# The Effects of Defined Linear Features on Surface Hygiene and Cleanability

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**DECLARATION**

This is to certify that the material contained in this thesis has been produced by the author and has not been accepted in substance for any other degree and is not currently submitted in candidature for any other academic award.

Adele Packer

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**ABSTRACT**

Hygienic food contact surfaces are inert, hard and easy to clean. Aggressive cleaning and disinfection regimes, and general usage and wear, may damage the integrity of the surface, and the resultant defects – pits or scratches – increase the roughness of the surface and potentially affect subsequent cleanability by retaining microorganisms and organic soil.

It is generally acknowledged that an increase in surface roughness, often measured using the $R_a$ parameter (the average departure of the surface profile from a centre line) increases the retention of microorganisms on a surface, although feature dimension may also have some influence. The retention of more amorphous organic (food) soil is less affected by the feature dimension, but is likely to be enhanced by any increase in $R_a$ value.

The aim of this project was to explore the relationship between surface topography and microbial cell retention on surfaces via the use of surfaces with defined linear features, and with defined chemical properties. Stainless steel is the most commonly used material for hygienic surfaces, but its surface chemistry can be complex. Thus, in order to explore the effect of topography in a controlled manner, test surfaces were coated with titanium, using plasma vapour deposition.

A novel impression technique was developed, using acetate softened with acetone pressed against in-use stainless steel surfaces, which when hardened could be removed and examined using atomic force microscopy and scanning electron microscopy. The diameter and profile of typical linear features were measured, enabling model surfaces to be constructed *in vitro*. Thirty micrometre diameter features were reproduced using nano-indentation, but microorganisms tended to be retained on the edges of the features, rather than within them, because there was accumulation of debris at the edges whose smaller feature size provided increased

surface area for microbial retention. Consequently, attention was focused on linear features of microbial dimensions approximating to one and 0.5 micrometer width. These were conveniently obtained by titanium-coating CDs (feature size 1.02 μm, $R_a$ 0.042 μm) and DVDs (feature size 0.59 μm, $R_a$ 0.024 μm) respectively.

*Escherichia coli* did not adhere well to the titanium-coated test surfaces. When stainless steel surfaces were coated with titanium, the same phenomenon was observed: thus it was the surface chemistry rather than topography which reduced microbial retention. In the presence of an organic (meat) soil, retention was again lower on the titanium surface. Thus *E.coli* was not used in subsequent work, although the potential for titanium coatings to reduce fouling by this species should be explored further.

*Listeria monocytogenes* and *Staphylococcus sciuri* were used subsequently, representing different shaped microorganisms related to food-borne illness (*S.scuiri* being related to *Staphylococcus aureus*). Retention of bacteria on the test surfaces was assessed by incubating cells and surfaces for 1h, gently rinsing, and examining and enumerating retained cells via scanning electron microscopy. Retention was related to cell size and feature size: the spherical staphylococci were preferentially retained on the 1.0 micrometer featured surfaces, being effectively wedged within the features, whilst *L.monocytogenes* was preferentially retained on the 0.5micrometer featured surfaces, because cell-surface contact was maximised by the increased density of 'peaks' on the surface, with the rod-shaped cells lying across and along the linear features. Epifluorescence microscopy was attempted, after staining attached cells with acriding orange, but the relationship between cells and surface features could not be visualised. The strength of attachment rather than the amount of attachment was measured using atomic force microscopy, by application of an increasing lateral force

on attached cells, and assessment of the number of scans required to remove cells. Results were similar to those obtained in retention assays, with the *S. sciuri* retained in highest numbers on the 1 μm features and the least on the 0.5 μm features, emphasising the importance of the relationship between cell size and feature size. Again *E.coli* could not be used, since it did not adhere: when combined with organic material, the AFM probe could not be used.

A more realistic physical removal strategy was applied via repeated physical 'wipes' with a mechanised device and water, subsequent to fouling of surfaces with soil, or cells, or a cell-soil mixture. Different fluorescent stains were applied that stained either soil or cells, enabling differential analysis of the area of a microscopic field covered by cells or soil. Whether there was a single fouling event, or sequential fouling-cleaning events, increasing wipes removed increasing amounts of cells and/or soil, and wipes applied along surface features were more effective at removal than wipes applied across the features.

Results have revealed that the relationship between cell size and linear feature width and orientation is key to determining whether or not cells are retained on surfaces: the $R_a$ value is of less importance. The direct relationship that is often proposed to exist between $R_a$ value and cell retention is only likely to be true within particular ranges: if the surface features are larger than microbial cells, the cells may not be retained; similarly with features smaller than the diameter of cells. If features are of microbial dimensions, then enhanced retention might be anticipated. Organic food soil is more heterogeneous, thus is retained in features irrespective of feature size, although removal is improved from larger features. Thus rather than merely measuring the $R_a$, it would appear to be important to assess feature dimension in relation to the size of the microorganism of concern in a given environment.

The range of methods used in this study have helped interpretation of a complex interaction between cells, soil, surface and its topography and chemistry. The work described will be useful for exploring these phenomena further, and in the assessment of the effectiveness of putative novel antimicrobial surfaces and/or cleaning regimens used in different environments.

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# CHAPTER 1

# THE INFLUENCE OF SURFACE CHEMISTRY AND TOPOGRAPHY ON MICROBIAL RETENTION

## 1.1 INTRODUCTION

Hygienic surfaces are hard, inert, and easy to clean. Stainless steel remains the surface of choice in most instances in the food industry, since it is stable at a variety of temperatures, is inert, relatively resistant to corrosion and may be treated electrolytically or mechanically to achieve functionally and aesthetically improved surfaces (Verran et al., 2000). Wear of steel tends to result in linear features (scratches), with occasional pits. Glass/ceramics are similarly ‘‘hygienic’’ and ceramic tiles may be used on walls or floors. ‘‘Wear’’ of such ceramic surfaces tends to present as fractures. Plastics, epoxy resins, and rubbers are softer, more flexible, and much more problematic in terms of hygienic status and cleanability, although they are essential for some appliances such as gaskets and conveyors in some parts of the food processing plant. Loss of flexibility through excess wear, and resultant cracking increases the potential for penetration of contaminating microorganisms into the material.

Most ‘‘open’’ surfaces tend to be exposed to liquid only intermittently, for example during cleaning, thus the attached cells do not form a true biofilm as would be seen on ‘closed’ surfaces at a solid–liquid interface such as within pipework, although microcolonies might be observed if conditions are suitable for any multiplication, and areas of poor accessibility and surfaces of increased porosity/flexibility would facilitate the accumulation of microorganisms. In addition to changes in topography due to wear, surface hydrophobicity will affect drying kinetics (Whitehead and Verran, 2007), with moisture droplets being more prominent

on hydrophobic surfaces, taking longer to dry, thus promoting cell deposition at the solid–liquid–air interface. Vertically orientated hydrophobic surfaces will facilitate liquid run-off, leaving a relatively clean surface—a phenomenon employed in part by the ‘‘Lotus effect’’ (Furstner et al., 2005). This study focuses on open food contact surfaces, which are primarily metal.

## 1.2. FACTORS AFFECTING RETENTION

### *1.2.1 Surface topography*

One factor that significantly affects microbial retention on a surface is substratum topography. It is generally agreed that an increase in both the type and degree of surface roughness enhances the retention of microorganisms on a surface thereby providing a protected site from which subsequent colonisation may occur (Verran et al., 1991). Therefore substratum topography has significance in food hygiene, where fouling is enhanced and cleanability impeded.

Stainless steels often present a ‘‘finish’’, for example a brush finish (Fig 1.1), where parallel linear features of defined dimensions give a pleasing aesthetic to the naked eye, and reduce the visibility of fingerprints. It has been shown that the finish of stainless steels used in food processing does not affect their cleanability or hygienic status (Airey and Verran, 2007; Hilbert et al., 2003), although bacterial attachment (not retention) on electropolished surfaces has been shown to be less than that on rougher stainless steel finishes (Arnold et al., 2004).

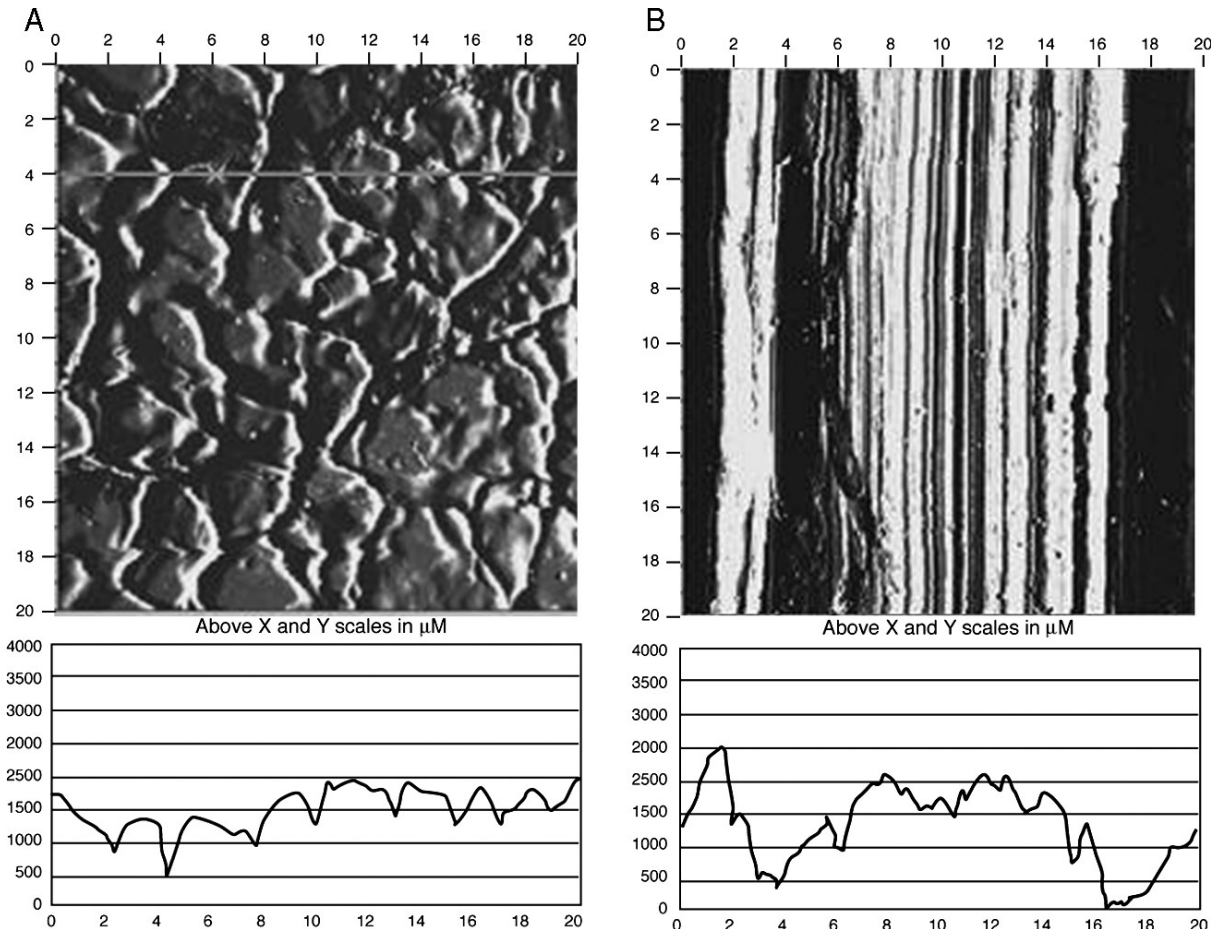



Fig. 1.1 AFM images of 304 stainless steel with (a) 2B and (b) brushed finish

However, wear of such surfaces inevitably results in the production of linear features, of differing dimensions and length, randomized across a surface (i.e., scratches), and ‘‘pits’’ (Fig. 1.2). Simulation of worn surfaces to assess cleanability *in vitro* has demonstrated that hygienic status of stainless steel and ceramic was not affected in terms of microbial retention, but cleanability, in terms of removal of organic (food) soil was reduced (Boyd et al., 2001; Frank and Chmieliewski, 1997; Verran et al., 2001; Verran and Whitehead, 2006).

*1.2.1.1 Surface roughness parameters*

All manufactured surfaces depart to some extent from absolute perfection in terms of presenting a ‘smooth’ surface. Imperfections on any surface take the form of a series of peaks and valleys which may vary in both height and spacing. Surface roughness exists in two principle planes (Thomas, 1999); perpendicular to the surface, described as height deviation, and in the plane of the surface, described by spatial parameters and identified as texture. Amplitude parameters are the most important parameters to characterise surface topography (Gadelmawla et al., 2002). They are used to measure the vertical characteristics of the surface deviations. There are a number of amplitude parameters for example $R_a$, $R_z$, $R_p$, $R_q$, for the purpose of this study the parameter chose to represent surface roughness was $R_a$. The $R_a$, also known as the centre line average is the most universally used roughness parameter for quality control (Verran and Maryan, 1997). In microbiological publications the $R_a$ is the most common descriptor of surface roughness (Verran and Boyd, 2001) perhaps because it is easy to define and measure.

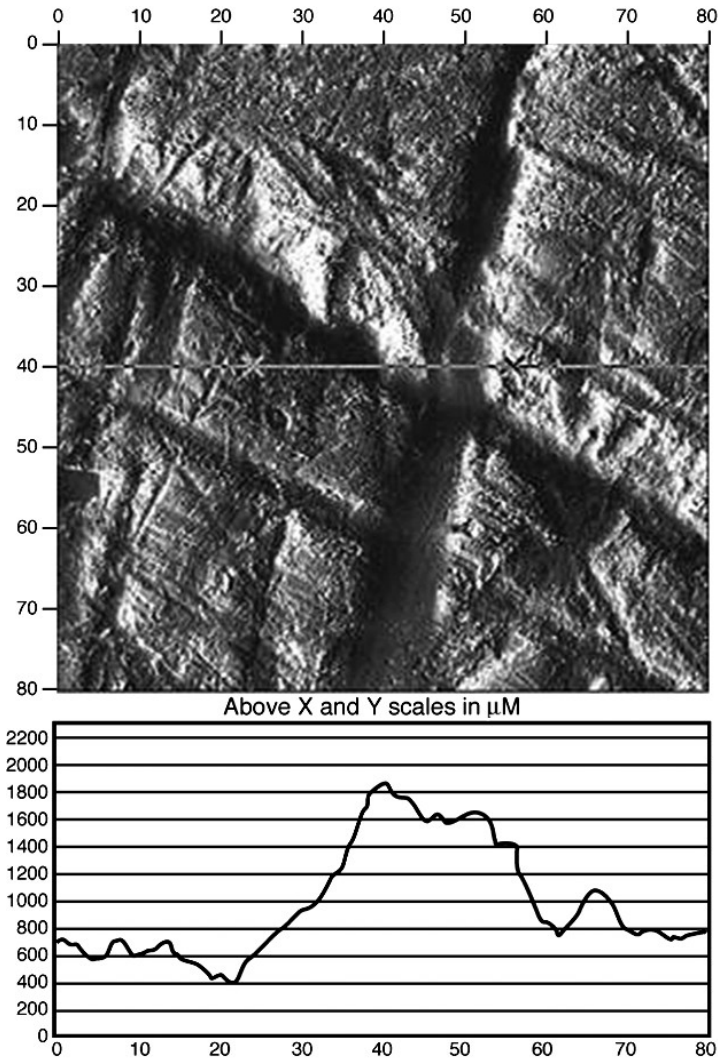



Fig. 1.2 AFM image of a replica of a worn stainless steel surface demonstrating linear features of differing dimensions and length, randomized across a surface (i.e., scratches), produced by wear on an in-use stainless steel surface.

However, although the $R_a$ value is not very sensitive to single large peaks or valleys, or changes in profile (Fig 1.3) it gives a good general description of height variations (Gadelmawla et al., 2002), on the assumption that features are evenly distributed across a surface (i.e. it is a statistical value). However information on the dimensions of individual features in not provided (Westberg, 1998). The $R_a$ measurement provides an indication of surface roughness, usually given in micrometres, describing the average departure of the surface profile from a constructed ‘‘centre line’’—in effect a two-dimensional measure of a three-dimensional parameter. An $R_a$ value of less than 0.8 μm is generally accepted as indicative of a hygienic surface. However, $R_a$ values can only be really representative if there is a regular surface topography, as for polished and brushed stainless steel, but not for worn surfaces whose aberrations tend to be of a random nature (Verran et al., 2000). Thus, in the food processing industry, wear of food contact surfaces through abrasion and impact damage will affect topography, but may not necessarily alter the key parameters used to measure surface roughness (Boulange-Petermann, 1996; Boyd et al., 2001b; Holah and Thorpe, 1990; Packer et al., 2007; Verran et al., 2000, 2001; Verran and Boyd, 2001). It is therefore important to visualize the surface as well as deriving an $R_a$ value, or any other statistically derived parameter. Attempts to examine the effect of surface topography on microbial retention have revealed apparently conflicting data. Some have observed no relationship between surface roughness (in terms of $R_a$) and the ability of bacteria to attach (Boulange-Petermann et al., 1997; Flint et al., 2000; Langeveld et al., 1972; Tide et al., 1999; Vanhaecke et al., 1990; Verran and Boyd, 2001).

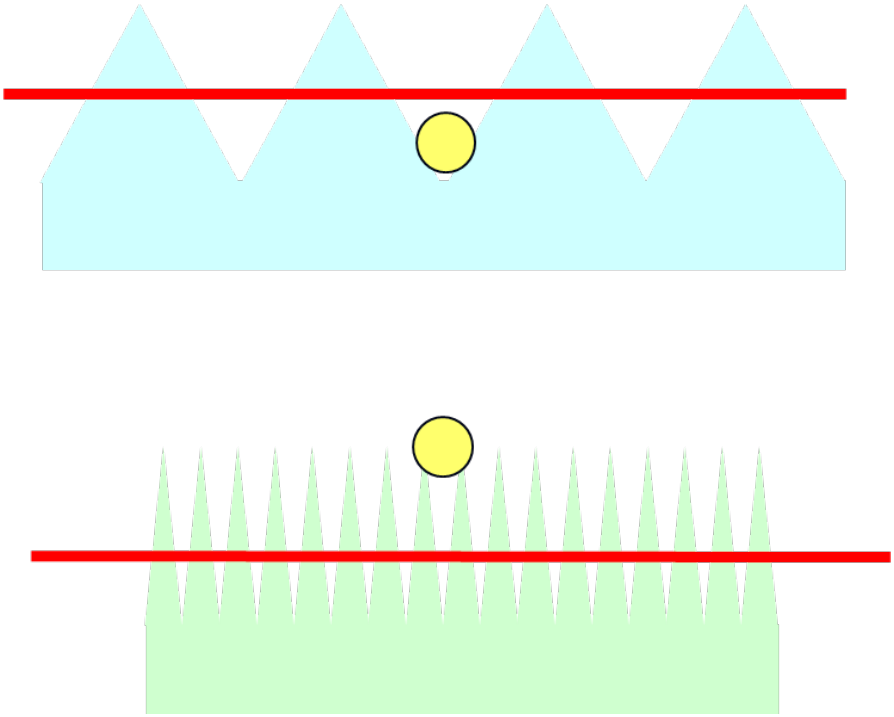


Fig 1.3 Different surface topographies with the same $R_a$ value.

$$R_a = \frac{1}{n}\sum_{i=1}^{n} |y_i|$$

Fig 1.4 Mathematical equation for the calculation of average roughness $R_a$

Others have suggested that the greater the degree of surface roughness, the greater the retention of microorganisms (Bollen et al., 1997; Holah et al., 1990; Medilanski et al., 2002; Verran and Maryan, 1995). However, these apparent contradictions arise primarily from the use of different perspectives of scale (Verran and Boyd, 2001). If the surface irregularities are much larger than the microorganisms, passive retention will be minimal (Verran et al., 1991) unless within these macroflaws, micro or even nanosize features existed (Verran and Boyd, 2001). If the features are of microbial dimension or slightly smaller, then retention will be enhanced. However, surfaces with a regular nanotopography have been shown to reduce microbial attachment, due to the lack of sufficient area for contact between the cell and the substratum (Cousins et al., 2007; Li et al., 2004; Whitehead and Verran, 2006). On worn, hygienic food contact surfaces, all of these topographical features are likely to be present. Their separate impact on surface cleanability and cell retention has yet to be investigated, requiring the fabrication of surfaces with defined topography. Thus, microbial retention assays have been carried out in our laboratories on a range of engineered surfaces with controlled topographical features of dimensions comparable to those of microbial cells (e.g., pits (Whitehead et al., 2005) and grooves (Packer et al., 2007; Scheuerman et al., 1998)). Whitehead et al. (2005) demonstrated that with a range of differently sized unrelated microorganisms, the size of circular surface defects was important with respect to the size of the cell, and its subsequent retention.

*1.2.1.1.1 Macro roughness, $R_a$ ~ 10µm*

Surfaces with high $R_a$ values of the macro scale tend to be found where the impact of hygiene and infection are less significant, for example in water distribution systems (Verran and Boyd, 2001).

*1.2.1.1.2 Micro roughness $R_a$ ~ 1µm*

Intraoral surfaces have roughness values that fall into this category (Verran and Boyd, 2001). In a study of plaque formation, a cut off point of surface roughness for facilitating adhesion and colonisation was estimated to be $R_a$ = 0.2µm (Bollen et al., 1997). The surface finish of stainless steel contact surfaces used in the food industry and other hygienic applications should not have an $R_a$ of more than 0.8µm (ISO 4287 : 1997).

*1.2.1.1.3 'Nanoroughness', $R_a$ <1µm*

Small features may affect not only the topography, but also the hydrophobicity, and hence its interaction with microorganisms. However, unlike micro and macro surface features that may be present on a surface, features of such small size will have little effect on the $R_a$ or other roughness values, and are beyond the scope of this study.

