2} for dual cultures (Table 1), this indicated that the minimum sessile cell concentration required for bacterial transfer under the conditions used in these experiments was above 2.3×10^5 cfu·cm^{-2}; correct sanitary procedures can ensure that this level is not reached. Kumari and Sarkar [24] showed that *B. cereus* cell counts could reach up to 10^6 cfu·cm^{-2} on the surface of a stainless steel tank if inadequately cleaned, hence demonstrating that normal hygienic practices are extremely important in controlling biofilm formation by this organism. The spores of *Bacillus* have been shown, by AFM, to be more able to attach to stainless steel than the vegetative cells [25], indicating the importance of monitoring by culture techniques that allow spore germination.

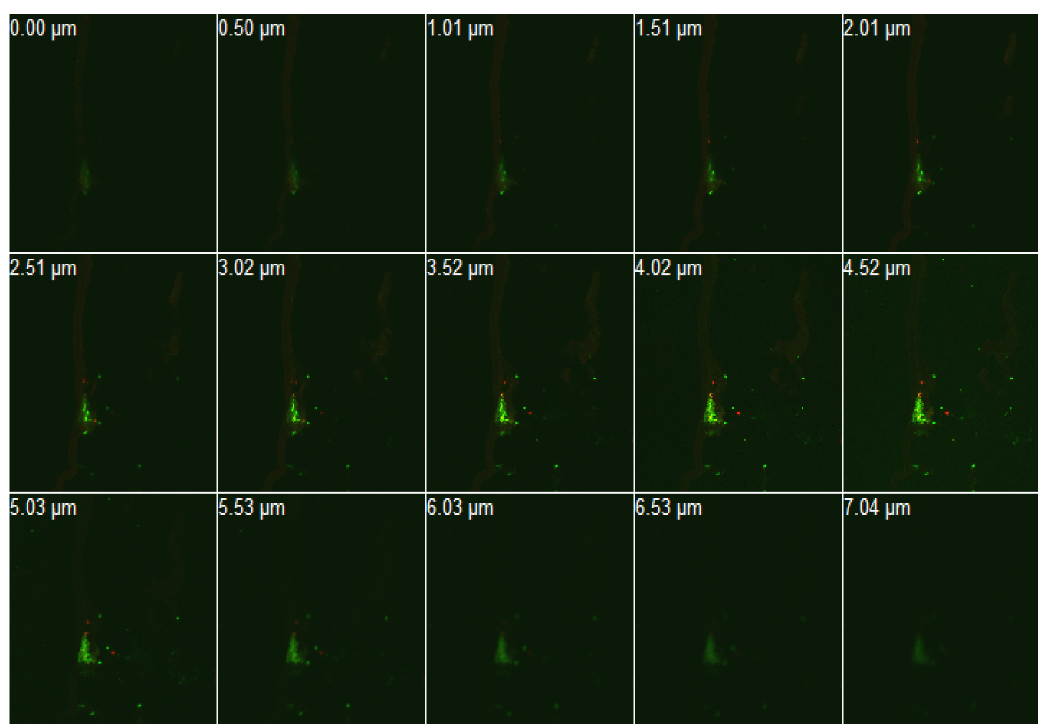

Figure 5. CSLM top to bottom Z-stack images of immunostained chicken drumstick cryosection after 4 hours incubation with *E. coli* O157:H7 and *S. typhimurium* co-culture. *E. coli* O157:H7 cells are shown in red and *S. typhimurium* in green. Biofilm thickness 7.04 µm and optical slices 0.50 µm apart.

The importance of surface contamination level on microbial cell transfer between materials has previously been pointed out by Montville and Schaffner [26], but they do not identify a minimum value. The only suggestion for such a parameter is contained in the empirical models of Sheen and Hwang [27], for slicing of ham. When the inoculum level of *E. coli* O157:H7 was ≥5log cfu on the

ham or blade, a predictive model could be proposed, but at ≤ 4log cfu transfer was too random to allow a model to be developed. These figures are similar to that determined as a minimum concentration for cell transfer of *B. cereus* in our experiments.

Table 2. Bacteria transferred to stainless steel after 5 min contact with contaminated chicken.