*1.2.2 Surface chemistry*

In food hygiene, the contact surface is required to be inert, so that transfer of any potential chemical contaminant from substratum to food does not occur. The chemistry of stainless steel, the material of choice, is complex, being modified in order to produce materials with properties relating to conditions of use: chromium for example enhances corrosion resistance. Other elements can be added, for example, nickel, manganese, molybdenum (Maller, 1998). Chromium reacts with the atmosphere to form a protective oxide layer (passivation) and it is this oxide layer that gives stainless steel its enhanced corrosion resistance. Because of the speed of re-passivation (sec or min) in normal ambient conditions it is difficult to determine the exact chemical makeup of the surface of stainless steel and a review of the experiments and *in situ* techniques concludes that the passive film is never static and that it changes across a surface (Olsson and Landolt, 2003). The effect of variations in

the surface chemistry of stainless steel on microbial retention has not been explored to date: for example, differences between grain boundary and the bulk surface properties may affect cell–substratum interactions over and above those of simple topography. However, in food processing, the substratum employed is rarely that of mill finish 2B, which is produced by cold rolling, annealing as has a pickled finish, with grain boundaries. Brushed or polished surfaces are in common use, providing a defined topography imposed on top of the grain boundaries. There have been studies assessing the effect of surface chemistry on microbial attachment, using surfaces of comparable topography but differing chemistry. For example, the adhesion of *Listeria monocytogenes*, *Staphylococcus aureus*, and *Escherichia coli* have been assessed using surfaces with varying chemical groups grafted onto the surfaces (hydrophilicity, hydrophobicity). The vast majority of individual cells retained (96.9%) was associated within surface features. It was shown that the bacterial attachment of *Listeria monocytogenes* and *Escherichia coli* was affected by the chemistry of the underlying substrate (Cunliffe et al., 1999). The underlying surface chemistry has also been shown to affect biofilm formation (Teughels et al., 2006). Wilks et al. (2005) found that the persistence and survival of *Escherichia coli* O157 was greatly reduced on copper alloys in comparison to stainless steel: However, the toxicity of copper would preclude its use on food contact surfaces (Airey and Verran, 2007). When a 2B finished stainless steel was coated with a titanium grid, *Pseudomonas aeruginosa* (Verran et al., 2003) and *Staphylococcus aureus* (Verran and Whitehead, 2005) were found to preferentially bind to the raised titanium features. In contrast, *Escherichia coli* would not attach to titanium coated surfaces (personal communication (K. Whitehead). This different behaviour of cells on surfaces might provide an

opportunity for coating surfaces at critical control points on equipment with a material less conducive to retention, enabling targeted hygiene control strategies

*1.2.3 Presence of organic matter*

At the solid–liquid interface surfaces are rapidly ‘conditioned’ with organic material. The biological surface presented to microorganisms by the conditioning layer provides an element of specificity via receptors for the adhesion of pioneer organisms (Verran and Boyd, 2001). On an open surface (solid–air interface), this might instead occur more nonspecifically via direct contact with food (Verran, 2002). Attached microorganisms may be retained in surface features mixed with organic material such as fats, carbohydrates or proteins, or detergent residue (Verran, 2002). Thus, the term ‘‘conditioning film’’ may not be appropriate especially where a more significant transfer of organic matter occurs (Verran, 2002). Relatively large deposits of organic soil may fill larger defects prior to microbial surface contamination (Frank and Chmielewski, 1997; Kumar and Anand, 1998; Milledge and Jowitt, 1980; Verran and Jones, 2000), and mask the underlying topography, while the formation of a thinner conditioning film (Carpentier and Cerf, 1993) on any surface in an aqueous environment may mask small topographical features. There is also evidence that nanoscale roughness enhances the adhesion of the conditioning layer to the substratum (Hanarp et al., 1999). Thus the presence of organic (and inorganic) material on a surface affects its cleanability, and also, potentially, its hygienic status since the ‘‘soil’’ can interfere with the activity of cleaning and disinfecting agents, by physically and ‘‘chemically’’ protecting microorganisms. This organic material may also potentially provide nutrients for the residual microorganisms, enabling multiplication and an increase in contamination of the surface. It has been shown that following continued cleaning and fouling cycles, stainless steel grain boundaries

become progressively more contaminated with organic soil (Verran et al., 2001; Verran and Whitehead, 2006). This cumulative soiling will inherently affect surface conditions and thus microbial attachment and retention.

## 1.3. CHARACTERISATION OF SURFACES

### *1.3.1 Topography*

There are several methods by which surface topography can be characterized, but the choice of method is dependent on the size of the samples and surface features. At the nanoscale surface topography is usually characterized using atomic force microscopy (AFM). The AFM has a sharp tip that can vary in shape but is around 10 nm in size, and can be used in a contact or noncontact mode when scanning a surface. The force applied to the tip and the tip shape can also be varied depending on whether hard or biological samples need to be imaged. Although the surface can be visualized in three-dimensions with excellent resolution, the area analysed is small, thus irregularities in the surface can greatly skew the results unless a large number of measurements are made (Verran and Boyd, 2001). Another disadvantage of this instrument is that many samples may be too rough to image. White light interferometry may be used at the nanoscale level, but can scan larger surface areas. It utilizes the deflection of light from surface irregularities to produce a three-dimensional noncontact image of the surface. The advantage of using a white light interferometer over AFM is that it is easy to operate and is relatively quick; however it is not always easy to image some polymers and translucent materials. In laser profilometers, again noncontact instruments use highly precise stages to create profiles and three- dimensional topographies. The solid stylus (probe radius 2–10 mm) profilometer traces across the surface producing a two-dimensional trace from which roughness values are calculated. However if an entire surface is to be mapped,

many lines must be scanned. There are also limits in lateral resolution, set by the size of the probe tip, which can range from 10–25 mm diameter. For both the solid stylus profilometer and the AFM, probe dimensions affect observed results. If the probe is physically incapable of reaching the bottom of narrow troughs, features are recorded as being more shallow than they are. It is also difficult to accurately chart highly curved, undercut, or convoluted surfaces with steep slopes.

### 1.4 AIM OF STUDY

The aim of this study was to identify factors that will improve the hygienic status of open food contact surfaces. The objectives were to:

I. Determine the dimensions of typical linear features (abrasions and scratches) on in-use stainless steel.

II. Reproduce typical controlled linear features in the laboratory.

III. Assess the effect of surface topography and chemistry on the retention of microbial cells and organic soil using surfaces that may be used in the food industry.

IV. Identify properties of the surface that will minimise fouling and maximise cleanability.

# CHAPTER 2

# PRODUCTION AND CHARACTERISATION OF SURFACES WITH DEFINED CHEMISTRY AND LINEAR TOPOGRAPHIES

## 2.1 INTRODUCTION

In the food industry, surfaces should be “in a sound condition and easy to clean” (European legislation, 1994). In the food plant environment, open working

surfaces are considered to be potential sources of microbial contamination, thus their hygienic status and cleanability is of paramount importance (Taylor and Holah, 1996). Viable microorganisms attached to the surface or retained within surface features may escape the cleaning and disinfection procedures and pose a biotransfer potential (Hilbert et al. 2003).

As has already been noted, the $R_a$ is a 2-dimensional measurement of the average departure of the surface profile from a mean centre line (Anon, 1988) and is used as a descriptor of surface roughness. The white light profilometer (WLP) measures the $S_a$ value which is the same measurement as the $R_a$ except it is taken across an area of the surface, rather than a line profile.

Neither $R_a$ nor $S_a$ differentiate between linear and circular features encountered by the profile probe (ie scratches and pits), but also the size of the probe scanning the surface will affect the measurement obtained, due to different probe resolutions. The advantage of using atomic force microscopy and white light profilometry is that they provide information on a scale more related to microorganisms than the more easily accessible solid stylus profilometer. Further, these methods allow visualisation of the surface under investigation.

Although information on the surface topography may be specified by the manufacturer, the new surface is only pristine for its first use, and inevitably changes to the topography will take place over time due to wear (Timperley et al. 1992), through abrasion, cleaning and impact damage. These newly introduced surface topographical features (Verran and Boyd, 2001; Whitehead et al. 2006) may increase the retention of both organic soil and microorganisms and hamper cleanability (Boyd et al. 2001). It is therefore of value to be able to monitor such wear, and also to use a worn surface during *in vitro* cleaning assays, which in turn requires simulation and

reproduction of *in situ* surfaces. Direct *in situ* examination of worn surfaces is difficult for several reasons encompassing production down time, potential contamination risks, and access to surfaces. Thus it is advantageous to take impressions of the surface which can be analysed remotely.

Stainless steel is the material of choice for use in the food industry. However, it is chemically heterogeneous, and thus it is difficult to specify the effect of surface chemistry on microbial retention. One approach is to maintain the surface topography, whilst producing a homogeneous surface chemistry, via deposition of a conformal coating on the surface using the physical vapour deposition (PVD) technique of unbalanced magnetron sputtering. Magnetron sputtering is an atomistic deposition process with concurrent bombardment of the growing film by energetic ions. This allows fully dense coatings, which are conformal to the substrate surface to be produced (Kelly and Arnell, 2000; Whitehead et al. 2004).

Titanium exhibits well-known properties such as very high resistance to corrosion, low specific weight, very low toxicity, and high biocompatibility (World Health Organisation, 1982), thus could safely come into contact with foodstuffs (Feliciani et al. 1998). Furthermore, titanium has previously been reported to have antibacterial properties (Yoshinari et al. 2000: 2001; Shibata et al. 2004). Nanophase titania and crystalline titanium oxide surfaces have been shown to reduce the adhesion of *Staphylococcus epidermidis* (Colon et al. 2006) and *Streptococcus spp*. respectively (del Curto et al. 2005).

The aim of this part of the study was to develop a rapid and simple non-destructive method for indirect characterisation of surface wear *in situ*, and to characterise the shape and dimensions of surface features in the range of typical microbial dimensions. Using these data, a titanium (TiOx) coating was then applied to

surfaces with features of dimension comparable to those found on the worn surface. These surfaces were then used to investigate their effect on the retention of microorganisms and organic soil.

## 2.2 MATERIALS AND METHODS

*2.2.1. Indirect examination of surfaces*

This technique enabled comparisons to be made between the roughness values obtained directly from a surface and indirectly via an impression of the surface thus validating the impression technique for subsequent sampling of surfaces *in situ*. A 10 mm x 10 mm square of pre-cut 180 μm thick cellulose acetate (Agar Scientific Ltd, Essex, UK) film was transferred to a glass Petri dish and softened for 30 seconds in 2 ml of acetone (BDH, UK). Once soft, the film was removed with tweezers and applied to the steel surface. A light fingertip pressure was applied. When the acetone had evaporated (15 min), the acetate film was peeled off using tweezers, yielding a negative replica of the template surface on the underside of the film. The film was inverted, placed onto a scanning electron microscope (SEM; Cambridge Stereoscan, Cambridge, UK) stub, and gold sputter coated. The coated replica of the original surface was removed from the stub after SEM analysis, for subsequent atomic force microscopy (AFM) and white light interferometry (WLI). The diameters, depth and profile of typical features were measured where possible.

*2.2.2 Surfaces*

*2.2.2.1 Stainless steel surfaces*

Type 304 fine polished stainless steel [FP] (Outokumpu Stainless Ltd, Sheffield UK) was cut into 10 mm x 10 mm squares using a guillotine. The samples were examined in a pristine as-manufactured state, with the protective plastic coating removed only directly before analysis. Representative worn stainless steel (type unknown) was sampled *in situ*, via an impression technique (described above), from a horizontal food preparation area in a canteen.

*2.2.2.2 Polished silicon wafers*

Polished silicon wafers obtained from Montco Silicon Technologies (Washington, US). Wafers were cut into approximately 1 $cm^2$ pieces using a diamond tipped cutting tool (Agar, Stansted, Essex, England). Dust particles were removed with Nitrogen gas (Whitehead et al. 2005).

*2.2.2.3 Production of surfaces with 30 micron features*

The larger features (30 μm wide) identified from the impression technique (2.2.1) were created using a Teer Coatings Ltd. ST3001 scratch tester. This was used in combination with a 200 micron radius diamond and a 2N load, to produce linear features with widths of the order of 30 microns. The 30 micron surfaces were then coated with titanium in a magnetron sputtering rig. Ten surfaces were checked for topographical continuity using Atomic Force Microscopy.

*2.2.2.4 Production of surfaces with microbial scale features*

Smaller features identified from the impression technique were represented most conveniently using unwritten compact discs (CD) and digital video discs (DVD) which provided a simple means for obtaining surfaces presenting regular 0.59 μm (DVD) and 1.02 μm (CD) width linear features. For the CD, the density of lines was approximately 5,000 per $cm^2$; 10,000 per $cm^2$ for the DVD. The protective coats were stripped from the discs after an overnight soak in 30 % sodium hydroxide (BDH, UK) followed by a rinse with sterile distilled water. The stripped CDs and DVDs were dried in a laminar flow hood, and cut into 1 $cm^2$ pieces (Whitehead et al. 2004). Surfaces presented specific topographical features; 'smooth' surfaces (i.e. $R_a$ value < 5 nm) and surfaces with unidirectional grooved features of regular size. All surfaces were coated with titanium using physical vapour deposition to ensure chemical homogeneity.

*2.2.3 Substrata coating*

*2.2.3.1Physical Vapour Deposition (PVD)*

The biological response of a material may be changed by modifying its surface topography and chemistry (Colligon et al., 1996). Physical vapour deposition (PVD) is the name given to coating processes where the transport of material to the substrate is effected by a physical driving mechanism (Hultman and Colligon, 1999). Such mechanisms include evaporation, sputtering and ion plating. Ejection of atoms from a surface is called sputtering, which was first reported by Grove in 1852 who observed formation of deposit in a glass in which a discharge had been running. He identified this to be the same material as one of the electrodes. This ejection of atoms from an ion-bombarded surface is now known as sputtering.

2.2.3.2 *Magnetron Sputtering*

In the basic sputtering process, a target (or cathode) plate is bombarded by energetic ions generated in a glow discharge plasma in front of the target. The bombardment of ions causes the removal of target atoms known as “sputtering”, these atoms may then condense on a substrate as a thin film. Secondary electrons are also emitted from the target surface as a result of the ion bombardment. These electrons help to maintain the plasma. Magnetrons make use of the fact that a magnetic field parallel to the target can constrain a secondary electron motion to the vicinity of the target. The magnets are arranged in such a way that one pole is positioned at the central axis of the target with a second pole formed by a ring around the outer edge of the target. The increases the probability that an ionising electron-atom collision may occur. Higher sputtering rates are achieved by the increased ion bombardment of the target.

2.2.3.3 *Unbalanced magnetron sputtering*

In an unbalanced magnetron the outer ring of magnets is strengthened relative to the central pole. In a conventional dc magnetron electrons are constrained to follow spiral orbits about the lines of magnetic flux which form closed paths onto the target. By changing the strength of the N and S magnets so that one is stronger than another not all the field lines are closed between the central and outer poles in the magnetron, but some are directed towards the substrata, and some secondary electrons are able to follow those field lines. Therefore the plasma is not confined to the target region but is also allowed to flow out towards the substrate.

*2.2.3.4 Deposition of titanium onto substrates*

In order for the effects of topography alone on microbial retention to be ascertained, a uniform surface chemistry was required. To achieve this, substrata with varying underlying topographies were coated with titanium via magnetron sputtering. The titanium coatings were deposited onto the substrate surfaces by biased magnetron sputtering in a modified Edwards E306A coating system rig. Sputtering took place from a single 150 mm diameter x 10 mm thick, 99.5% pure titanium target (base pressure 10-4 Pa; argon gas at a working pressure of 0.15 Pa; magnetron power of 0.5 kW; time 15 minutes). Prior to the deposition of the titanium coatings, the substrates were sputter cleaned at 1000V DC for 10 minutes. During deposition, the substrates were biased at -50V to ensure the formation of a dense, conformal film. This conformality was confirmed by fracturing coatings and examining cross-sections using SEM, which revealed thickness, structure and substrate coverage. The film was stable in aqueous medium. Due to the reaction of titanium in air the surfaces used in the subsequent microbiological experiments are TiOx.

*2.2.4. Atomic force microscopy (AFM)*

Atomic force microscopy (Quesant Instruments, CA, USA) was used to image and characterise the topography of the new surfaces, and the impressions of new and worn surfaces. The AFM was operated in contact mode using silicon nitride tips with a spring constant of 0.12 N/m. Two and three dimensional maps were imaged from the surfaces and $R_a$ values were produced by taking the average of the absolute deviation of the roughness irregularities from the mean line over one sampling length. Five samples were taken from three different areas therefore n = 15 for each surface.

*2.2.5. White light interferometry (WLI)*

Using WLI the topography of the new surfaces was measured directly and also indirectly from the surface replicas. Analysis of the surface roughness qualitatively (images) and quantitatively ($S_a$ values) were taken using a MicroXAM (phase shift) surface mapping microscope with an ADE phase shift XYZ 4400 ml system and an AD phase shift controller (Omniscan, Wrexham, UK). The image analysis system used was Mapview AE 2.17 (Omniscan, Wrexham, UK). Analysis was carried out using EX mode. Three samples of every surface were examined and five separate areas on each sample scanned to gain average values.

*2.2.6. Scanning electron microscopy (SEM) and energy dispersive X ray spectroscopy (EDX)*

Titanium coated surfaces were checked for conformity of film deposition using SEM and EDX characterisation. Images of substrata were obtained using a JEOL JSM 5600LV scanning electron microscope (Jeol Ltd, Herts, UK). Replicates were carried out in triplicate. Chemical analysis of substrata was carried out to a 1 μm

depth using a Link Pentafet detector (Oxford Instruments, Buckinghamshire, UK), with Inca software (Oxford Instruments, Buckinghamshire, UK).

Substrata plus retained cells were immersed in 4% (w/v) gluteraldehyde for 24 h at 4 ºC. After fixing, substrata were washed gently with distilled water and passed down an ethanol (BDH, Basingstoke, Hampshire, England) gradient at 30%, 50%, 70%, 90% and 100% each for 10 min. The samples were mounted onto stubs for gold sputter coating prior to examination. Images of substrata and cells were obtained as above. Ten fields per sample were counted.

*2.2.7 Contact angle measurements*

Contact angle measurements were determined at room temperature using the sessile drop technique and 5 µL volumes of solution on a Kruss goniometer and data analysis system. Five microlitres of HPLC grade water (BDH, Poole, UK) were deposited onto a horizontal sample using a syringe. Advancing contact angles of the droplet were calculated automatically using the analytical software.

*2.2.8. Maintenance and preparation of microorganisms*

*Escherichia coli* CCL 410 (a non-pathogenic 0157:H7 strain) and *Staphylococcus sciuri* CCL 101 were kindly provided by Brigitte Carpentier (AFSSA, France). *S. sciuri* was used as a representative of the Staphylococcus genus associated with the food industry. Stock cultures of microorganisms were stored at −80 °C and stored and maintained according to Cabellero et al., (2009).

In preparation for assays, stock cultures were inoculated onto nutrient agar (NA) (Oxoid, UK) and incubated at 37 ◦C for 24 h. Cultures were sub-cultured onto

fresh agar before use and were maintained at 4 ◦C. Fresh stocks were prepared from the frozen stock cultures every three months. A single colony of *E. coli* was inoculated from an agar plate into 100 ml of nutrient broth (Oxoid, UK) and incubated for 18 h with shaking at 37 ˚C. Cells were harvested by centrifugation (3600 × g for 12 min) and washed three times in 10 ml sterile distilled water. The resultant cell suspension was adjusted to an optical density (OD) 1.0 at 540 nm corresponding to concentrations of *E. coli* 5.73 ± 0.82 × $10^8$ and *S. sciuri* 0.64 ± 0.66 × $10^9$ colony forming units $ml^{-1}$ (CFU $ml^{-1}$).

Stock cultures of *L. monocytogenes* were inoculated on tryptone soya agar (Oxoid) and incubated at 30ºC overnight. *S. sciuri* was prepared in the same way except nutrient agar and nutrient broth (NB) (Oxoid) was used, and cells were grown at 37ºC. Cultures were stored at 4ºC and used within 1 month. Ten millilitres of TSB was inoculated with a single colony of *L. monocytogenes* and incubated at 30ºC overnight or ten millilitres of NB was inoculated with a single colony of *S. sciuri* and incubated at 37ºC overnight. One hundred microlitres of *L. monocytogenes* was used to inoculate 100 ml TSB which was incubated at 30C for 18 h or 100µl of *S. sciuri* was used to inoculate 100 ml of NB which was incubated at 37C for 18 h. Following incubation, cells were harvested at 716 g for 10 min and were washed three times, by resuspension in sterile distilled water, vortexing for 1 min and then centrifugation at 716 g for 10 min. Cells were resuspended to an OD (optical density) 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 ± 0.58 x $10^8$ colony-forming units (cfu) $ml^{-1}$ for *Listeria monocytogenes* and 1.64 ± 0.96 x $10^8$ cfu $ml^{-1}$for *Staphylococcus. sciuri.*

*2.2.9. Meat exudates*

The method was kindly provided by Brigitte Carpentier (AFSSA, France). One kilogram of fresh rolled beef brisket (COOP, UK) was cut into 10 mm x 10 mm pieces. The meat pieces were put into a stainless steel tray and covered in aluminium foil. The meat was covered by another tray and weighed down with 8.4 kg of stainless steel sheets and frozen at -20ºC for 24 h. The meat was defrosted at room temperature and the meat exudates were poured off and the meat squeezed to recover surplus exudates. The meat exudates were stored in 20 ml aliquots at -20ºC until needed.

*2.2.10. Retention assays and differential staining (Whitehead et al., 2009)*

Three replicate test substrata were coated with 10 µl of meat exudate and dried for one hour in a class 2 flow hood. These conditioned surfaces were then placed horizontally in a glass Petri dish, to which 25 ml of standardised cell suspension were added, and incubated for 1 h at 37ºC without agitation. Test substrata were removed, rinsed once, for 5 s, gently with 5 cm3 distilled $H_2O$, with a distilled water bottle with a 3 mm nozzle at a 45◦ angle. Samples were air dried in a microbiological class 2 containment hood for one hour. Retained cells and meat exudate were stained according to the method of Whitehead et al. (2009) with 10 µl of 4′,6-diamidino-2-phenylindole (DAPI) (Sigma, UK) which had been dissolved in sterile distilled water at a stock concentration of 0.3 g/ml and a working concentration of 0.1 g/ml (Excitation 340, Emission 488). This 10 µl was applied to the samples and spread across the surface using a sterile plastic spreader. Ten microlitres of 9-(2-carboxyphenyl)-6-diethylamino-xanthen-3-ylidene]-diethyl-azanium chloride (Rhodamine B) (Invitrogen, Scotland) were dissolved in acidic ethanol at a stock concentration of 0.1 g/ml, and a working concentration of 0.1 mg/ml (Emission 554, Excitation 627) was applied to the sample as above to stain the meat exudate. Stains were not washed off between applications. Substrata plus adherent microorganisms

and meat exudate were visualised using epifluorescence microscopy (Nikon Eclipse E600, Nikon, Surrey, England). The microscope was mounted with an F-View II black and white digital camera (Soft Imaging System Ltd., Helperby, UK, supplied by Olympus, Hertfordshire, UK). This system used a Cell F Image Analysis package (Olympus, Hertfordshire, UK). To obtain surface coverage data for cells and soil separately, an area of each surface was selected at random and an image captured, first using one UV light wavelength stain filter, and then a second image was captured using a second filter. The threshold of the images was set and the percentage area of a microscopic field covered by stained material (cells or organic soil) was recorded individually for forty fields.

*2.2.11. Statistical analysis*

Statistical tests were carried out using a two - tailed distribution *t*-test with two sample homoscedastic variance. The results are reported as mean ± standard deviation. The differences observed between the substrates were considered significant at $p < 0.05$.

## 2.3 RESULTS AND DISCUSSION

To determine the integrity of the cellulose acetate replication method, impressions were taken of a range of 'in-situ' worn surfaces and new pristine surfaces (Table 1). The use of cellulose acetate softened with acetone for taking impressions of hygienic surfaces proved successful in reproducing surface topography and enabling wear damage to be measured and visualised at a microbiologically relevant scale. The new fine polished stainless steel surface was replicated using the cellulose acetate technique and it was demonstrated that using WLI (Fig. 2.1) and AFM, that the impression material clearly reproduced the topographic features. White light interferometry and AFM measurements of a surface with known dimensions demonstrated that the cellulose acetate method gave reproducible inverse replicates of the surface topography. The fine polished stainless steel presented linear surface grooves, consistent with the polishing method. The WLI image revealed unidirectional surface features in the range of 1 μm - 10 μm width and 2 μm depth, with lengths > 60 microns. The widths of many surface features were of bacterial cell dimensions (≥ 1 μm), although larger surface features (macrotopographies) were observed (20 μm width), within which further features of microbial dimensions (1 μm – 10 μm) were also observed. AFM images (not presented) of the fine polished surface linear features supported the WLP findings. Feature widths were in the range of bacterial cell dimensions (≥ 1 μm), but larger surface features were also observed (20 μm width) with depths up to 0.25 μm.

Using the SEM, the cellulose acetate replica of the original surface was imaged (Fig. 2.2a). This image was then converted into a negative replica of the image (Fig. 2.2b), to give a true representation of the surface. Large surface scratches with widths of approximately 1 μm - 10 μm could be determined across the length or width of the worn surface. Some scratches of > 200 microns in length were observed.

Table 1 Widths and depth of features following cellulose impressions of *in situ* surfaces.

| Feature width (µm) | Depth (nm) | Location |
|---|---|---|
| 24 | 700 | Wear scratch on industrial surface |
| 20 | 600 | Wear scratch on industrial surface |
| 8 | 300 | Wear scratch on industrial surface |
| 30 | 725 | Manufactured scratch |
| 35 | 700 | Manufactured scratch |
| 8 | 300 | Fine polished finish brand coupon. |
| 30 | 825 | Created surface scratch tester |
| 8 | 275 | Created surface nanoindenter |

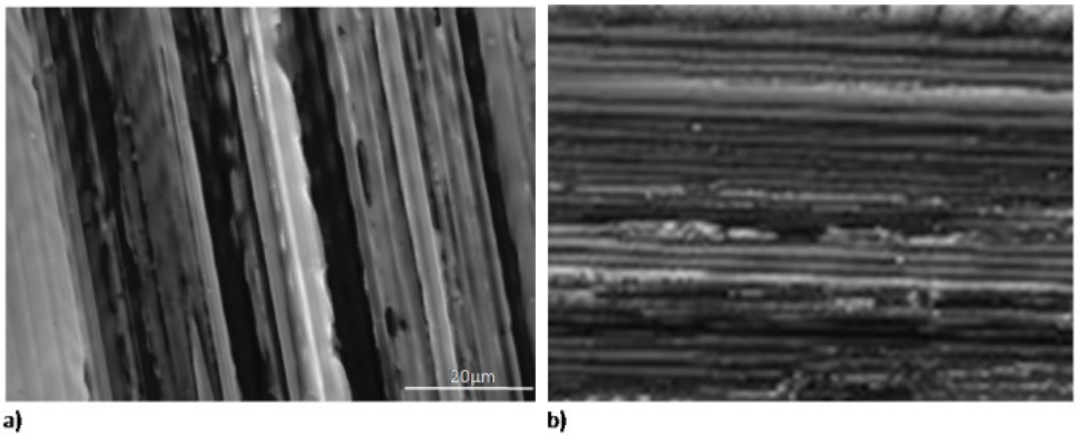



Fig. 2.1 White light image of (a) new fine polished steel ($S_a$ = 0.2 µm) (b) replica of the same sample of new fine polished steel (Sa = 0.2 µm). Scan size 80 µm x 60 µm.

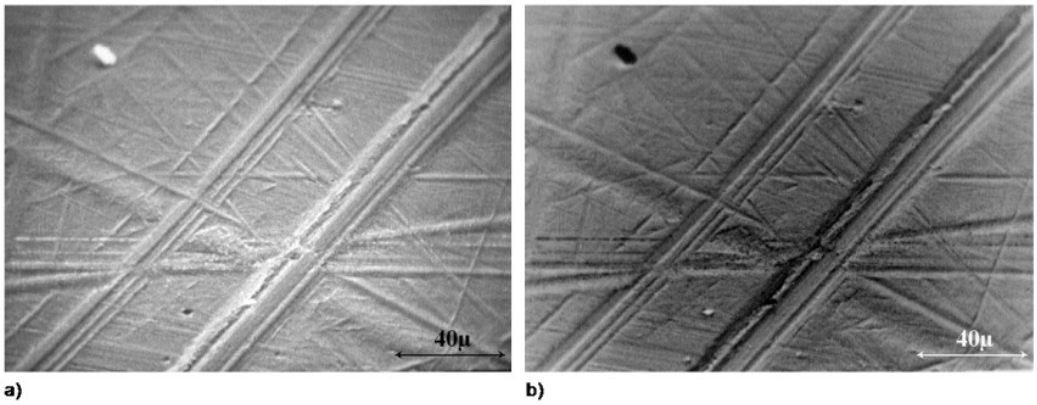



Fig. 2.2 Scanning electron micrograph of a cellulose acetate replica of a worn surface (a) the replica – a negative of the surface (b) a negative of the replica, thus a representation of the original surface.

SEM images of the worn surface demonstrated that a true representation of the original surface had been made. However, the SEM cannot provide feature depths or roughness values of the worn surface, for example $R_a$ and Sa values.

AFM images of the cellulose acetate replicas of the worn stainless steel surface revealed surface features of <0.5 µm - 20 µm width with depths up to 2.5 µm. Using AFM and WLP, roughness measurements ($R_a$ and Sa) were derived for the fine polished and worn surfaces (Fig. 2.3). There was no significant difference for the Sa values for the fine polished, or worn surfaces ($p > 0.05$). However, significant differences were observed for the $R_a$ values between the two surfaces ($p < 0.001$). AFM and WLP measurements allowed the surface topographies to be visualised and the $R_a$ and Sa values to be determined. Both measurements were used, because they can image different sized areas and provide complementary data. However, a larger area was imaged using the WLI (600 µm x 800 µm) perhaps explaining why there was no difference observed in Sa values between the surfaces. Using the AFM imaging of a smaller area (80 µm x 80 µm), $R_a$ values between the fine polished and worn surfaces were significantly different ($p < 0.001$). The polished surfaces were selected for subsequent work because their surface features were representative of the smaller surface features found on the worn surfaces (Chapter 3), and the nature of the features were relatively regularly distributed across the surface facilitating reproducibility.

Dairy grade 304 2B stainless steel surfaces were also used to determine whether modifying the surface chemistry would affect microbial retention. Titanium coatings were deposited onto these surfaces by magnetron sputtering in order to produce fully dense coatings, which were conformal to the substrate surface (Fig. 2.4).

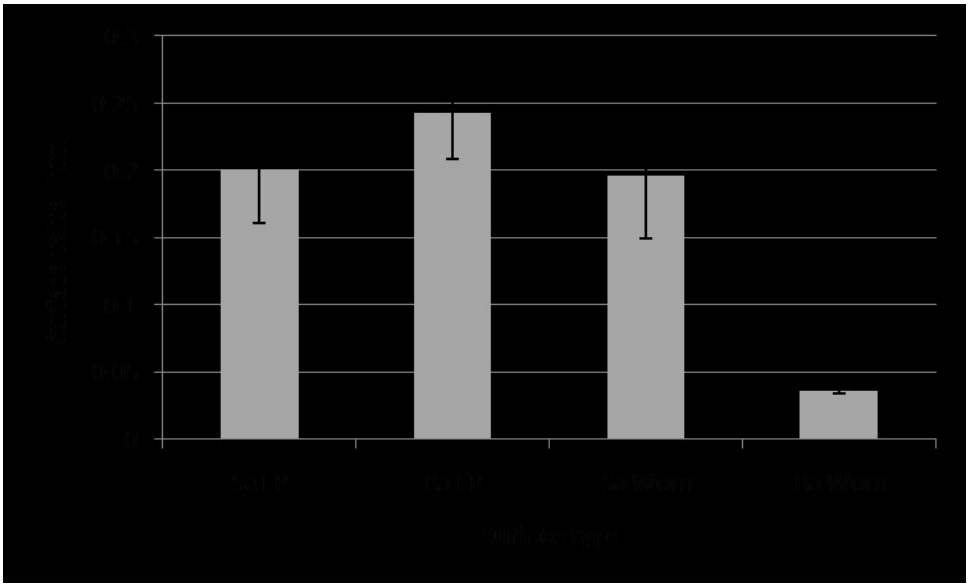


Fig. 2.3 Roughness values of surfaces using a) WLI (Sa) and b) AFM (Ra). FP = fine polished; worn = worn stainless steel surface. Each surface was scanned fifteen times n(15) and an average calculated.

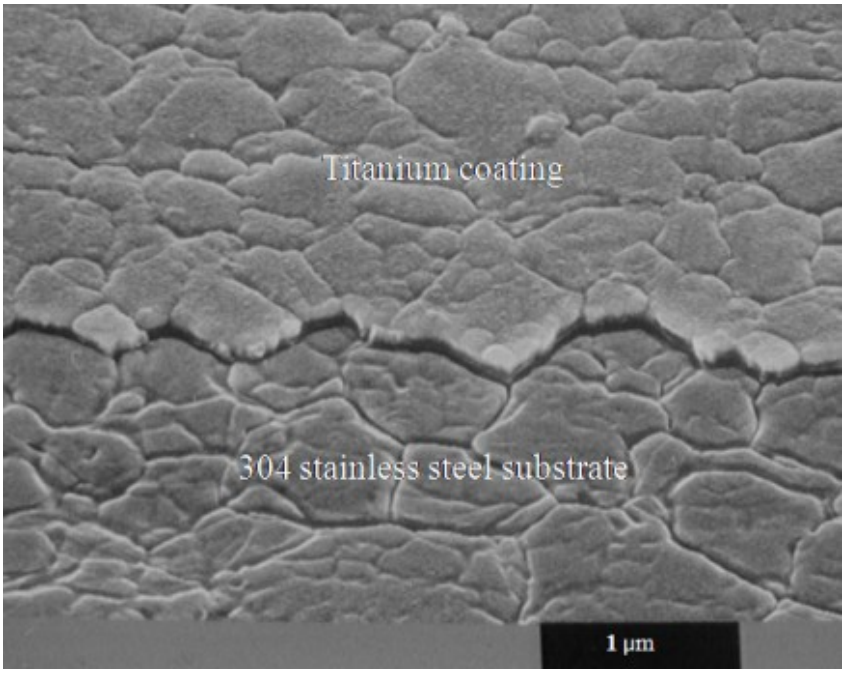



Fig. 2.4 Electron microscope image of titanium coated 304 2B stainless steel demonstrating the dense surface and conformity of the coating. The grain boundaries are still clearly visible.

There was no significant difference in surface topographies or $R_a$ values observed in the fine polished surfaces before and after sputtering with titanium. Surface wettabilities of the stainless steel and titanium coated (TiOx) stainless steel were in the range of 86 ± 3° (data not shown), with no significant difference ($p > 0.05$) between samples. All the surfaces had $S_a$ or $R_a$ values below 0.8 μm, implying that they would be easy to clean (Flint et al. 1997; Verran et al. 2001; Whitehead et al. 2005). It has previously been shown on fine polished stainless steel surfaces, that microorganisms were retained in the smaller surface grooves and grain boundaries (Fang et al., 2002), which was speculated to be due to a lowered cell-surface binding energy at those sites (Whitehead and Verran, 2007). Linear surface features, such as scratches and abrasions observed may therefore enhance bacterial and soil retention. Indeed, WLP images revealed features of microbial cell dimension within larger features, and these smaller features can enhance cell retention (Packer et al., 2010)

Retention assays were carried out in order to determine the amount of *Escherichia coli* retained on the stainless steel and titanium coated (TiOx) fine polished stainless surfaces (Fig. 2.5). Cells were retained on the stainless steel coated surface (Fig. 2.5a), but not on the titanium coated (TiOx) surface (Fig. 2.5b). To further investigate this phenomenon, cells and meat soil were added to the surface and differentially stained (Fig. 2.6). Both the cells (Fig. 2.7a) and the meat soil (Fig. 2.7b) were retained in lower amounts (in terms of surface area coverage) on the titanium coated (TiOx) surfaces.

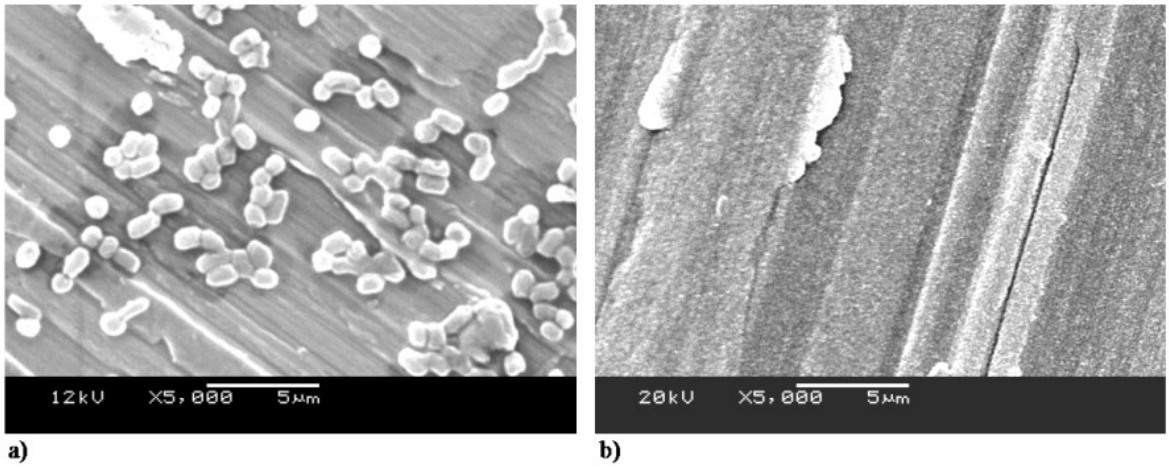



Fig. 2.5. SEM images of *Escherichia coli* retained on a) uncoated stainless steel surface and b) titanium coated (TiOx) brushed stainless steel surface demonstrating that cell numbers were clearly lower on the titanium coated (TiOx) surface.

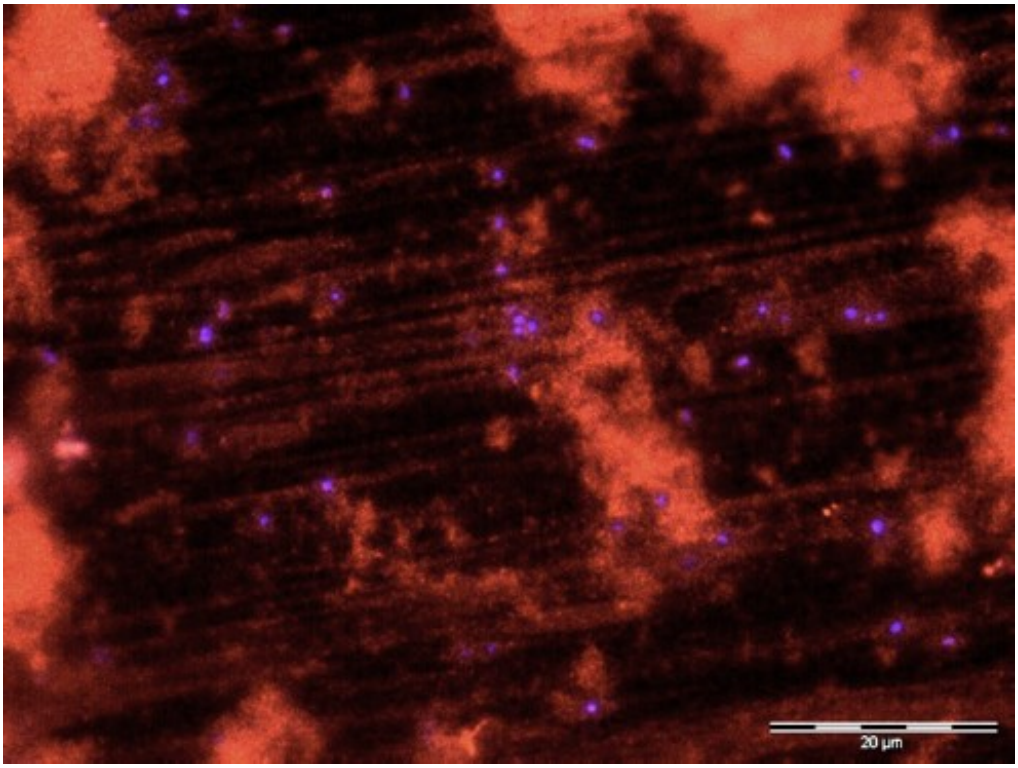
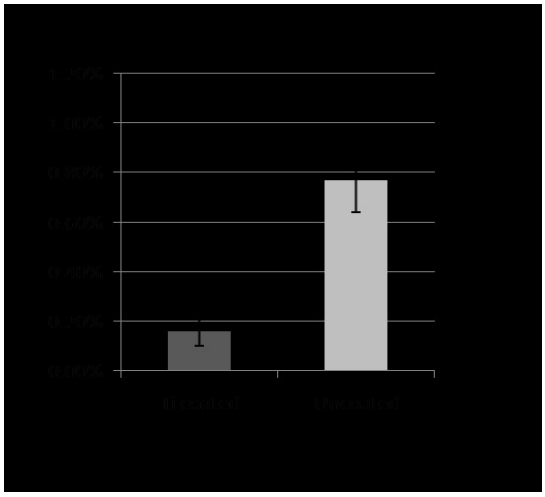
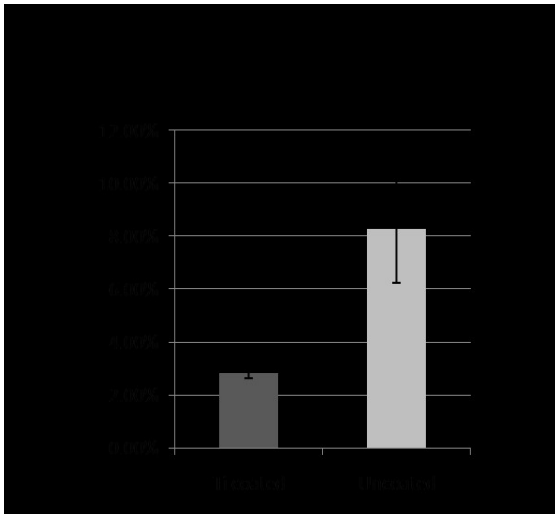



Fig. 2.6. Differentially stained epifluorescence microscopy image *E.coli* (blue) and meat extract (red) on the fine polished steel demonstrating the pattern of distribution of meat soil and cells retained across the surface.



a)



b)

Fig. 2.7. Percentage coverage of a) cells and b) meat soil retained on uncoated and titanium coated (TiOx) stainless steel surfaces determined using epifluorescence microscopy.

The use of the PVD technique of magnetron sputtering allowed the surface features of the stainless steel to be maintained whilst producing a homogeneous titanium (TiOx) coating. Previous work using this method has demonstrated that conformal and fully dense coatings are produced (Kelly and Arnell, 2000; Whitehead et al. 2004). These chemically uniform surfaces allowed the effect of surface chemistry on microbial retention to be assessed. The work demonstrated that, in spite of the presence of topographic features, coating with a regular titanium (TiOx) chemistry reduced *Escherichia coli* and meat extract retention. In agreement with our work, Jeyachandran et al. (2007) demonstrated that surface chemistry of a titanium oxide film retained fewer bacteria than other materials, and suggested that surface chemistry was a more important factor than surface roughness. Work by Ma et al. (2008) has demonstrated that the heterogeneous chemistry of a surface may provide 'sticky' points for bacterial retention and thus surfaces should have a highly uniform surface chemistry, again asserting that the effect of surface chemistry on microbial retention needs to be further investigated. In addition, the durability of the coatings clearly needs to be explored.

Although coverage of surfaces by cells and soil was low, with coverage of soil considerably higher than that of cells, there was nevertheless a significant reduction observed in both instances on the titanium (TiOx) surface. One would hope that in a processing plant, surfaces would be effectively cleaned and disinfected, thus residual material would be minimal. It is this residual fouling that could be reduced by the utilisation of a less retentive surface – hence our simulation of this scenario, with a very gentle washing procedure. However, the method described is amenable to variations in substratum, and inoculum size and nature (different cell and soil types).

The cellulose replication technique allows for examination of the same area by different techniques. It is also possible to monitor wear of specific sites on a surface over time using this method. These impressions allow surface analysis of both localised and larger areas using a range of techniques which allow identification of surface features that are important in microbial retention. The titanium (TiOx) coating on the stainless steel discouraged the retention and enhanced the removal of both *Escherichia coli* cells and the meat conditioning film. This work indicates that coating stainless steels with titanium (TiOx) may increase the hygienic properties of a food contact surface under specified conditions.