Bacteria on chicken surface	Number transferred to stainless steel coupon ($cfu \cdot cm^{-2}$)
Single cultures	
E. coli O157:H7	1.2×10^3
S. typhimurium	2.6×10^4
S. aureus	0.2×10^1
P. aeruginosa	3.2×10^5
Dual cultures	
E. coli O157:H7	1.0×10^2
S. typhimurium	1.4×10^3
E. coli O157:H7	2.4×10^3
S. aureus	Not detected*
E. coli O157:H7	1.2×10^2
P. aeruginosa	6.4×10^4

*Detection limit is theoretically 1 $cfu \cdot cm^{-3}$, equivalent to 6.25 $cfu \cdot cm^{-2}$

Although *S. aureus* did form biofilms on both stainless steel and chicken, an undetectable number of cells was transferred from chicken to the steel coupons, again indicating the lower adhesive capacity of Gram positive bacteria. Generally, a one-log difference in transferred numbers was found for each of the bacteria when in co-culture, compared with single species (Table 2).

DGGE analysis of biofilms transferred to stainless steel from chicken slices (Figure 6) confirmed the results of viable counts.

In dual species experiments, both *P. aeruginosa* and *S. typhimurium* bands were visually stronger than *E. coli*, while the *S. aureus* band was weaker. The data in Figure 6b presents numerical profiles of the band densities. The profiles were obtained in the ImageJ software by rotating the image in Figure 6a through 82° clockwise, defining a region-of-interest (ROI) covering the horizontally oriented lane; plotting a profile on the ROI; and saving the numerical data to an Excel spreadsheet. Corrections were applied to compensate for the slight distortions in the DGGE gel, so that the 16S-RNA fragments from the same organism would appear at the same position in the figure. This numerical representation of the density of the bands (Figure. 6b) showed that bands representing *P. aeruginosa* and *S. typhimurium* were stronger than the band for *E. coli* O157:H7 and that for *S. aureus* weaker. This band density correlates with the relative numbers of the different species indicated by plate counts and CLSM, which all show higher numbers of *P. aeruginosa* and *S. typhimurium* and lower numbers of *S. aureus* in comparison to *E. coli*. A correlation has also been

shown between total aerobic plate counts and the number of bacterial genera/species determined by DGGE on meat slicers [28]; the same publication used DGGE to demonstrate that members of the genus *Pseudomonas* were the most common bacteria to be found on slicers. DGGE proved to be useful for semi-quantitative determination of biofilm organisms, giving it an advantage over the fluorescence *in-situ* method, which can identify the exact location of an organism in the biofilm, but is less easily quantified.