# CHAPTER 3

# RETENTION OF *STAPHYLOCOCCUS SCIURI* AND *LISTERIA MONOCYTOGENES* ON SURFACES WITH DEFINED LINEAR FEATURES

## 3.1 INTRODUCTION

**Substratum surface physical and chemical properties are known to influence the extent and form of microbial adhesion and colonization. Biofouling and biofilm formation are major concerns in industries which require hygienic surfaces, such as the food industry. The tendency for a surface to facilitate the retention of microorganisms is undesirable because the presence of the microorganisms poses a biotransfer potential – that is, the ability to be transferred from the inert substratum to another, such as food, or personnel, where multiplication and infection might result (Verran 2002). Surfaces which are to be used in hygienic applications must be easy to clean, thus minimizing contamination of the product through corrosion or by the build up of harmful bacteria (Connolly et al., 1970). It can therefore be seen that hygienic quality is linked to cleanability (Mettler and Carpentier 1999), which in turn is linked to the characteristics of the surface. The adhesion of bacteria to a surface depends on a number of chemical, physical and microbiological factors. It has been hypothesised that bacteria preferentially stick to surfaces for three reasons: (i) a higher surface area available for attachment; (ii) protection from shear forces; and (iii) chemical changes that cause preferential physicochemical interactions. Increased surface roughness will additionally provide features such as pits and**

**scratches which will increase the area for attachment (Scheuerman et al., 1998), and protect from shear forces (Lehtola et al., 2007; Nejadnik et al., 2008).**

Focusing on topography, in more detail, it can be postulated that surface features whose dimensions greatly exceed those of the microorganisms will have little effect on retention because cells will be washed out fairly easily – unless within the large features there are micro and nano-topographies that will provide attachment points for the microbial cells (Tebbs et al., 1994; Flint et al., 2000; Edwards and Rutenberg 2001; Medilanski et al., 2002; Whitehead et al., 2005).

**In contrast, some studies have shown that there appears to be a minimum roughness value below which microbial cells are not retained (Verran et al., 2003). Standard worn surfaces reproducing those observed *in situ* were produced *in vitro*, were found to have $R_a$ values ranging from 23 to 900 nm (Verran et al., 2001) and were shown not to significantly affect retention of microorganisms. Stainless steels are widely used in hygienic applications, so it is useful to know that routine wear does not affect cleanability in terms of removal of microorganisms, in the absence of food soil. However topographical features are found on worn and new steel surfaces. These typically linear features are produced on *in-situ* surfaces by scratching but also on manufactured brushed steels to provide an aesthetic appearance. In contrast to the work by Verran et al., (200l), it has also been shown that surfaces with many defects of uneven dimension and distribution have poor cleanability (Jullien et al., 2003), and are more likely to remain more soiled than those with a more 'designed' topography, because of an increased number of retention sites for soiling components and microorganisms (Taylor et al., 1998). Thus, it is essential to be able to clearly define the nature of the topography of a surface in order to attain optimal**

**cleanabilty as hygienic quality and cleanability are closely linked to surface topography (Mettler and Carpentier 1999).Without precise surface characterisation, such predictions are likely to be incorrect and over simplistic.**

Thus, it is apparent that there is a relationship between the hygienic status of surfaces and their roughness within a specific $R_a$ range. It is also apparent that, whilst $R_a$ remains useful as a general guideline of surface texture, it does not provide sufficient information to describe the hygienic nature of a surface. It would therefore be of value to create defined features, which are of microbial dimension in order to investigate their effect on the retention of microorganisms and to understand the fundamental mechanisms influencing bacterial retention. Characterization of the effect of surface topography on microbial retention may enable the definition of a cut off value for a given substratum-microbial combination, below which retention will not occur (Medilaniski et al., 2002)

The aim of this work, was to determine the effect of linear features of defined width on the retention of different sized bacteria associated with the food industry. Surfaces were produced with the same surface chemistry but different surface topographies, with linear features on the surfaces of widths 30, 0.5 and 1 micron. Smooth titanium coated (TiOx) silicon wafers were also included as smooth controls. The objective of this work was to carry out retention assays on surfaces of defined topography to determine how cells of different sizes and shapes interacted with different sized features.

## 3.2 METHODS

### *3.2.1 Substrata*

Substrata with 30µm, 1µm and 0.5µm features were selected and produced as described in Chapter 2.

### *3.2.2 Microbiology*

#### *3.2.2.1 Microorganisms*

*Listeria monocytogenes* ScottA and *Staphylococcus sciuri* CCL 101 (kind gift from Dr Brigitte Carpentier (Agence française de sécurité sanitaire des aliments (AFSSA), Maisons-Alfort, France)) were used in the study. *Escherichia coli* was omitted due to its lack of adhesion onto surfaces described in Chapter 2.

#### *3.2.2.2 Preparations for retention assays*

Stock cultures of *L. monocytogenes* were inoculated on tryptone soya agar (TSA) (Oxoid, Hampshire, UK), and incubated at 30 ºC overnight. *S. sciuri* was prepared in the same way except nutrient agar (NA) and nutrient broth (NB) (Oxoid, Hampshire, UK) were used and cells were grown at 37 ºC. In preparation for retention assays plate cultures were stored at 4 ºC and used within one month. Ten milliliters of TSB were inoculated with a single colony of *L. monocytogenes* from a fresh culture and incubated at 30 ºC overnight or ten milliliters of NB were inoculated with a single colony of *S. sciuri* and incubated at 37 ºC for 24 hours. One hundred microlitres of *L. monocytogenes* was used to inoculate 100 ml TSB which was incubated at 30 ºC for 18 h or 100 µl of *S. sciuri* was used to inoculate 100 ml of NB which was incubated at 37 ºC for 18 h.

Following incubation, cells were harvested at 716 x *g* for 10 min and were washed three times, by re-suspension in sterile distilled water, vortexing for 1 min, and then centrifugation at 716 x *g* for 10 min. Cells were re-suspended to an optical

density (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 $1.07 \pm 0.58 \times 10^8$ colony forming units (cfu) $ml^{-1}$ for *L. monocytogenes* and $1.64 \pm 0.96 \times 10^8$ cfu $ml^{-1}$ for *S. sciuri.*

*3.2.3 Retention Assays*

The titanium coated (TiOx) test substrata were placed in sterile Petri dishes to which 25 ml of standardised cell suspension was added. The surfaces were then left to incubate horizontally at 37 ºC (*S. sciuri*) or 30 ºC (*L. monocytogenes*) without agitation for one hour. After the incubation period the surfaces were removed with sterile tweezers and each washed gently along the grooves, once with 5 $cm^3$ distilled $H_2O$, from a plastic bottle held at a 45º angle, with a 3 mm nozzle (Whitehead *et al.,* 2007). Substrata were then placed in a laminar flow hood, to allow any retained cells to air dry. Repeat experiments were performed with each sample tested in triplicate.

*3.2.4 Epifluorescence Microscopy*

Epifluorescence microscopy was used to observe the pattern and extent of retention of the cells on the surfaces. From the images produced the patterning of the cells on each surface could be seen reflecting the underlying topographic features. In order to locate and count the cells to be examined with respect to the interaction with surface features a method with higher magnification was required, the surface features themselves could not be seen using epifluorescence microscopy. Scanning electron microscopy allows for high resolution images to be achieved showing of the cells on the surface in relation to the features, allowing for percentage coverage of the surfaces by cells to be calculated.

*3.2.5 Preparation of microbial samples for Scanning Electron Microscope (SEM)*

The substrates with attached cells were immersed in 4 % v/v gluteraldehyde (Agar, Essex, UK) for 24 h at 4 ºC. Samples were thoroughly rinsed with 100 $cm^3$

distilled $H_2O$ as previously described. Samples were then dried in a class 2 flow hood for one hour, prior to being stored at room temperature in a phosphorous pentoxide (Sigma Aldrich, Dorset, UK) dessicator for at least 48 h. The samples were fixed to stubs for gold sputter coating, which was carried out using a Polaron E5100 (Milton Keynes, UK) SEM sputter coater. 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 scanning electron microscope (Jeol Ltd, Herts, UK) (Whitehead et al.,2005).

3.2.6 *Orientation of rod shaped cells on substrata*

Images were taken (n=10) of the surfaces with defined linear features. The number of cells on each of the surfaces was counted so a total cell count was achieved. The total number per unit area of rod shaped cell orientated along the grooves was also recorded. From these results the percentage of cells orientated in the direction of the grooves was also calculated. This was not necessary for the cocci. Ten images were taken for each surface. The number of attached cells was counted for each image, and the number of cells per $cm^2$ calculated.

*3.2.7 Statistics*

The statistical test carried out was a two-sample Student's *t*-test. Data were considered significant at the 95 % confidence level ($p < 0.05$). Error bars indicate the standard deviation of the data.

## 3.3 RESULTS AND DISCUSSION

*3.3.2 Surfaces of defined topography*

An understanding of how substratum surface properties affect the adhesion of bacteria assists in designing or modifying surfaces which discourage adhesion (Flint et al., 2000). The wear of food contact surfaces through abrasion, cleaning and impact damage increases the surface roughness (Verran and Boyd, 2001), thus can introduce topographical features which may increase retention of both organic soil and microorganisms (Boyd et al., 2001). This work investigated the effect of defined linear features that were representative of worn and new stainless steel surfaces, on the retention of microorganisms. The selection of the size and shape of the linear features on worn surfaces was based on previous work in our laboratories (Verran et al., 2009). Previous work has also demonstrated that typical wear of hygienic food contact surfaces does not necessarily affect their cleanability in terms of removal of microorganisms (Verran et al., 2001). However, in that work, surfaces were produced by abrasion and features were randomised across the surface to give topographies comparable with surfaces visulalised *in situ*. Thus surface feature sizes were irregular and ill defined. Other work in our laboratories has shown that surface features of new stainless steel were in the range of microbial dimensions (Verran et al., 2000; Whitehead and Verran 2007).

Surfaces with poor cleanability have many surface defects. These surfaces are more likely to remain more soiled because of an increased number of attachment sites for soiling components and microorganisms. Surface roughness provides niches in which microorganisms are protected from shear forces and hygiene and cleaning measures, thus allowing the entrapped microbial cells time to attach irreversibly to a surface (Taylor et al., 1998). A width of 30 microns was identified as being a typical 'large' feature, and the nanoindenter successfully produced surfaces with these relatively shallow, wide valleys either closely aligned, or more distant. Retained

microbial cells were distributed evenly (Fig 3.1), but where the features were closely aligned, retention was significantly enhanced at the 'peaks', where there had been some 'pile-up' of substratum material (Fig 3.2). Thus smaller surface features at the peaks had a greater effect on microbial retention than the larger features the smooth wide valleys at the base of the grooves.

Surfaces with a controlled titanium (TiOx) surface chemistry and smooth, 0.59 μm width linear features or 1.02 μm width linear features were produced, which were representative of smaller linear features apparent on in –use surfaces (Fig 3.3). These surfaces had significantly different ($p < 0.001$) $R_a$ values of 0.001 μm (smooth titanium (TiOx) surface), 0.0024 μm (0.59 μm width featured surface) and 0.0042 μm (1.02 μm width featured surface). Throughout this work, the surfaces are referred to as 0.5, and one micron surfaces respectively.

Smooth silicon wafers were also used for the retention assay as a comparison with a featureless surface. All surfaces were titanium coated using magnetron sputtering. AFM images (20μm scans) (Fig. 3.3) of these Ti coated substrates show the surface features. As the feature width increased there was an increase in the $R_a$ of the surfaces. However all the substrata had $R_a$ values far below the 0.8μm hygienic level (Fig. 3.4).

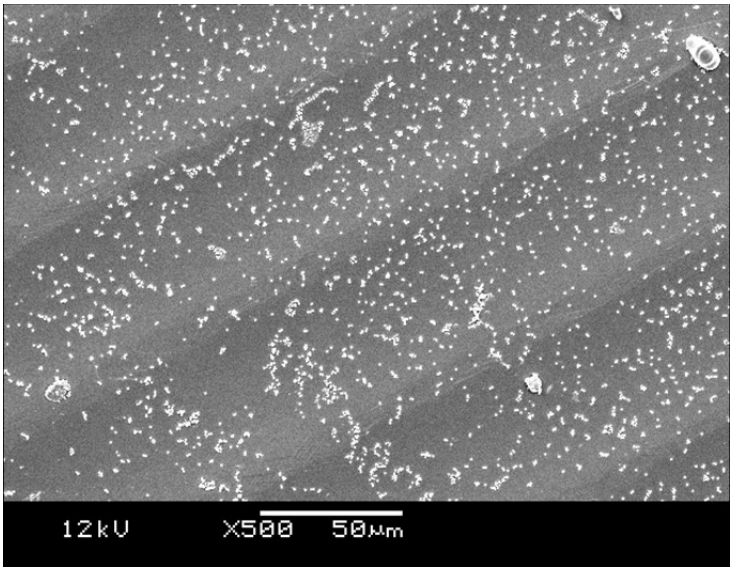



Fig 3.1 Thirty micron grooves with spacing showing *S. sciuri* cells are distributed evenly

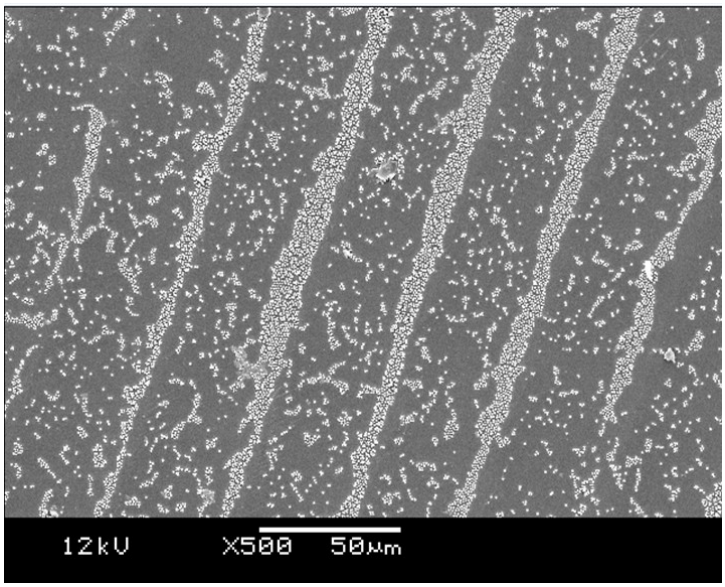



Fig. 3.2 Thirty micron grooves with minimal spacing showing *S. sciuri* retained in grooves

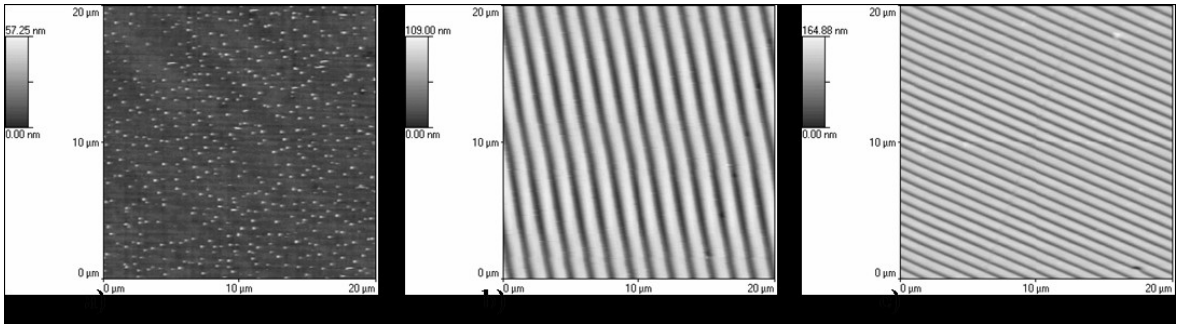



Fig. 3.3 AFM images of titanium coated (TiOx) surfaces a) silicon b) 1 micron wide groove c) 0.5 micron wide groove.

Fig. 3.4 $R_a$ values for surfaces of defined topography coated with titanium (TiOx).

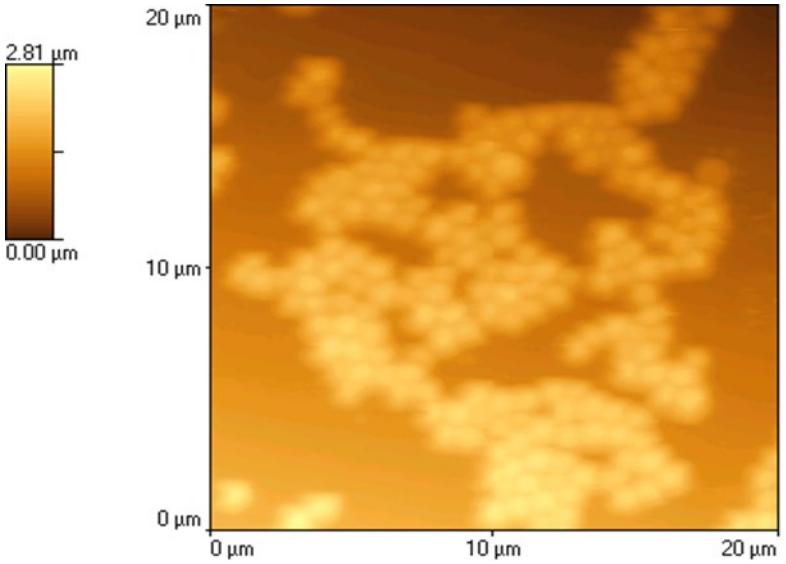
3.3.2 *Cell retention on surfaces*

AFM images (Fig. 3.5) of the bacteria were used show the rod and coccal shapes, with cell sizes being 2.5 µm x 1 µm for *L. monocytogenes* and 1 µm diameter for *S. sciuri*.

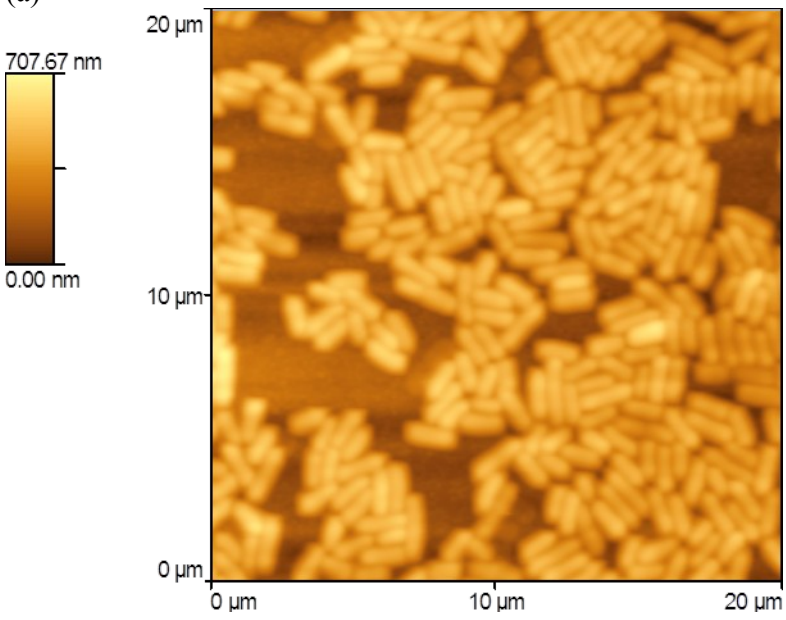
Retention assays on the surfaces of defined topography and chemistry using *S. scuri* (Fig. 3.6a) showed that on the smooth Ti coated silicon surface cells clustered together in clumps across the substrate surface. On the substrata with defined linear features (0.5, 1.0µm) (Fig. 3.6 b,c) microorganisms were clearly lodged into the surface features in smaller clusters and are more evenly distributed across the surfaces.

Following retention assays on the surfaces of defined topography and chemistry retention of the *L. monocytogenes* showed a similar pattern of distribution to that of the *S. sciuri.* Microorganisms on the smooth Ti coated silicon surface (Fig. 3.7a) were again organised in clusters across the smooth surface. The microorganisms on the 0.5µm (Fig. 3.7b) and 1µm (Fig. 3.7c) grooved substrata were more evenly distributed across the surface. The majority (81%) of the microorganisms on the 0.5µm surface are aligned across the grooves.

The number of microorganisms retained on the surfaces for *S. sciuri* (Fig. 3.6) was related to the size of surface features present. The highest retention of microorganisms was present on the surface with the largest features (1.0µm) and the lowest retention occurred on the surface with the smallest features (0.5µm) ($p<0.001$). The surfaces were cleaned along and across the grooves, but the cleaning direction was found to produce no significant difference ($p>0.05$) in the removal from *S. sciuri* for the 0.5µm and 1.0µm surfaces.




(a)




(b)

Fig. 3.5 AFM image (20 x 20µm square) scan of bacteria on silicon a) *S. sciuri* b) *L. monocytogenes.*

Fig. 3.6. Images illustrating microorganisms on surfaces of defined topography a i) Epifluoresence *S. sciuri* on Ti coated silicon a ii) SEM *S. sciuri* on Ti coated silicon b i) Epifluoresence *S. sciuri* on 0.5 micron groove b ii) SEM *S. sciuri* on 0.5

micron groove c i) Epifluoresence *S. sciuri* on 1 micron groove c ii) SEM *S. sciuri* on 1 micron groove.

Fig. 3.7 Images illustrating microorganisms on surfaces of defined topography a i) Epifluoresence *L. monocytogenes* on Ti coated silicon a ii) SEM *L. monocytogenes* on Ti coated silicon b i) Epifluoresence *L. monocytogenes* on 0.5 micron groove b ii) SEM *L. monocytogenes* on 0.5 micron groove c i) Epifluoresence *L. monocytogenes* on 1 micron groove c ii) SEM *L. monocytogenes* on 1 micron groove.

a)

b)

Fig. 3.8 Number of microorganisms retained on titanium coated (TiOx) substrate surfaces following retention assays dependent on washing direction a) along b) across linear features.

The highest numbers of *L. monocytogenes* retained were on the surface with the 0.5μm features and the least retention was present on the smooth silicon surface ($p<0.005$). The surfaces were cleaned along and across the grooves, but the cleaning direction was found to produce no significant difference ($p>0.05$) in the removal of *L. monocytogenes* for the 0.5μm and 1.0μm surfaces.

*3.3.3 Orientation of rod shaped cells on substrata*

Due to their rod shape *L. monocytogenes* cells might lie along or across the grooves. Using SEM images the cells on each of the substrata with defined features were visualised and counted. The orientation of the cells with respect to the direction

of the linear feature was also noted. The majority of cells on the 0.5µm wide grooves (81% washing along grooves, 77% washing across grooves) are aligned across the grooves. This implies that the cells remaining on the surface have the greatest retention when they are orientated across the groove.

Previous work (Medilanski et al., 2002) has hypothesised that for grooves smaller than the *L. monocytogenes* cells, the cells will orientate themselves across rather than along a scratch in order to maximise cell-surface contact. However just over half (57% washing along grooves, 53% washing across) of the *L. monocytogenes* on the 1µm grooved surface are aligned with the surface grooves. These grooves are of the order of the width of the microorganism, increasing the surface area for contact. However the binding energy appears to be greater for *L. monocytogenes* on the 0.5µm surface as the overall retention is greater. There is no significant difference in the wash direction ($p > 0.05$) on the orientation of cells for either the 0.5 or 1.0 micron grooved surface.

In conclusion using surfaces with defined linear topographic features and chemistry, retention of a microorganism was shown to be affected by the width of features present relative to the shape and size of the bacterial cell features of the same dimension enhance retention. Thus the $R_a$ value alone may not be enough to quantify the hygienic status of a surface, with surface feature size relative to cell size being an important factor. The retention assays described in this Chapter provide useful comparison regarding the amount of retention, but it is the strength of attachment which is of most concern. If high numbers of cells are easily removed, then that is of less importance than if low numbers of cells were strongly retained. The following Chapter explains this phenomenon.

# CHAPTER 4

# STRENGTH OF ATTACHMENT OF *STAPHYLOCOCCUS SCIURI* AND *LISTERIA MONOCYTOGENES* ON SURFACES WITH DEFINED LINEAR FEATURES

## 4.1 INTRODUCTION

The previous Chapter showed that features of defined topography affected the amount of retention of cells dependant on the shape and size of the cells and their relationship with the feature size. The aim of this work was to assess the strength of attachment by determining the lateral force required to remove coccal and rod shaped bacteria from surfaces with varying defined topographical features. The objective of this work was to use Atomic Force Microscopy (AFM) to measure the force of removal to displace two differently shaped bacteria, *Staphylococcus sciuri* and *Listeria monocytogenes* from three chemically and topographically defined surfaces; smooth silicon surface, 0.5 micron wide grooved surface, 1 micron wide grooved surface.

The environment in food preparation plants is considered as a significant source of microorganisms, where open work surfaces in particular are points frequently involved in the contamination of food products (Taylor et al., 1996). Microbial cells in this environment can stay viable, adhere to the equipment surfaces and contaminate any substance which comes into contact with them (Bower et al., 1996). In appropriate conditions these attached bacteria are then able to grow and colonise the surface as a biofilm (Kumar et al., 1998). Therefore the removal of these bacterial cells and prevention of growth is important. The adhesion of a microorganism to a surface is influenced by various factors related to the structural and physiological characteristics of the cell and the physical and chemical properties

of the surface (Jullien et al., 2002). For example, it has been shown that surface topography may influence removal and retention of microorganisms from the surface (Flint et al., 2000).

Measurement of the amount of cell attachment to surface has traditionally been quantified by cell counting methods *in situ* using microscopy/image analysis or culture (An et al., 1997). Flow cells have been commonly used in studies to calculate the strength of attachment to a surface. Cell adherence has also been calculated by applying a shear force across the surface and monitoring the cell detachment (Sjollema et al., 2001). Adhesion forces required to detach cells have also been calculated from surface tension effects caused by a passage of air bubbles across a surface (Gomez-Suares et al., 2001). These techniques are however limited because the measurement of the bacterial adhesion is based on an estimation of the critical force applied (Senechal et al., 2004). The AFM can be used to measure the force required to displace bacterial cells because it can image down to the nanometer level with high force resolution. The AFM tip can be used to displace individual bacterial cells by increasing the usually low perpendicular tip- surface force whilst the AFM tip can move in a raster fashion and dislodge weakly adhered cells (Boyd et al., 2002).

The effect of substratum topography on the removal of microorganisms will depend on the type shape and size of the microorganism. This work aimed to quantify the lateral force required to displace rod and coccal cells attached to topographically defined surfaces.

## 4.2 METHODS

### *4.2.1 Substrata*

Surfaces presented specific topographical features; 'smooth' surfaces (i.e. $R_a$ value < 5 nm) and surfaces with unidirectional grooved features of regular size (0.5 μm width grooves (DVD) and 1.0 μm width grooves (CD) were all produced as described in Chapter 2.

*4.2.2 Substrata coating*

Surfaces were coated with titanium as described in Chapter 2.

*4.2.3 Substrata coating*

In order for the effects of topography on microbial retention to be unambiguously ascertained, uniform surface chemistry is required. To achieve this, the selected substrata were coated in titanium via magnetron sputtering to provide surfaces of uniform chemistry, but varying underlying topography. Titanium coatings (typically 1 μm thick) were deposited onto the substrate surfaces by biased magnetron sputtering in a modified Edwards E306A coating system rig. Sputtering took place from a single 150 mm diameter x 10 mm thick, 99.5% pure titanium target. Prior to the deposition of the titanium coatings, the substrates were sputter cleaned at -1000V DC for 10 minutes. During deposition, the substrates were biased at -50V to ensure the formation of a dense conformal film.

*4.2.4 Contact angle measurements*

Contact angle measurements were determined at room temperature using the sessile drop technique (see Chapter 2).

*4.2.5 Scanning electron microscopy (SEM) and Energy Dispersive X ray (EDX)*

Titanium coated surfaces were used for SEM and EDX characterisation. Chemical analysis of substrata was carried out to a 1 μm depth using a Link Pentafet

detector (Oxford Instruments, Buckinghamshire, UK), with Inca software (Oxford Instruments, Buckinghamshire, UK). Analysis used a windowless system with a resolution of 133 eV. Images of substrata were obtained using a JEOL JSM 5600LV scanning electron microscope (Jeol Ltd, Herts, UK). Replicates were carried out in triplicate.

*4.2.6 Maintenance and preparation of microorganisms*

Cells were prepared as described on Chapter 2.

*4.2.7 Strength of cell attachment*

Using an AFM microscope, the force required to remove cells under liquid from a surface can be quantitatively measured by using a perpendicular force applied to the tip of an AFM cantilever as a tool. Ten microliters of bacteria were added to the test surfaces and dried onto the surfaces for one hour in a microbiological class 2 laminar flow hood and for the remaining twenty three hours at room temperature in a sterile container.

An Explorer AFM was used for the cell strength of attachment force measurements (Veeco Instruments, Cambridge, UK). The cantilevers were pyramidal probes with a manufacturer's spring constant of 0.05 $Nm^{-1}$, and front and back angles of 35$^{o}$ (Veeco Instruments Ltd., Cambridge, UK). A schematic diagram showing the cantilever assembly is given in Fig 4.0. Before each experiment the spring constant of the cantilever was determined. AFM was operated in contact mode and measurements were carried out at a rate of 20.03 μm $s^{-1}$ at a scan size of 20 μm ×20 μm. Substrata with dried cells were positioned on the AFM and a dry scan of the sample was taken to ensure the presence of cells in the area of analysis. 0.1 ml of HPLC grade water (BDH, UK) was placed on the sample and the AFM laser was re-aligned. The cantilever was brought into contact with the surface and a measurement of the force

applied to the cantilever was obtained from force distance curves. To convert the cantilever deflection to a perpendicular force, the spring constant, the value of the gradient in the constant compliance region of the force curve and the zero of the force was defined (Bowen et al., 2000).

The cantilever deflection (d) is then converted into a force (F) using Hooke's law:

$$F = - kd \qquad (1)$$

where k is the cantilever spring constant, and d is the cantilever deflection. The curve can be corrected by plotting F as a function of (z − d), where z is the vertical displacement of the piezoelectric scanner (Dufrene et al., 2001). Further calculations were carried using the methodology of Deupree and Schoenfisch (2008), to determine the lateral force of interaction of the cantilever tip with the cell. The applied force normal to the plane of interaction can be calculated from the equation;

$$Fapp = -kd \sin(\theta + Ø) \qquad (2)$$

where the angles θ and Ø are parameters of probe geometry and cantilever orientation respectively (Deupree and Schoenfisch, 2008). The lateral component of the applied force was determined using;

$$Flat = Fapp \cos(\theta) \qquad (3)$$

giving the value of the shear lateral force that may detach cells from the surface (Deupree and Schoenfisch, 2008). To determine the force required for bacterial removal, scans were repeated with increasing force applied to the cantilever tip. After each scan the remaining bacteria were counted and plotted as a percentage as a function of the lateral force applied.

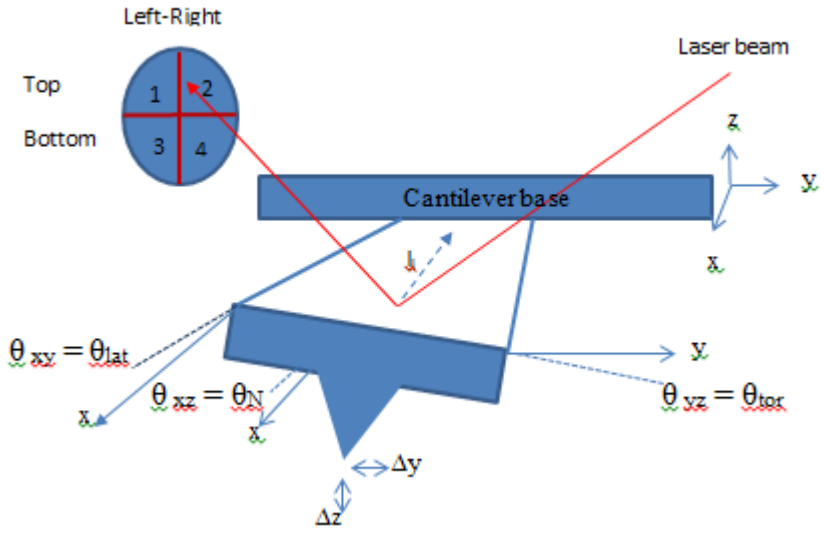



Fig 4.0 AFM diagram of cantilever with angles showing torsional and lateral deflections.

*4.2.8 Statistics*

Statistical tests were carried out using a two - tailed distribution t-test with two sample homoscedastic variance. The results are reported as mean ± standard deviation.

## 4.3 RESULTS AND DISCUSSION

AFM has been used to measure cell to surface adhesion by attaching bacterial cells to the cantilever tip and measuring the force required to detach the cell from the surface (Lower et al., 2000). This technique measures the perpendicular force applied to the surface. In order to replicate the cleaning process the lateral force required to displace bacterial cells must be investigated. The lateral force has been shown to be affected by surface topography (Busscher et al., 2001) where the lateral forces to displace the bacteria are considerably lower than that of the perpendicular force. It has been also demonstrated using AFM that microbial adhesion to linear featured surfaces which were smoother or had a rougher $R_a$ = 0.16 μm value gave rise to increased microbial retention (Medilanski et al., 2002).

### *4.3.1 Surface analysis*

SEM analysis demonstrated that the sputtered coatings were conformal and uniform in composition across the sample surfaces (Fig. 4.1). The surfaces were analysed using AFM and were shown to have $R_a$ values of 0.003 μm ± 0.0001 μm for the smooth titanium surface (TiOx), 0.024 μm ± 0.001 μm for the 0.5 μm featured surface and 0.026 μm ± 0.001 μm for the 1 μm featured surface (Fig. 4.2). The depth of the features on both grooved surfaces was 0.8 μm ± 0.04 μm. No significant variation ($p < 0.001$) in surface wettability was observed, with an average contact angle of 91˚ being determined for all three samples. This compares with the contact angle of 86° ± 7° for titanium oxide (Zhu et al., 2003).

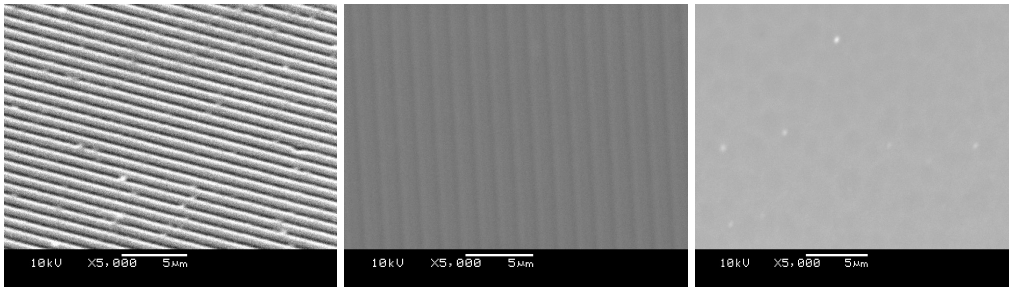



Fig. 4.1 SEM analysis demonstrating that the sputtered coatings were conformal and uniform in composition across the sample surfaces.

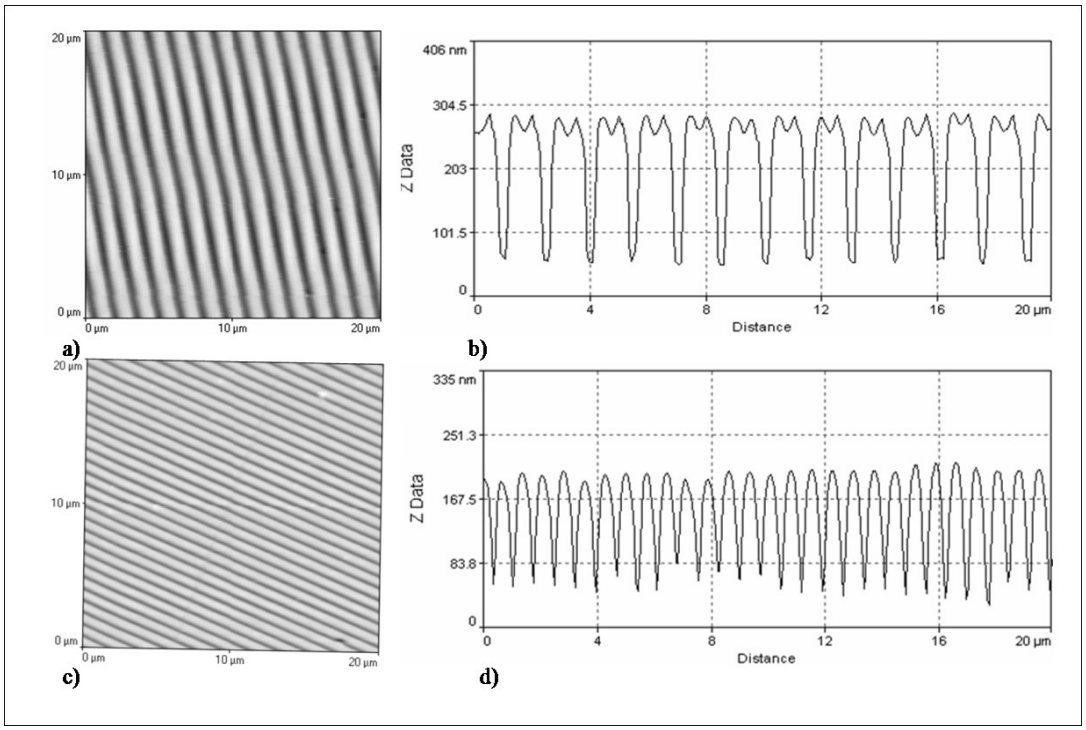



Fig. 4.2 AFM analysis of the surfaces to demonstrate the surface topographies a) 1 micron surface b) 0.5 micron surface.

*4.3.2 AFM force measurements*

The aim of this study was to compare the strength of adhesion of *E. coli*, *S. sciuri* and *L. monocytogenes* cells to substrata of defined topography *in-situ* via AFM. Use of the Explorer AFM allowed work to be carried out to determine the force required to remove *S. sciuri* or *L. moncytogenes* cells from 0.5µm, 1µm and smooth titanium surfaces (TiOx). Through the use of a liquid cell the force measurements were carried out under liquid, where the microorganisms were pushed off the surface if the applied force was great enough. The substrata with the dried cells was mounted on a metallic stub and positioned on the AFM magnetic sample area. The AFM was set up in contact mode and 0.1 ml of distilled water was applied to the surface of the substrate and the AFM laser realigned.

*4.3.2.1 Escherichia coli*

The strength of attachment of *Escherichia coli* could not be investigated because the cells were not adhered to the surface in the presence of water, so no force results could be obtained.

*4.3.2.2 Staphylococcus sciuri*

The AFM was used to determine the effect of increasing tip force on the removal of *S. sciuri* from smooth Ti coated Silicon (Fig. 4.3) 0.5µm (Fig. 4.4) and 1 µm (Fig. 4.5), titanium coated (TiOx) surfaces. Using the AFM , bacteria were visualised on all three of the substrate surfaces. Bacteria were clearly visible with a tip force of 1 nN. The effect of increased tip force on the removal of bacteria from the substrates is illustrated (Fig. 4.6, 4.7, 4.8).

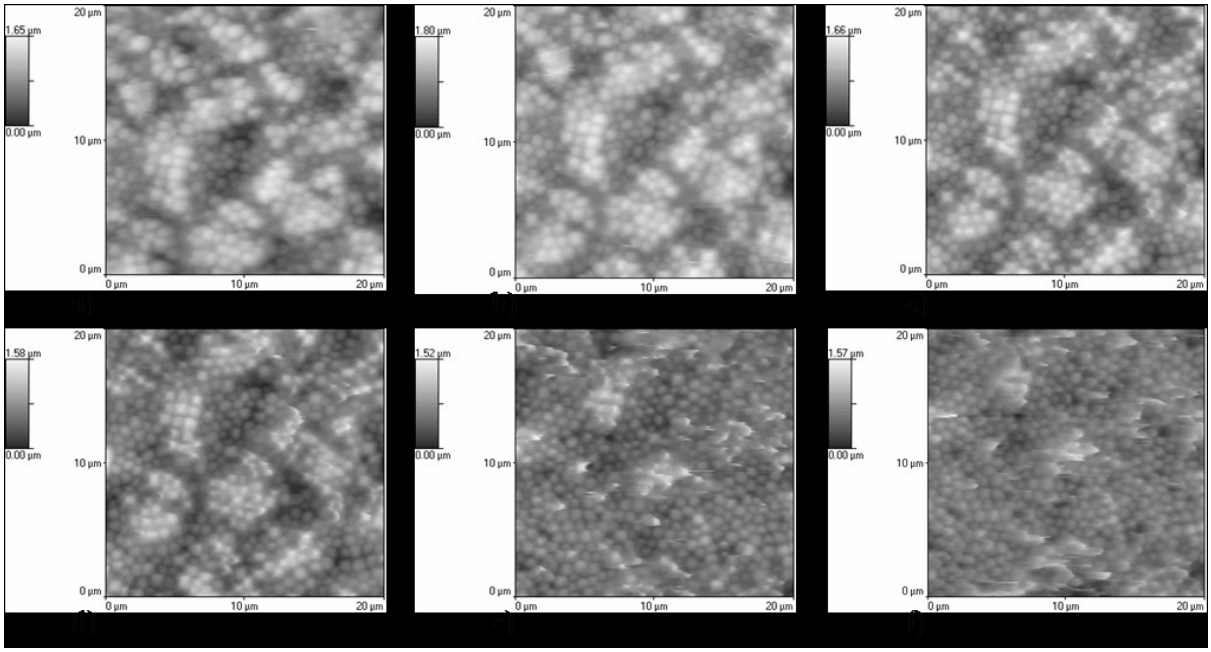


Fig. 4.3. AFM image of the removal of *S. sciuri* from a titanium coated (TiOx) smooth silicon surface with increasing tip force (nN) (a) 1 (b) 5 (c) 13 (d) 18 (e) 26 (f) 28

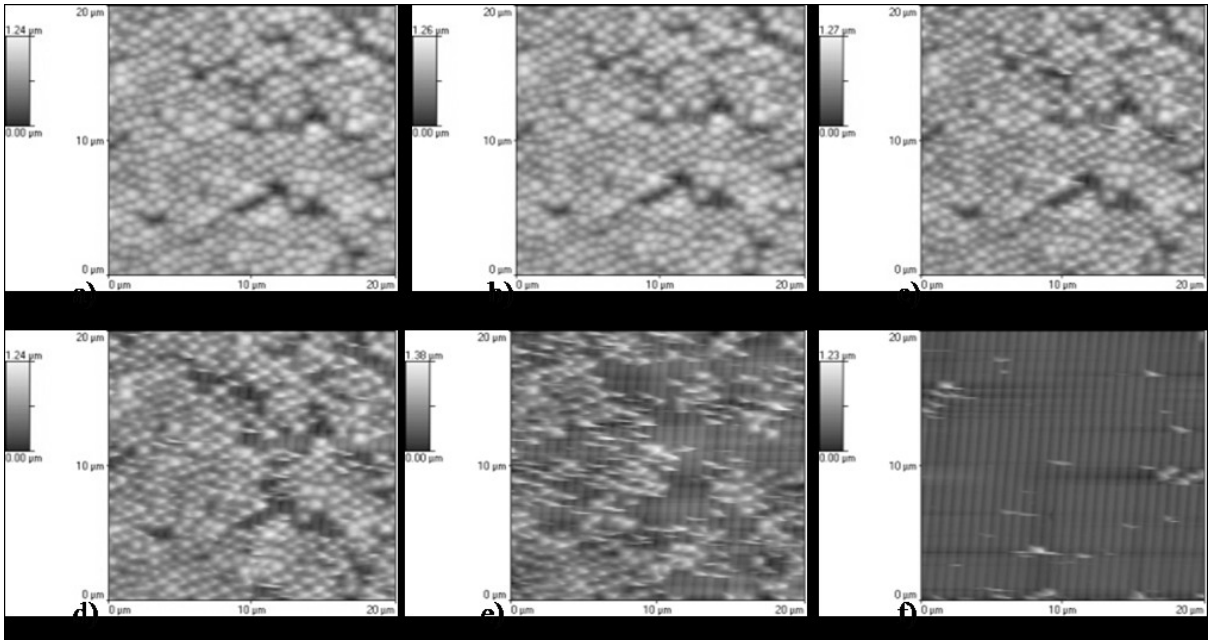


Fig. 4.4. AFM images after removal of *S. sciuri* from a titanium coated (TiOx) 0.5 micron wide grooved surface with increasing tip force (nN) (a) 3 (b) 6 (c) 8 (d) 12 (e) 16 (f) 19

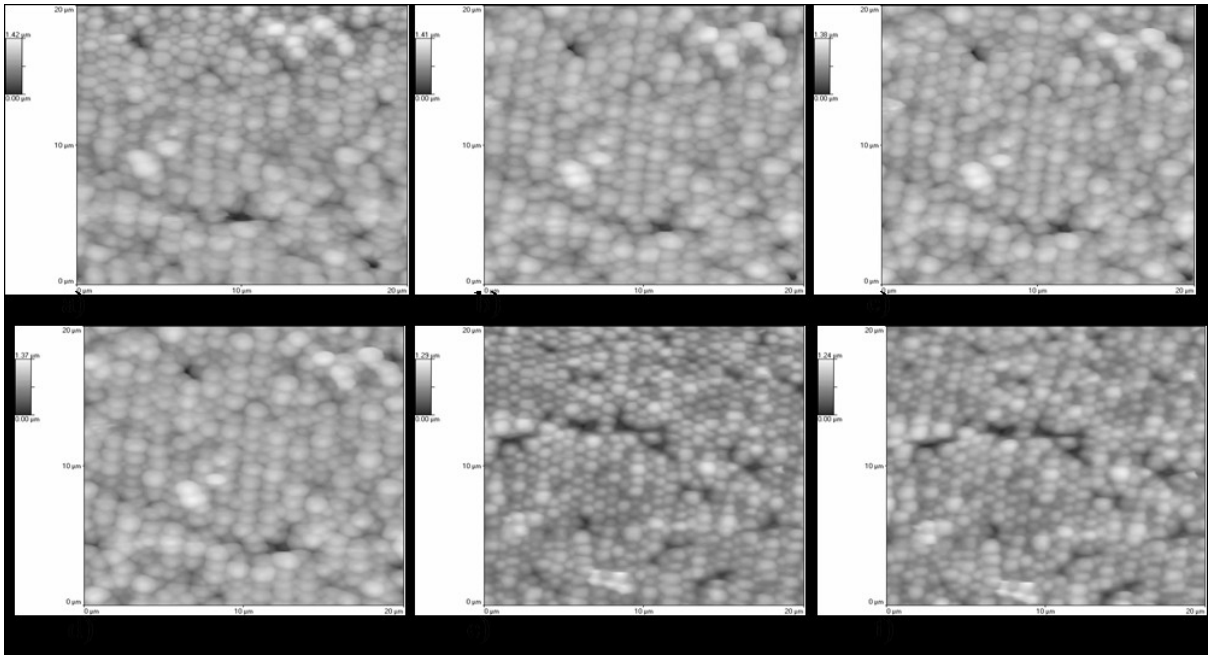


Fig. 4.5. AFM images of the removal of *S. sciuri* from a titanium coated (TiOx) 1 micron wide grooved surface with increasing tip force (nN) (a) 3 (b) 5 (c) 8 (d) 12 (e) 17 (f) 21

Fig. 4.6 The removal of *S. sciuri* from a smooth titanium coated (TiOx) silicon surface with increasing tip force. The experiment was repeated three times (n=3).