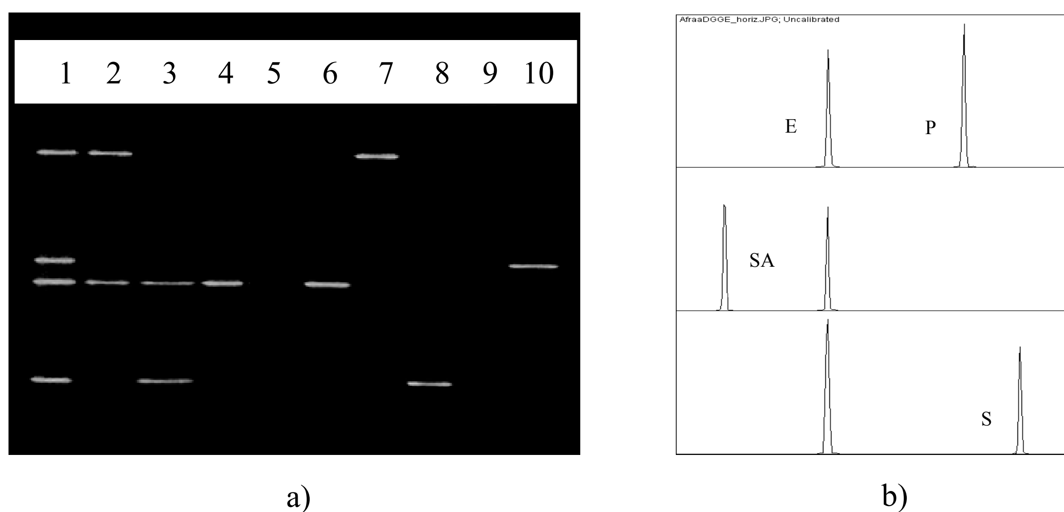

Figure 6a. DGGE of PCR products from single and dual culture biofilms transferred from chicken slices to stainless steel after 5 min contact. Lane 1 control ladder (mixture of DNA from individual strains); Lane 2 *E. coli* O157:H7 + *P. aeruginosa*; Lane 3 *E. coli* O157:H7 + *S. typhimurium*; Lane 4 E. *coli* O157:H7 + *S. aureus*; Lane 5 negative control; Lane 6 *E. coli* O157:H7; Lane 7 *P. aeruginosa*; Lane 8 *S. typhimurium*; Lane 9 *S. aureus;* Lane 10 Positive control (*B. cereus*). **Figure 6b.** Scanned DGGE gel showing comparative density of lines from amplified DNAs from biofilms containing *E. coli* (peak E) with *S. aureus* (S—bottom scan), *S. typhimurium* (SA—middle scan) or *P. aeruginosa* (P—top scan).

3.4. Transfer from stainless steel to chicken

CLSM proved unsatisfactory for routine analysis of chicken tissue (see earlier section) and so only culture techniques were used to assess the efficiency of transfer of biofilm cells from stainless steel to raw chicken slices. The results are shown in Table 3.

S. typhimurium and *P. aeruginosa* were the most efficient bacteria in transferring from steel to chicken (29.1 and 18.75% transfer, respectively, in single species biofilms), with *E. coli* a poor third at 2.0%. The same was true when the bacteria were mixed with *E. coli*, but transfer rates were greatly reduced (0.5 and 0.95%, respectively). *E. coli*, in fact, showed the highest transfer from dual culture biofilms (1.8%), but this may be accounted for by the fact that this level was achieved in the biofilm

with *S. aureus*, which did not transfer any detectable cells to the chicken tissue and did not, apparently, compete with *E. coli* in biofilm formation.

Table 3. Transfer from stainless steel biofilms to chicken within 5 min contact time.

Type and number of bacteria on stainless steel surface ($cfu·cm^{-2}$)	Number transferred to chicken slice ($cfu·cm^{-2}$)	Percentage transfer
Single cultures:		
E. coli O157:H7		
1.8×10^6	3.6×10^4	2.0
S. typhimurium		
2.2×10^6	6.4×10^5	29.1
S. aureus		
5.0×10^5	1.2×10^3	0.24
P. aeruginosa		
3.2×10^7	6.0×10^6	18.75
Dual cultures:		
E. coli O157:H7		
1.1×10^6	1.8×10^3	0.16
S. typhimurium		
1.8×10^7	9.0×10^4	0.5
E. coli O157:H7		
1.2×10^6	2.2×10^4	1.8
S. aureus		
2.2×10^4	undetectable	0
E. coli O157:H7		
2.0×10^6	4.0×10^3	0.2
P. aeruginosa		
4.0×10^7	3.8×10^5	0.95

4. Conclusion

CLSM, DGGE and standard microbiological methods were used to show that both stainless steel and chicken slices could be colonized by Gram negative and Gram positive bacteria that can be associated with food processing equipment. Gram negative bacteria produced much greater biofilms on stainless steel than Gram positives and attachment to chicken was lower for all species studied, under the conditions used in these experiments. *S. aureus* and *B. cereus* produced thin, or no, biofilms on chicken when tested as pure or dual cultures and transfer of these organisms from chicken to stainless steel could not be detected. Transfer from steel to chicken seemed to require a minimum of 10^5 $cells·cm^{-2}$ in the biofilm for a 5 min contact transfer, a level that should be readily avoidable by routine sanitary measures. The most aggressive colonisers transferred from chicken to steel were *P. aeruginosa* and *S. typhimurium*, followed by *E. coli* O157:H7 and *S. aureus*. Without a doubt, *P.aeruginosa* had a considerably higher colonizing ability than the other genera tested in our

experiments, both for chicken meat and for stainless steel, a phenomenon probably associated with the high levels of EPS produced by this species. This would be a suitable organism for use in the testing of anti-adhesive and anti-microbial colonization treatments in the food industry and CLSM, once optimized for the particular substrate of interest, would be an excellent method for evaluating the results.

Acknowledments

We wish to thank Dr Vitaly Zinkevich for help with DGGE and Dr Claudia Coutinho for allowing access to CLSM.

Conflicts of Interest

All authors declare no conflicts of interest in this paper.

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