(a)

(b)

Fig. 4.7. The percentage removal of *S. sciuri* from a 0.5 micron grooved Ti coated (TiOx) surface with increasing tip force, with cleaning a) along the grooves, b) across the grooves. The experiment was repeated three times (n=3).

(a)

(b)

Fig. 4.8. Removal of *S. sciuri* from a 1 micron grooved Ti coated (TiOx) surface with increasing tip force, with cleaning a) along the grooves, b) across the grooves. The experiment was repeated three times (n=3).

As the tip force increased the cells began to be removed from the Ti coated silicon 'smooth' surface and the Ti coated 0.5 μm wide grooved surface (Fig. 4.7). However the number of cells attached to the Ti coated 1 μm wide grooved surface remained constant (Fig. 4.8). Significantly more ($p < 0.001$) cells were removed from the Ti coated silicon and the 0.5 μm wide grooved surface than the 1 μm wide grooved surface at an applied force of 20nN. The 0.5 μm wide grooved surface displayed the lowest level of retention, where a force of 20nN was sufficient to remove 95% of the cells from the surface (Fig. 4.7). Removal of the cells from the Ti coated smooth silicon surface was much lower with 35% of the cells removed at a force of 20nN (Fig. 4.6). However the majority of the cells remained attached to the 1 μm wide grooved surface with only 2% of the cells removed from the surface after an applied force of 20nN (Fig. 4.8).

*4.3.2.2 Listeria monocytogenes*

Cells were attached to the same three titanium coated (TiOx) surfaces of defined topography, silicon (Fig. 4.9), 0.5μm groove (Fig. 4.10) and 1 μm groove (Fig. 4.11). The effect of increased lateral force on the adhesion of *L. monocytogenes* to silicon, 0.5μm groove and 1 μm groove is as illustrated (Fig. 4.12, 4.13 a and b, Fig. 4.14a and b).

Fig. 4.9. AFM image of the removal of *L. monocytogenes* from a smooth titanium coated (TiOx) silicon surface with increasing tip force (nN) (a) 2.3 (b) 5.9 (c) 13.4 (d) 15.45 (e) 24.5 (f) 29.8

Fig. 4.10 AFM image of the removal of *L. monocytogenes* from a 0.5μm grooved titanium coated (TiOx) surface with increasing tip force (nN) (a) 0.9 (b) 6.9 (c) 12.1 (d) 15.2 (e) 19.3 (f) 27.4

Fig. 4.11. AFM image of the removal of *L. monocytogenes* from a 1 micron titanium coated silicon (TiOx) surface with increasing tip force (nN) (a) 2.3 (b) 6.2 (c) 9.5 (d) 11.8 (e) 15.96 (f) 21.87

Fig. 4.12. The removal of *L. monocytogenes* from smooth titanium coated (TiOx) silicon surface with increasing tip force. The experiment was repeated three times (n=3).

(a)

(b)

Fig. 4.13 The removal of *L. monocytogenes* from a 0.5µm grooved titanium (TiOx) coated surface, with cleaning a) along the grooves, b) across the grooves. The experiment was repeated three times (n=3).

(a)

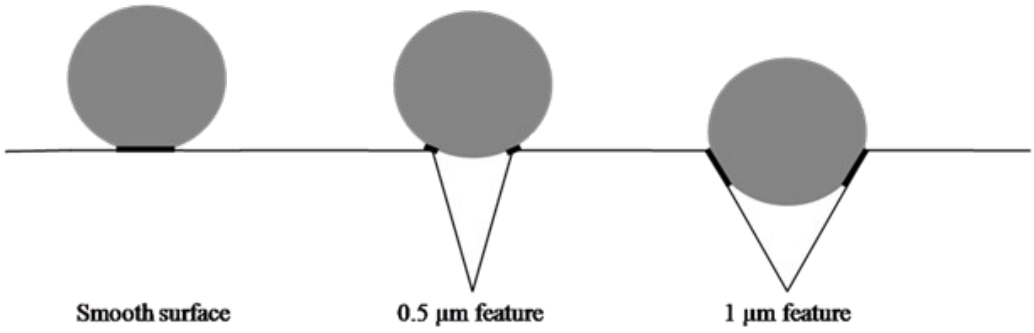
(b)

Fig. 4.14. Graph to illustrate the removal of *L. monocytogenes* from a 1 µm grooved titanium coated (TiOx) surface, with cleaning a) along the grooves, b) across the grooves. . The experiment was repeated three times (n=3).

The smooth silicon surface showed the lowest removal of microorganisms and the 0.5µm groove surface the highest removal. 7 % of cells were removed from the silicon (smooth surface) and 18% of cells were removed from the 1 µm groove surface whereas 92% of the cells were removed from the 0.5µm surface. Thus there was a significantly greater ($p < 0.0001$) removal of cells from the 0.5µm surface than the 1µm surface or the silicon surface. The removal of cells from the 1µm surface was significantly greater ($p < 0.0001$) than the Ti coated (TiOx) silicon surface. The removal of rod shaped cells from smooth titanium coated silicon has been shown to be significantly greater than the removal of rod shaped cells from 0.5µm surfaces, with 80% of cells removed from the 0.5µm titanium (TiOx) surface compared with 34% from the titanium coated (TiOx) silicon surfaces (Whitehead et al., 2006).

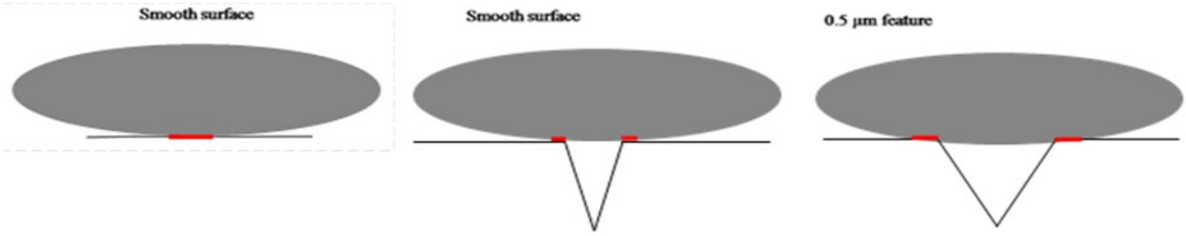
It is possible to explain the results with respect to the shape and size of the microorganism in relation to the width of the defined surface linear features. The titanium coated (TiOx) silicon (Fig. 4.12) and 0.5µm (Fig. 4.13) grooved surfaces

force curves show that as force increases the number of cells removed increases. The 1µm force curves (Fig. 4.14) display steps where an increase in force does not result in a removal of any cells. This lack of cell removal may be explained by the location of the cells on the surface. Cells which are orientated across the linear features may be easier to remove, hence the initial increase in cell removal. However those lying along the linear features may require a greater force for removal, thus resulting in a stationary phase until a higher more favourable removal force is attained. It appears that there was a relationship between the area of the cell in contact with the surface and the ease of removal of the microorganism. The smooth titanium coated (TiOx) silicon surface provides a small area of contact at the base of the coccal shaped *S. sciuri* (Fig. 4.15) providing a small area of cell – surface contact. However the rod shaped *L. monocytogenes* is much larger that the *S. sciuri* and is in contact with the surface along its length (Fig. 4.16), therefore providing a greater cell – surface contact than present for the *S. sciuri*. The 0.5µm surface grooves are much smaller than either the *L. monocytogenes* or the *S. sciuri,* therefore providing little cell – surface area for contact. Therefore the cell – surface contact is low on the 0.5µm surface grooves for both of the microorganisms, facilitating removal with increasing tip force. The 1µm wide surface groove is the optimal sized feature for the *S. sciuri* being of the same width, the thus providing a large area of cell – surface contact. The *L. monocytogenes* cells lie across the grooves on the 0.5 micron features (Chapter 3) decreasing the cell-surface contact from that observed on the smooth silicon surface. It is this cell surface contact that affects the force required for the removal of the microorganisms.




(a) (b) (c)

Fig. 4.15. The surface area of coccal shaped bacteria attached to a) smooth Ti coated (TiOx) silicon, b) 0.5µm titanium coated (TiOx) surface, c) 1µm titanium coated (TiOx) surface.




(a) (b) (c)

Fig. 4.16. The surface area of rod shaped bacteria attached to a) smooth Ti coated (TiOx) silicon, b) 0.5µm titanium coated (TiOx) surface, c) 1µm titanium coated (TiOx) surface.

A cell with low surface area contact will be removed with a lower applied force. On the 1 micron feature there were fewer rods retained (Chapter 3). However, they were predominantly retained within/along the features, so were hard to remove. It has been hypothesised (Edwards et al., 2001) that the binding strength of a cell is reduced by small grooves, but increased when the groove radius is close to the bacterial size. This hypothesis has been illustrated with respect to the microorganisms size in this study.

Using the AFM under liquid, cells were removed from a surface by applying an increasing defined tip force. The removal of cells was dependant on the feature size in relation to the shape and size of the microorganism. There was a significant difference ($p < 0.001$) in the removal of *S. sciuri* from all the surfaces. An increase in tip force was enough to remove cells from the 0.5µm grooved surface. However an increase in tip force was not significant enough to remove microorganisms from the 1µm grooved surface. There was a significant difference ($p < 0.001$) in the removal of *L. monocytogenes* from all surfaces with increasing tip force. More *L. monocytogenes* were removed from the 0.5µm micron surface than the smooth titanium coated (TiOx) silicon surface. Surface features smaller than the microorganism required the least tip force for removal irrespective of the shape of the microorganism. Cleaning along or across the grooves of the surfaces did not alter the removal of microorganisms with increasing tip force. Through use of an AFM under liquid the application of an increasing lateral force led to the removal of coccal cells. The shape of the microorganism in relation to the shape and dimension of the surface feature affected the ease of cell removal from a surface, depending on the surface area in contact with the cell. The presence of micron and sub-micron grooved features on a surface strongly influences bacterial adhesion and potentially, surface cleanability.

In contrast to estimation of the amount of retention, the AFM was used to determine the strength of attachment. The results for *S. sciuri* for retention and strength of attachment show the same patterns of remaining cells dependant on the underlying topography present, with the 1μm surface being the most difficult to remove cells from and the 0.5 μm surface the easiest. The *L. monocytogenes* provides results which differ between the retention assays and AFM measurements. Cells on the smooth silicon surfaces proved harder to remove using the AFM, whereas the cells on 0.5 μm surfaces proved easier to remove with increasing tip force.

Cell shape and size with respect to the shape and size of surface features determined the number of cells removed. *S. sciuri* and *L. monocytogenes* cells were more easily removed from the 0.5 μm surfaces. This effect is due to the shape of the microorganisms, as contact was lost with the surface across the groove diameter and confirms the model of Whitehead et al on adhesion of *S. aureus* and *P. aeruginosa* (Whitehead *et al* 2006). The *S. sciuri* cells were wedged within the 1μm features. These results support the hypothesis that both bacterial size and shape with respect to size and shape of the surface have an effect on the strength of cell attachment to the surface.

Therefore this work suggests that it is the strength of attachment that is important. If few cells are hard to remove, this is of more concern than lots of easily removed cells. It is therefore important to kill cells through post-cleaning disinfection and sanitation. It is also important to look at the effect of organic soil on cell adhesion (Chapter 5).

# CHAPTER 5

# THE DETECTION AND REMOVAL OF *E. coli* AND/OR MEAT SOIL ON SURFACES WITH DEFINED LINEAR SURFACE TOPOGRAPHIES

## 5.1 INTRODUCTION

Food and food contact surfaces can become contaminated with pathogenic and non pathogenic microorganisms through contact with soil, water, fertilizers, equipment, humans, aerosols, and animals (Verran et al., 2008). The presence of bacteria on stainless steel surfaces is commonplace in the food industry and can be considered an important source of potential contamination for any food, leading to economic and hygienic problems (Carpentier and Cerf 1993; Hilbert et al., 2003; Zottola et al., 1994). Not only is there concern surrounding the retention and transfer of potential microbial pathogens, but also increasingly, the effect of retained organic material on surface hygiene and cell retention properties is being investigated (Whitehead et al., 2010).

In an industrial plant where continual cleaning and soiling occurs, the detection of residual organic soil and cells is necessary to ensure that build up is minimal (Whitehead et al., 2010). Surfaces in industrial systems are critical components in the initiation of biofouling because they serve as the interface between the biological and mechanical environments where cells and organic material may attach to and be retained in inert surfaces (Whitehead et al., 2009). An open surface presents a solid–air interface, where surface conditioning may be “non specific”, resulting from the passive transfer of food material from substrate to substratum, as the food passes through the processing plant, or is handled in the domestic environment (Verran et al., 2008). The development of adsorbed layers, termed

conditioning films on a surface are considered to be the first stage in biofilm formation. On open food contact surfaces, once microbial attachment has occurred, a 'true' biofilm, that is a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, and that are embedded in a matrix of extracellular polymeric substances that they have produced (Donlan and Costerton, 2002), is unlikely to develop. However, the transfer of cells and soil to the surface, a process known as biofouling, may result in a gradual build up of material on the surface unless it is removed using a cleaning process. Attached microorganisms may be retained in surface features mixed with organic material such as fats, carbohydrates or proteins, or detergent residue. Thus, the term ''conditioning film'' may not be appropriate, especially where a more significant transfer of organic matter occurs (Verran, 2002), and surface coverage is uneven: ''soiling'' may be a more appropriate term.

*E. coli* O157:H7 can be transmitted to humans through indirect or direct contamination of foods (Bouvet et al., 2001). Undercooked ground beef and raw milk have been implicated in foodborne infection (Armstrong et al., 1996). Many strains of Shiga toxigenic *E. coli* are human pathogens causing illness ranging in severity from mild diarrhoea to severe renal complications that can result in death (Rivas et al., 2007). Cross-contamination during processing and subsequent handling and preparation of foods leads to the entry of these pathogens into the food chain (Hood and Zottola, 1997; Kumar and Arand, 1998). With increasing concerns over biotransfer potential and with the low minimum infectious doses for pathogens such as *E. coli* O157:H7, the detection of low levels of contamination is becoming increasingly important (Davidson et al., 1999).

The presence of conditioning films or food soil on a surface is known to affect the hygienic status of a surface (Jullien et al., 2008; Whitehead et al., 2008), yet the effect of conditioning films on cell attachment and retention is still unclear (Al-Makhlafi et al., 1994; Hood and Zolotta, 1997; Gram et al., 2007). Organic material on surfaces may provide a potential nutrient source which may lead to pathogen growth, transfer of microbial cells and subsequent food contamination and increased challenge for cleaning. The type of soil retained on the surface and the surface properties are important in influencing cell retention and surface hygiene. It has been shown that carbohydrate based organic fouling of a pastry site was almost completely eliminated by cleaning operations, whereas surfaces in meat and milk sites did not retain their initial surface properties indicating that food soil material remained on the surface. (Mettler and Carpentier 1998). Fat components are likely to interact with the hydrophobic regions of stainless steel (Snijders et al. 1985), and stainless steels fouled with proteins or fatty acids are more difficult to disinfect (Snijders et al. 1985). Thus, when surfaces interact strongly with organic material, the effectiveness of the subsequent cleaner or reagent used to clean or disinfect the surface may be reduced.

Regular cleaning of the equipment is required to prevent the build up of adsorbed organic material and microorganisms (Verran et al., 2010). In order to determine the most effective cleaning and sanitizing protocols it is important to assess the behaviour of these two components (soils and cells) on surfaces separately and in combination. There are many methods described for assessing the fouling of surfaces by microorganisms or by organic material, but a relatively simple method for assessment of the components in combination is not readily available (Verran et al., 2002). The accurate monitoring of surface cleanliness in terms of bacterial contamination is usually carried out using methods such as plate counts or replica

plating. However these methods take at least eighteen hours to obtain results and do not determine the presence or amount of residual organic material on a surface, which may interfere with cleaning and disinfection. One of the easiest methods given appropriate equipment is staining and visualization using epifluorescence microscopy. Using the differential staining technique previously described (Chapter 2), it was possible to visualize the distribution of cells and organic material on a surface before and after cleaning, and to measure surface coverage by using two stains via image analysis. Work described in Chapter 2 demonstrated reduced retention of *E. coli* and organic soil via the use of a titanium coating.

The aim of this Chapter was to extend this work using a fine polished stainless steel surface, a titanium coated (TiOx) fine polished stainless steel surface, and the two titanium coated (TiOx) surfaces of defined topographic defined linear feature or dimensions of 1 μm, 0.5 μm diameter. Surfaces were either fouled with single components (cells or soil) or mixed components (cells plus soil) and were subjected to wipe cleans. The effect of a one-off soiling event as well as sequential fouling – cleaning cycles were investigated. Results will further elucidate the effect of surface topography and chemistry on surface fouling and cleanabilty.

## 5.2 METHODS AND MATERIALS

### *5.2.1 Microorganisms (method from Whitehead* et al., *2010 IJFM)*

*Escherichia coli CCL410* was a kind gift from Brigitte Carpentier (AFSSA, France). This strain was 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 fecal samples by the laboratory of Dr C. Vernozy-Rozand (Unité de Microbiologie alimentaire et prévisionnelle, Ecole vétérinaire de Lyon, France). This strain was selected since this work was carried out as part of the EU PathogenCombat project which involved a number of members in the work package who, to ensure continuity needed to work with the same strain. Stock cultures were stored at -80 °C in freezer mix according to Caballero et al. (2009). In preparation for attachment assays *E. coli* was inoculated onto tryptone soya agar (TSA) (Oxoid, Hampshire, UK), and incubated at 37 °C overnight. Cultures were stored at 4 °C. Ten millilitres of tryptone soya broth (TSB) was inoculated with a single colony of *E. coli* and incubated at 37 °C overnight. One hundred microlitres of this culture was used to inoculate 100 ml TSB, which was incubated at 37 °C for 18 h with shaking (200 rpm). Following incubation, cells were harvested at 716×g for 10 min and washed once, by re-suspension in sterile distilled water, vortexing for 30s, and then centrifugation at 716×g for 10 min. Cells were resuspended to an optical density (OD) of 1.0 at 540 nm in sterile distilled water corresponding to $0.68 \pm 0.22 \times 10^8$ colony forming units/ml (cfu/ml).

### *5.2.2 Meat exudates (method taken from Whitehead* et al., *2011 Biofouling)*

The method was kindly provided originally by Brigitte Carpentier (Agence´ Francaise de Securite´ Sanitaire des Aliments (AFSSA), France). One kilogram of fresh rolled beef brisket (CO OP, UK) was cut into 10 mm×10 mm pieces. The meat

pieces were put into a stainless steel tray and covered in an aluminium foil. The meat was covered by another tray and weighed down with 8.4kg 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.

*5.2.3 Wipe cleaning*

Cleaning assays were carried out on a) titanium coated (TiOx) 0.5 μm linear featured surfaces b) titanium coated (TiOx) 1 μm linear featured surfaces c) fine polished stainless steel and d) titanium coated (TiOx) fine polished stainless steel. Surfaces (10 mm x 10 mm) were inoculated with the cells, meat soil or cell/meat soil mixture and dried in a microbiological class 2 cabinet.

A crockmeter was adapted for the wipe clean method to ensure that each wipe across the stainless steel surface was standardised to ensure repeatable results (Verran *et* al, 2001a; Airey and Verran, 2007). Inoculated coupons were placed on the steel specimen holder stage of a crockmeter (A.A.T.C.C Crockmeter, Model CM1, Atlas Electric Devices Co., Chicago, USA). A blue wipe cloth (WYPALL® x80, Hydroknit®, cleaning clothes - folded blue, Kimberley-Clark, Surrey, 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. 1 ml of sterile distilled water was pipetted onto the cloth. The hand crank was turned the correct number of times to simulate a wipe cycle. Substrata were either continually cleaned or re-inoculated after each wipe cycle with either the cells, meat soil or the cell-meat soil mixture and air dried for one hour before being re-cleaned to complete the next wipe cycle (Smith et al., 2011 personal communication).

*5.2.4 Preparation of stains*

Rhodamine B ([9-(2-carboxyphenyl)-6-diethylamino-xanthen-3-ylidene]-diethyl-azanium chloride) was made to a stock solution of 0.1 g $ml^{-1}$ in acidic ethanol and was used at a working concentration of 0.1 mg $ml^{-1}$. DAPI (4',6-diamidino-2-phenylindole) was made to a stock solution of 0.3 g $ml^{-1}$ in sterile distilled water and used at a working concentration of 0.1 g $ml^{-1}$.

*5.2.5 Differential staining of meat soil and E. coli*

For soiling the surfaces with cells and soil, 10 µl of cells and 10 µl of soil were mixed together in an Eppendorf tube, vortexed for 5 s and the preparation was pipetted onto a stainless steel coupon. The preparation was spread across the surface with a sterile plastic spreader and dried in a class 2 flow hood at room temperature. Ten microlitres of 4′,6-diamidino-2-phenylindole (DAPI) dissolved in water (0.1 g/ml) was added to the samples and spread across the surface using a sterile plastic spreader, then 10 µl of rhodamine B dissolved in acidic EtOH (0.1 mg/ml) was added to the sample and applied in the same manner (Whitehead *et al*., 2009). DAPI was used since it is a non intercalated DNA specific stain, whilst Rhodamine B is a widely used biological stain that will highlight proteins and carbohydrates. Following staining all samples were thoroughly, but gently, rinsed with at least 10 $cm^3$ distilled $H_2O$, (using a bottle held at a 45° angle, with a 3 mm nozzle), and were dried at room temperature in a microbiological class 2 flow hood in the dark.

*5.2.6 Epifluorescence microscopy*

Substrata were visualised using epifluorescence microscopy (Nikon Eclipse E600, Surrey, UK). The microscope was mounted with an F-View II black and white digital camera (Soft Imaging System Ltd, Helperby, UK, supplied by Olympus, Hertfordshire, UK). This system used a Cell F Image Analysis package (Olympus, Hertfordshire, UK). The percentage coverage area of the stained material was measured to determine the surface coverage of the organic material (n = 40). To obtain data for cells and soil separately, an area of the surfaces was selected and an image captured first using one UV light wavelength stain filter, and then a second image was captured using a second filter. The threshold of the images was set and the percentage coverage of the material stained was recorded individually.

### *5.2.6 Statistics*

Statistical tests were carried out as mentioned previously (*2.2.1.1*).

## 5.3 RESULTS AND DISCUSSION

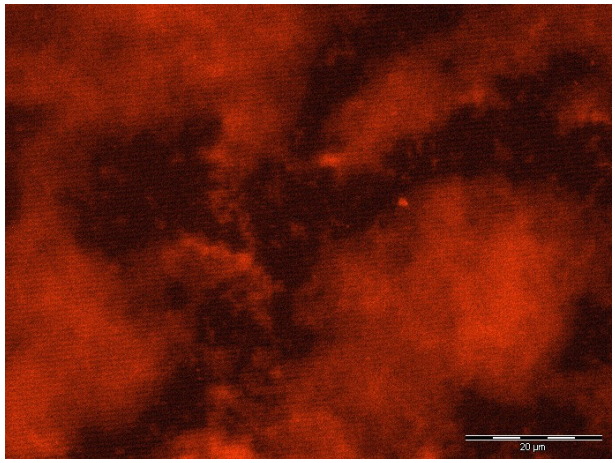
Following inoculation of either 0.5 µm or 1 µm titanium coated (TiOx) linear surfaces, or fine polished stainless steel of titanium coated (TiOx) fine polished stainless steel with either meat, *E. coli* or meat and *E. coli*, surfaces were wiped clean following one, or a number (≥10) of fouling events.

### *5.3.1 One initial fouling event*

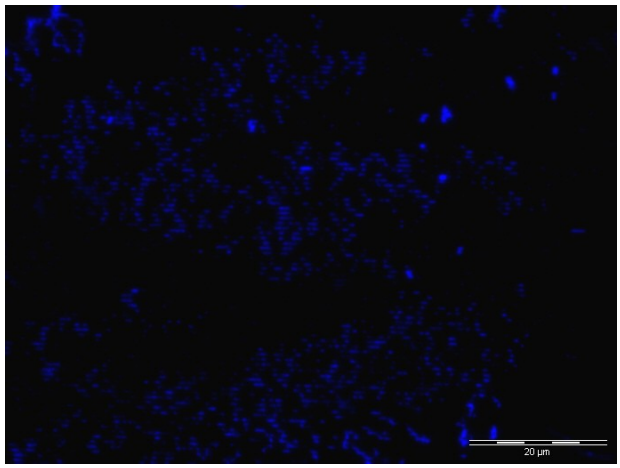
#### *5.3.1.1 0.5µm linear featured surface*

On the 0.5 µm linear surfaces the presence of a meat soil (Fig. 5.1a), *E. coli* (Fig. 5.1b) or meat soil and *E. coli* (Fig. 5.1c) was detected using differential staining before a wipe clean. The meat soil did not follow the contours of the linear surface features (Fig. 5.1a), and was heterogeneously spread across the surface. However, the cells appeared to follow the linear features of the surface in the absence (Fig. 5.1b) or presence (Fig. 5.1c) of the meat soil.

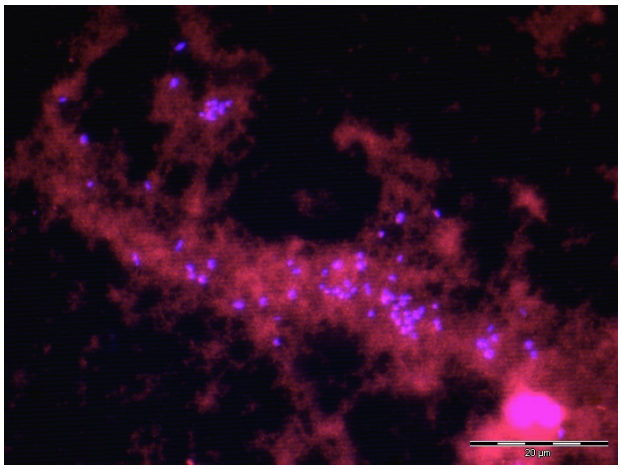
Following one initial fouling event and 0, 1, 5 and 10 repeated crockmeter wipes in a direction along and across the linear surface features, at higher number clean cycles, the meat and *E. coli* cells were difficult to detect, therefore data for only cleans at 0, 1 and 5 are shown. On the 0.5µm linear surface coverage of this surface by the meat soil decreased with increased wipes (Fig. 5.2). There was no significant difference found between the soil coverage after one wipe clean when those done across or along the direction of features were compared. However, a significant difference was observed after five wipe cleans. More meat soil was removed when the wipe was carried out along the surface features.




a)




b)




c)

Fig. 5.1 a) Meat soil stained with rhodamine (red) b) *E. coli* cells (DAPI stained blue) c) meat and cells retained together on 0.5 µm linear surfaces at 0 wipe demonstrating the presence of meat soil and cells. The cells that were retained followed the direction of the surface features

Fig. 5.2. Percentage coverage of meat soil remaining on 0.5μm linear surface features following 0, 1 and 5 repeated crockmeter wipes in a direction along and across the linear surface features, following one initial fouling event.

A few cells remained after a wipe clean in either direction (1.44 – 0.12%) (Fig. 5.3), and there was no significant difference between the percentage coverage of cells retained after one wipe, but with 5 wipes: more cells were removed from the surface when the wipe clean was carried out 'along' the surface features.

When cells and meat soil were combined and added to the surface, again more cells and soil were removed from the surfaces following the wipe clean along the surface features (Fig. 5.4). Insufficient data were obtained to enable comparison.

*5.3.1.2 1µm linear featured surface*

On the 1 µm linear surfaces meat soil (Fig. 5.5a), *E. coli* (Fig. 5.5b) or meat soil and *E. coli* (Fig. 5.5c) were detected before a wipe clean similar to the 0.5 µm linear surfaces in that the cells followed the linear features of the surface in the absence (Fig. 5.5b) or presence (Fig. 5.5c) of the meat soil. The meat (Fig. 5.5a), was heterogeneously spread across the surface.

When a meat soil was added and wiped from the 1µm linear featured surface it was removed with increasing numbers of wipes (Fig. 5.6). There was no significant difference between the effect of direction on wipe one, but, there was a significant difference between the along and across feature wipe results following the five and ten wipes, with more material retained when wiped along the features after 5 wipes.

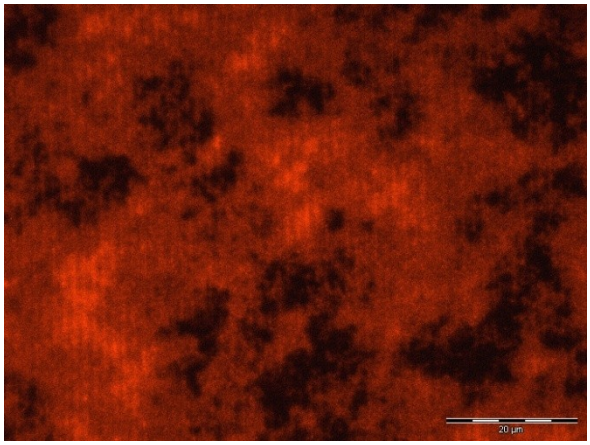
When *E. coli* cells were added and wiped from the 1µm linear featured surface, the results demonstrated that there was a very low percentage coverage of cells (0.8 %) remaining, but the coverage of cells retained on the 1µm linear featured surface decreased with increased cleans (Fig. 5.7). There was no significant difference between the percentage coverage of cells retained on the 1µm linear featured surface after along and across feature cleaning.

Fig. 5.3. Percentage coverage of *E. coli* cells remaining on 0.5μm linear surface features following 0, 1 and 5 repeated crockmeter wipes in a direction along and across the linear surface features following one initial fouling.

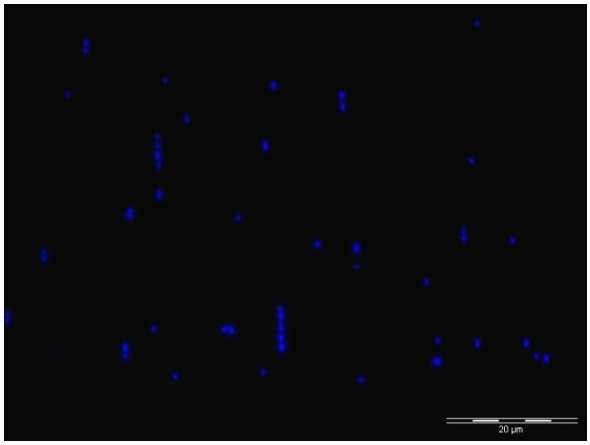
a)

b)

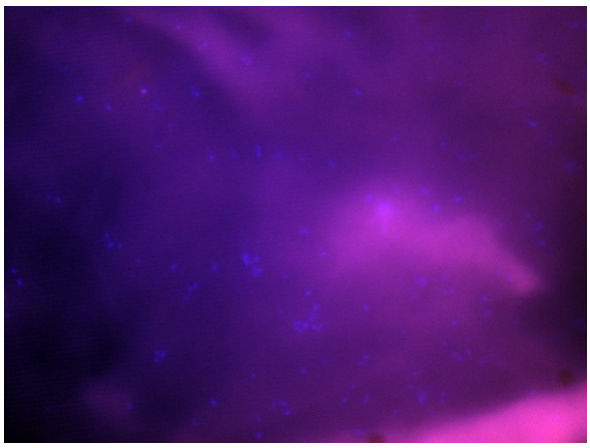
Fig. 5.4. Percentage coverage a) meat soil and b) *E. coli* cells remaining on 0.5µm linear surface features following a mixed application to the surface and 0 and 1 repeated crockmeter wipes in a direction along and across the linear surface features following one initial fouling event.




a)




b)



c)

Fig. 5.5 a) Meat soil stained with rhodamine (red) b) *E. coli* cells (DAPI stained blue) and c) the two combined on 1µm linear surface

Fig. 5.6. Percentage coverage of meat soil remaining on 1µm linear surface features following 0, 1, 5 and 10 repeated crockmeter wipes in a direction along and across the linear surface features following one initial fouling event.

Fig. 5.7. Percentage coverage of *E. coli* cells remaining on the titanium (TiOx) 1µm linear surface features following 0, 1, 5 and 10 repeated crockmeter wipes in a direction along and across the linear surface features following one initial fouling event.

Following the fouling of the surfaces with a mixed meat soil and *E. coli*, the amount of meat soil present decreased with increased wipes (Fig. 5.8). There was a significant difference between the amount of meat retained and the direction of wipe: surfaces cleaned in the direction (along) the surface features retained less meat soil. There was a low initial percentage coverage of cells (5.38%) at 0 wipe on the 1 µm linear featured surfaces (Fig. 5.8b), which was reduced further by cleaning (<0.36%). After wipe one, more cells had been removed in the across direction, whereas at wipe five more cells had been remove in the along direction. The number of cells retained after ten wipes was the same in both directions, but coverage was extremely low in all cases. When the coverage by cells in the presence and absence of soil was compared, at these low coverage values (Fig 5.9), it appeared that soil enhanced cell retention.

*5.3.1.3 Meat soil and E. coli on fine polished stainless steel surfaces*

Due to the difficulty encountered in retaining *E. coli* on the titanium (TiOx) surfaces (Chapter 2 and 4) (Verran et al., 2010) the percentage coverage of the mixed cell – soil inoculum were compared following one fouling event and repeated cleans (compared to Chapter 2 one clean) on fine polished titanium coated (TiOx) stainless

steel and fine polished stainless steel, in order to compare the effect of surface chemistry and repeated cleaning on retention.

On stainless steel coverage of the surface by meat soil and cells decreased with increased cleans (Fig. 5.10) (Fig. 5.11a and b) decreased with increased cleans. At wipe 0, the surface coverage for the meat soil was 81.74% and 19.49% surface coverage for the cells. However, by ten wipes the percentage coverage of meat soil had decreased to 1.39% (along) and 3.60% (across) and 0.18% (along) and 0.32% (across) for the cells. More cells and soil were removed when wiping along the linear features.

a)

b)

Fig. 5.8. Percentage coverage of a) meat soil and b) *E. coli* cells remaining on 1µm linear surface features following a mixed application of meat soil and cells following 0, 1, 5 and 10 repeated crockmeter wipes in a direction along and across the linear surface features following one initial fouling event.

Fig. 5.9. Comparison of cells remaining on a 1µm linear surface features following 0, 1, 5 and 10 repeated crockmeter wipes in a direction along and across the linear surface features in the presence and absence of meat soil following one initial fouling event.

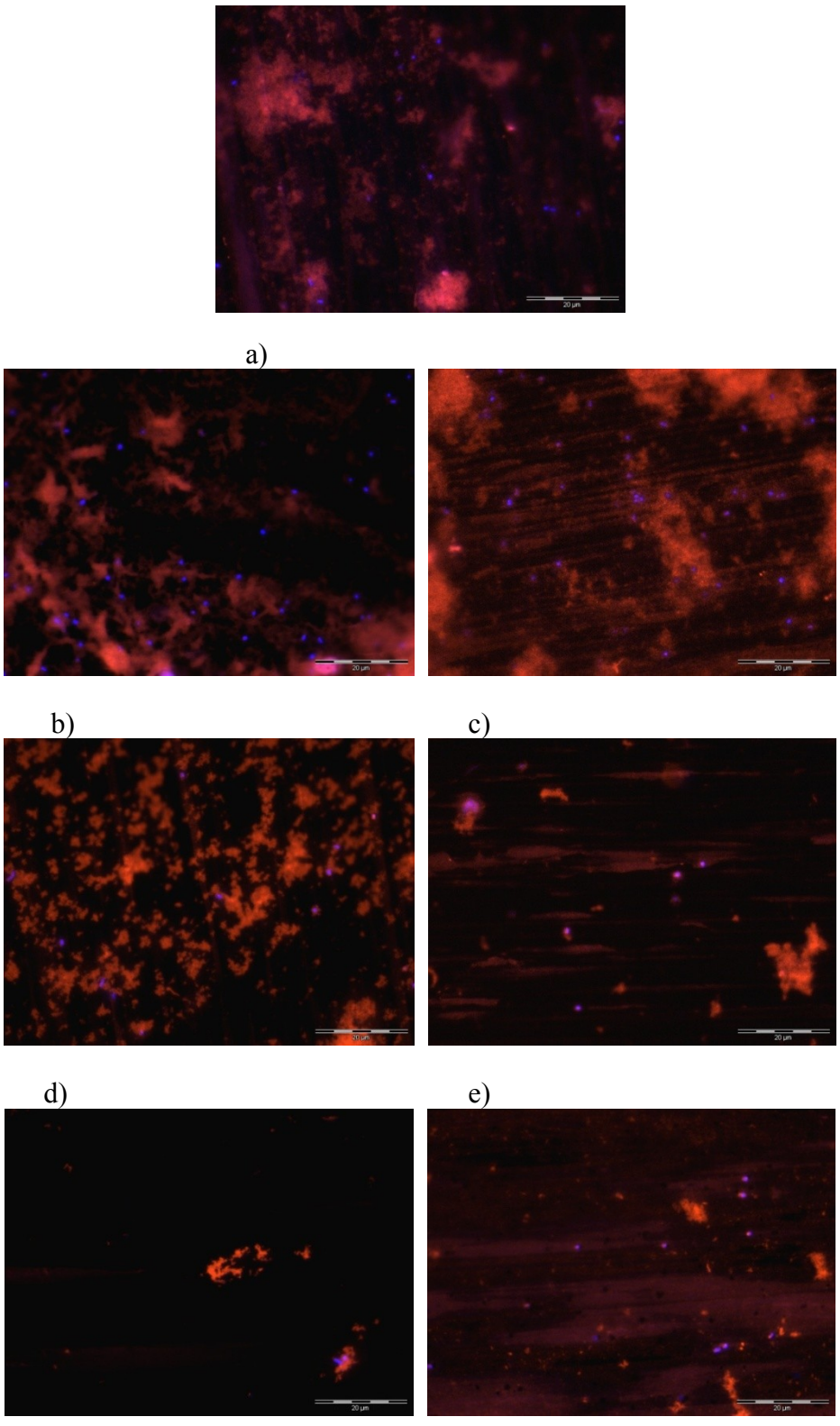



Fig. 5.10. Coverage of meat soil (red) and *E. coli* cells (blue) remaining on fine polished stainless steel surface following a) 0 wipe, b) one wipe clean along, c) one

wipe clean across, d) five wipe cleans along, e) five wipe cleans across, f) ten wipe cleans along and g) ten wipe cleans across.

a)

b)

Fig. 5.11. Percentage coverage of a) meat soil and b) *E. coli* cells remaining on fine polished stainless steel surface features following 0, 1, 5 and 10 repeated crockmeter wipes in a direction along and across the linear surface features following one initial fouling event.

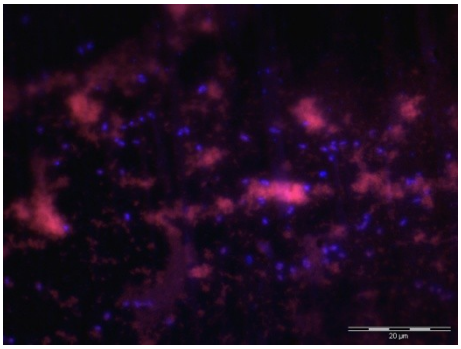
*5.3.1.4 Meat soil and E. coli on titanium coated, fine polished stainless steel surfaces*

Similarly, using cell – soil inoculum on titanium coated (TiOx) fine polished stainless steel, a surface with unidirectional grooves. Meat soil was heterogeneously spread across the surface, with coverage reducing with increasing wipes (Fig. 5.12) (Fig. 5.13). There was no trend in removal of the meat soil or cells when the surface were wiped along or across surface features. The presence of soil increased the very low retention of *Escherichia coli* on the titanium (TiOx) surface that was observed in Chapter 2, but again removal was more effective from the titanium coated (TiOx) surface (Fig. 5.14).

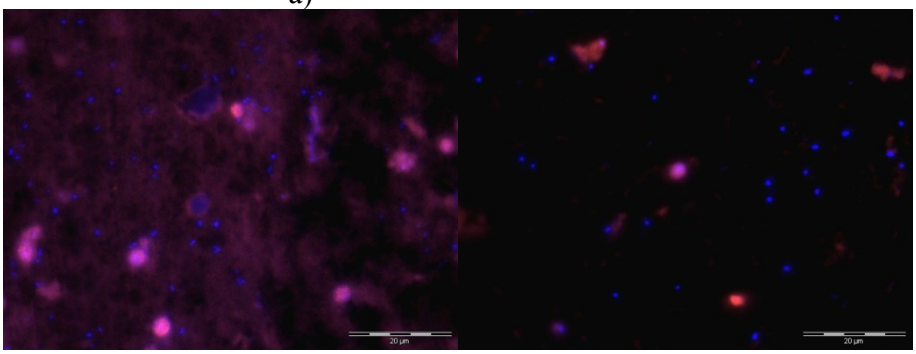
*5.3.2 Repeated fouling events*

*5.3.2.1 0.5µm linear featured surface*

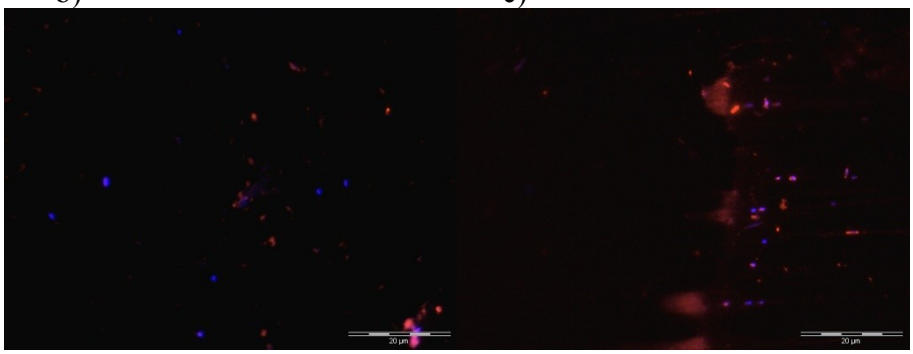
Following fouling of the 0.5 µm linear featured surfaces, it was again demonstrated both qualitatively (Fig. 5.15) and quantitatively (Fig. 5.16) that with increased wipe cleans, even with re-fouling, that an increased amount of meat soil and cells were removed from the surfaces with increased cleans. After one wipe clean more meat soil or cells were retained on the surfaces that had been cleaned in the direction across the surface features, than was removed following one wipe clean along the surface features. Although, after 10 cleans this was not the case. The additional inoculum applied after each wipe did not increase overall coverage: indeed coverage was considerably lower in this case, indicating the obvious value of physical cleaning on cumulative fouling.



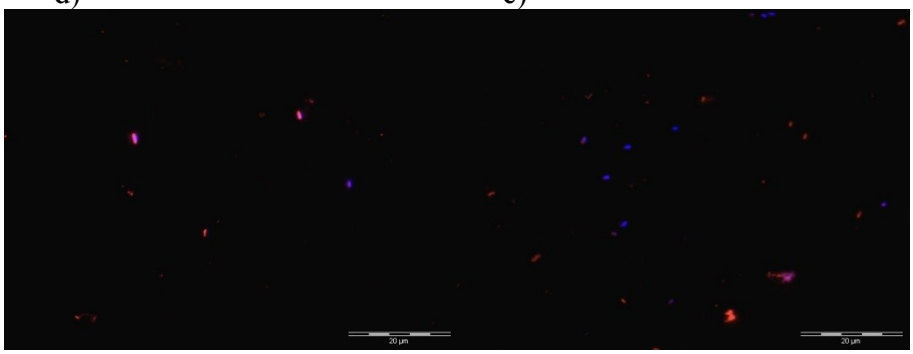
a)



b) c)



d) e)



f) g)

Fig. 5.12. Coverage of meat soil (red) and *E. coli* cells (blue) remaining on titanium coated (TiOx) fine polished stainless steel surface following a) 0 wipe, b) one wipe

clean along, c) one wipe clean across, d) five wipe cleans along, e) five wipe cleans across, f) ten wipe cleans along and g) ten wipe cleans across.

a)

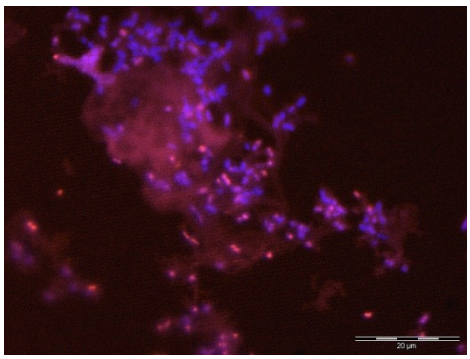
b)

Fig. 5.13 Percentage coverage of a) meat soil and b) *E. coli* cells remaining on titanium coated (TiOx) fine polished stainless steel surface features following 0, 1, 5 and 10 repeated crockmeter wipes in a direction along and across the linear surface features following one initial fouling event.

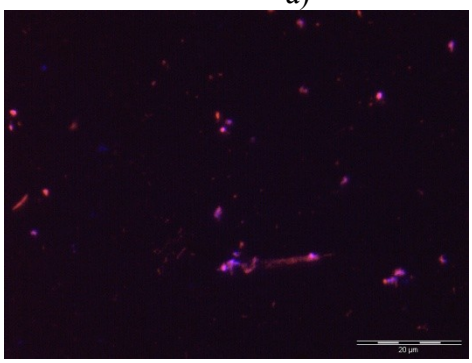
a)

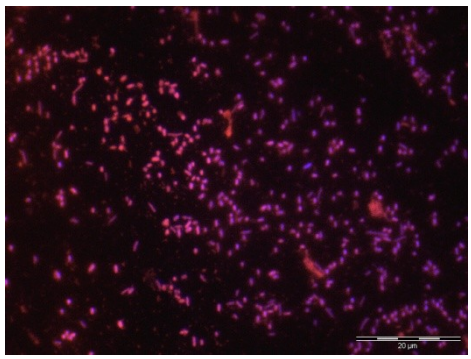
b)

Fig. 5.14. Comparison of the results for the percentage coverage of a) meat soil and b) *E. coli* cells remaining on titanium coated (TiOx) fine polished stainless steel and fine polished stainless steel surfaces features following 0, 1, 5 and 10 repeated crockmeter wipes in a direction along and across the linear surface features following one initial fouling event.

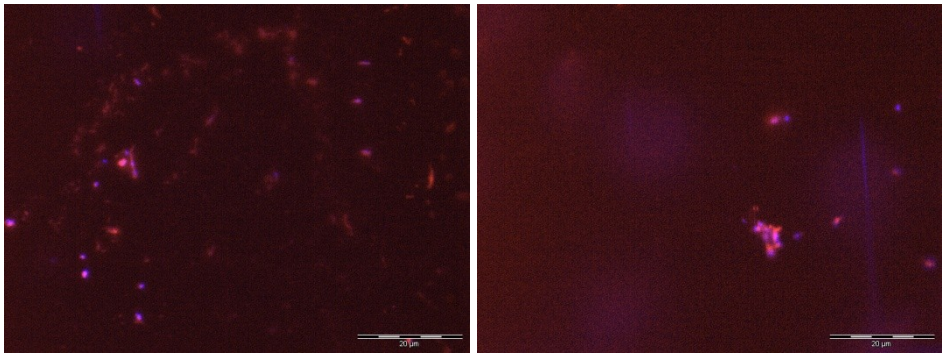


a)





b) c)



d) e)

Fig. 5.15 Coverage of meat soil (red) and *E. coli* cells (blue) remaining on titanium coated (TiOx) 0.5 µm linear featured surfaces following repeated crockmeter wipes in a direction along and across the linear surface features with repeated fouling events following each wipe clean. a) 0 wipe cleans b) 1 and c) 10 cleans in along direction, d) 1 and e) 10 cleans in across direction. The distribution of meat soil and cells on the surfaces.

a)

b)

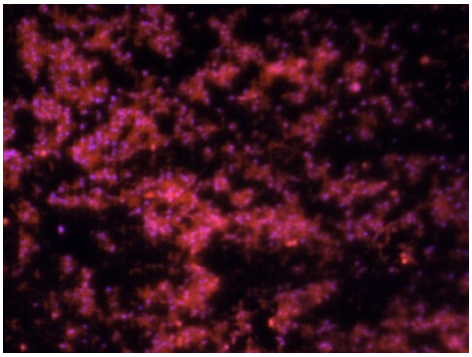
Fig. 5.16. Percentage coverage of a) meat soil and b) *E. coli* cells remaining on titanium coated (TiOx) 0.5 µm linear featured surface following 0, 1 and 10 repeated crockmeter wipes in a direction along and across the linear surface features with repeated fouling events following each wipe clean.

*5.3.2.2 One micron linear featured surface*

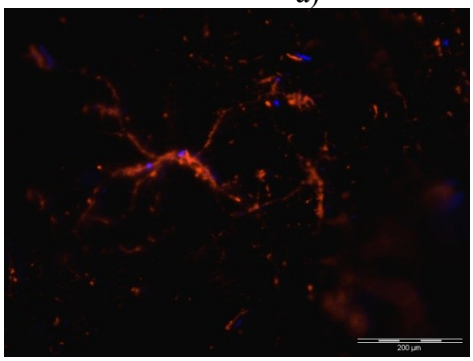
Unlike some of the previous epifluorescence images, it was difficult to state whether the amount of meat soil and cells retained on the surfaces increased with increased cleans (Fig. 5.17). However, it could be evidenced that following 10 cleaning cycles that cells were still evident on the surfaces (Fig. 5.17c/e). Thus in contrast to the previous work, an increase in cleans across the linear surface feature direction, resulted in an increased amount of meat soil (Fig. 5.18a) and cells (Fig. 5.18b), with the exception of the percentage coverage of the meat soil when the wipe clean was carried out along the linear surface features. There was a significant difference between the amount of cells and meat soil retained on the surfaces following 10 wipe cleans, when the direction of cleaning (along vs across) was compared. There was also a significant difference for the amount of retained meat soil on the surfaces following one wipe clean when the direction of cleaning was compared. After one wipe clean more meat soil or cells were retained on the surfaces cleaned along the linear surface features, whereas after ten cleans, more meat soil or cells were retained on surfaces cleaned across the direction of the linear surface features.

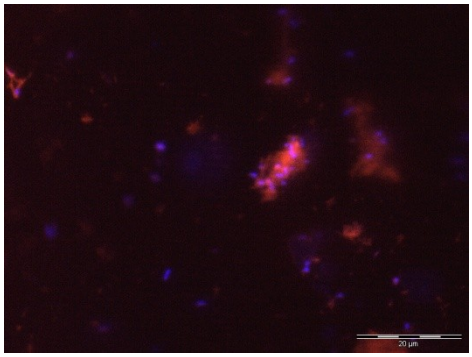
*5.3.2.3 Comparison of fine polished stainless steel and titanium coated fine polished stainless steel surfaces following ten wipe cleans*

Less meat soil (Fig. 5.19) (Fig. 5.20a) and cells (Fig. 5.20b) was retained on the titanium coated (TiOx) fine polished stainless steel surfaces when cleaned in either the direction of, or across the linear surface features ($p < 0.05$).

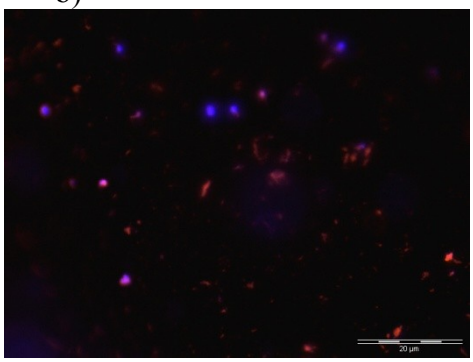


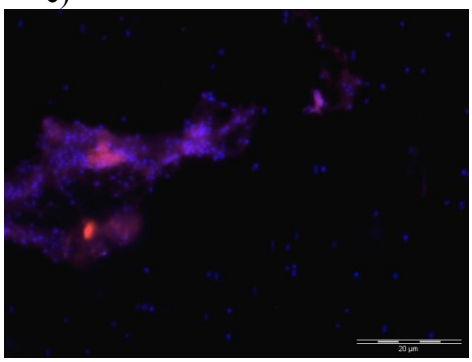
a)





b) c)





d) e)

Fig. 5.17. Coverage of meat soil (red) and *E. coli* cells (blue) remaining titanium coated (TiOx) 1 µm linear featured surfaces following repeated crockmeter wipes in a direction along and across the linear surface features with repeated fouling events following each wipe clean. a) 0 wipe cleans b) 1 and c) 10 cleans in along direction, d) 1 and e) 10 cleans in across direction.

a)

b)

Fig. 5.18. Percentage coverage of a) meat soil and b) *E. coli* cells remaining on titanium coated (TiOx) 1 μm linear featured surface following 0, 1 and 10 repeated crockmeter wipes in a direction along and across the linear surface features with repeated fouling events following each wipe clean.

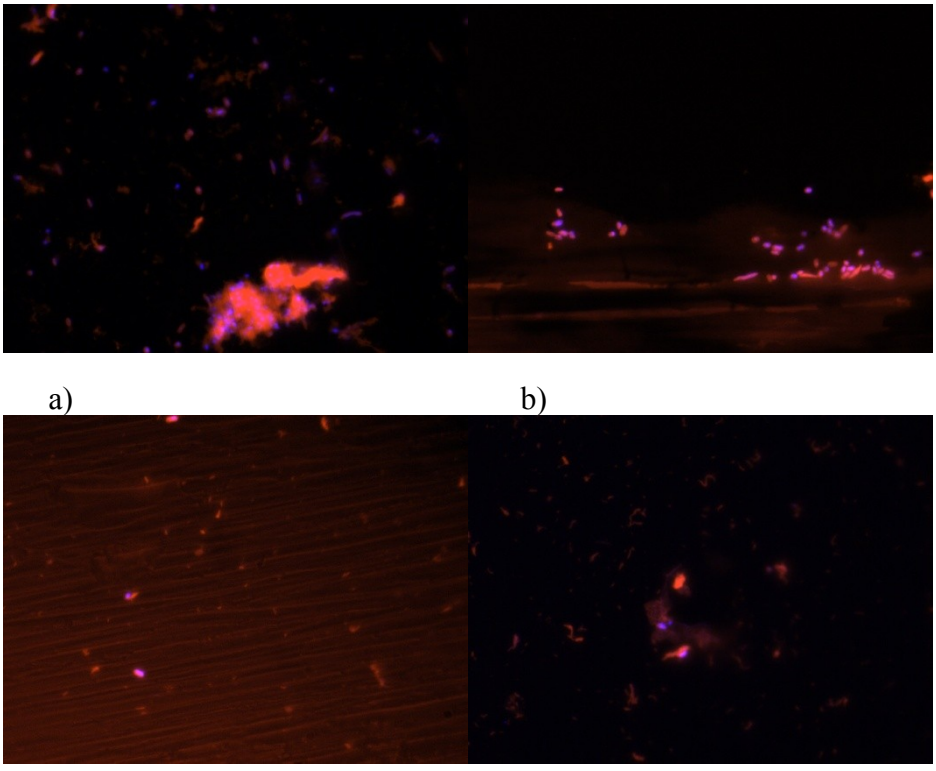



Fig. 5.19. Coverage of meat soil (red) and *E. coli* cells (blue) remaining on the a) fine polished stainless steel cleaned in a direction along the linear surface features b) fine polished stainless steel cleaned across the linear surface feature direction c) titanium coated (TiOx) fine polished stainless steel cleaned in a direction along the linear surface features and d) titanium coated (TiOx) fine polished stainless steel cleaned in a direction across the linear surface features surfaces following 10 repeated crockmeter wipes with repeated fouling events following each wipe clean.

a)
b)

Fig. 5.20 Percentage coverage of a) meat soil and b) *E. coli* cells remaining on the fine polished stainless steel (FPSS) and titanium coated (TiOx), fine polished stainless steel (TiFPSS) following ten wipe cleans in a direction along or across the linear surface features with repeated fouling events following each wipe clean.

This series of experiments provided a simulated 'clean' more closely resembling the environment *in situ*, rather than a gentle rinse (Chapter 2) or an AFM probe (Chapter 4) applying a removal force. Only water was used, rather than any chemical agent, to assess the effect of a physical force.

In all cases, coverage of surfaces by organic soil was high, at around 70%, with cell coverage being well below 20%. Cleaning always reduced coverage irrespective of surface or fouling/cleaning methods used. In the vast majority of cases (two exceptions) more soil and cells, separately or in combination, were removed when the wipe force was applied along the linear features. With repeated fouling-cleaning cycles, it appeared to be easier to remove soil and hence cells, soil enhanced cell retention – from larger features presumably due to improved accessibility to the wipe process and a greater amount of soil not attached to the surface of the features, thus reducing adhesion for the same topography ( ie fine polished stainless steel) a titanium coating (TiOx) improved cleanability in terms of soil and cell removal fro single and repeated fouling.

# CHAPTER 6

## SUMMARY AND CONCLUSIONS FROM THIS WORK

For hygienic, open, food contact surfaces, the retention and survival of viable microorganisms pre- and post cleaning and disinfection is of key concern since the presence of viable, if not necessarily multiplying, cells on open surfaces poses a biotransfer potential. The presence of organic (and inorganic) material on a surface affects its cleanability, and also, potentially, its hygienic status since the soil can interfere with the activity of cleaning and disinfecting agents, by physically and chemically protecting microorganisms.

Work in our laboratories has demonstrated that repeated fouling and cleaning cycles result in accumulation of organic soil on surfaces, but not of microorganisms, especially in surface features on simulated 'worn' surfaces, where linear features were randomised across the surface. (Verran et al., 2001; Verran et al., 2006). Using defined linear features with parallel orientation cells and meat soil were best removed with a wipe along, rather than across surface features. This occurred regardless of whether the cells and meat soil had been applied separately or together.

It has been suggested that relatively large deposits of organic soil may fill larger defects prior to microbial surface contamination (Frank and Chmielewski, 1997; Kumar and Anand, 1998; Milledge and Jowitt, 1980; Verran and Jones, 2000), and mask the underlying topography, while the formation of a thinner conditioning film (Carpentier and Cerf, 1993) on any surface in an aqueous environment may mask small topographical features (Boyd et al., 2000). There is also evidence that nanoscale roughness enhances the adhesion of the conditioning layer to the substratum (Hanarp et al., 1999). This cumulative soiling will inherently affect surface conditions and thus

microbial attachment and retention. It was evident from this work that although a build up of meat soil was not demonstrated with increased cleans, once fouled a surface always retains some organic material, and this retention was more evident on surfaces with larger defects. Work by the authors has shown that in a commercial bakery, residual material on surfaces generally consists of organic material with negligible microbial presence (Whitehead et al., 2009). The organic material tended to be embedded in the surface features of the work surface, and retained at low concentrations (Moore and Griffith, 2002). Thus, since surfaces once used are never again pristine, the retention and detection of organic material on surfaces is of importance. The soil will ultimately affect subsequent cell attachment and retention to the surfaces, and its presence should, therefore be taken into consideration when designing suitable cleaning regimes.

### *6.1 Concluding comments*

Substrata used in *in vitro* cleaning and disinfection assays should provide a realistic and reproducible challenge. This work used surfaces that reproduced those in the food industry, and via controlled surfaces with defined features that had been designed following analysis of the surface finishes and topographies used in the food industry (Chapter 1). Findings reveal that the size and shape of the surface features affects the cleanability of the surface, and also suggests that the directionality of cleaning affects efficacy. It has previously been shown on fine polished stainless steel surfaces, that microorganisms were retained in the smaller surface grooves and grain boundaries (Fang et al., 2002), which was speculated to be due to a lowered cell-surface binding energy (Whitehead and Verran, 2007). However organic soil was omitted from that work, and its presence has been shown to affect microbial retention,

presumably by providing some more amorphous ‘glue’ to strengthen the attachment (Chapters 2 and 5). In the absence of organic soil, retention of the microbial cells could be directly related to the surface feature size (Chapter 2 and 3).Organic soil additionally affected retention and removal of *E. coli*. Work from the retention assays (Chapter 3) also showed that on smaller features, retention was attributed to the maximum area of contact between cells and substratum being attained, with cocci being embedded in 1 μm-width grooves, and rods aligned along (and across) the densely packed parallel 0.5 μm grooves. Results from the cleaning assays support these findings that the surface topography affects cell attachment and retention, but the larger surface features were more easily cleaned than the smaller ones.

This work using PVD magnetron sputtering of titanium onto a fine polished stainless steel surface to produce chemically uniform surfaces allowed the effect of surface chemistry on microbial retention to be assessed. Conformal and fully dense coatings are produced (Kelly and Arnell, 2000; Whitehead et al., 2004). Here despite the presence of topographic features known to affect retention and cleanabilty, coating with a regular titanium chemistry reduced *E. coli* and meat soil retention (Chapter 2 and 5) (Verran et al., 2010).

Microbial and organic fouling of surfaces in the food processing industry is of key importance in terms of hygiene and cleanability. By defining precisely the interactions occurring between microorganisms and organic soil on well characterized surfaces, modifications may be made to ensure minimal adhesion and/or maximum cleanability (minimal retention). This multidisciplinary and multifactorial approach to the topic, addressing interactions occurring between the substratum, topography and chemistry, the microbial cell and food material, cleaning protocol has enhanced understanding of factors contributing to surface cleanability, and will thus facilitate

the development of strategies to minimize the retention of microorganisms and organic material retained on food contact surfaces.

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## APPENDICES

*List of articles*

a) *Peer reviewed articles*

## APPENDIX 1

Verran, J., Airey, P. J., Packer, A. and Whitehead, K. A. (2008) Microbial retention on food contact surfaces. *Advances in Applied Microbiology* 64 223-246

## APPENDIX 2

Verran, J., Packer, A., Kelly, P. and Whitehead K. A. (2010) Use of the Atomic Force Microscope to determine the strength of bacterial attachment on grooved surface features. *Journal of Adhesion Science and Technology* 24 (13-4) 2271-2285

**APPENDIX 3**

Verran, J., Packer, A., Kelly, P. and Whitehead K. A. (2010) The Retention of bacteria on hygienic surfaces presenting scratches of microbial dimensions *Letters in Applied Microbiology* 50 258-263

**APPENDIX 4**

Verran, J., Packer, A., Kelly, P. and Whitehead K. A. (2010) Titanium-coating of stainless steel may improve cleanability. *International Journal of Food Microbiology* 141 S134-S139

b) *Conference papers*

**APPENDIX 5**

Packer, A., Kelly, P., Whitehead K. A. and Verran, J. (2007) Effects of Defined Linear Features on Surface Hygiene and Cleanability Society of Vacuum Coaters 50th Annual Technical Conference Proceedings ISSN 0737-5921 pp 90-93

**APPENDIX 6**

Packer, A., Kelly, P., Whitehead K. A. and Verran, J. (2008) Influence of Varied Defined Linear Features on the Retention of Microorganisms Proc. SVC 51st Annual Technical Conference, Chicago, April , 243-246

**APPENDIX 7**

Packer, A., Kelly, P., Whitehead K. A. and Verran, J. (2009) Use of the Atomic Force Microscope to Determine the Effect of Defined Linear Features on the Adhesion of Bacteria. *Society of Vacuum Coaters 52nd Annual Conference*

**APPENDIX 8**

Verran, J., Packer, A., Kelly, P. and Whitehead, K.A. (2010) Outcomes from EU project PathogenCombat. Surface topography and organic soil factors affecting the hygienic status of open food contact surfaces. Fouling and Cleaning in Food Processing Conference Proceedings, Cambridge March.1-6

**APPENDIX 1**

Verran, J., Airey, P. J., Packer, A. and Whitehead, K. A. (2008) Microbial retention on food contact surfaces. *Advances in Applied Microbiology* 64 223-246

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**APPENDIX 4**

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