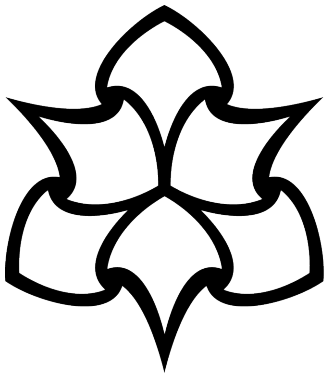

The Effect of Conditioning Films on the Hygienic Status of Titanium Based and Antimicrobial Surfaces

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Abstract

Biofouling and contamination of surfaces is of critical importance to both the food and medical industries, causing not only huge economic burden, but also increased health risks to consumers and patients. The choice of surfaces which can help mitigate these risks is of critical importance. Antimicrobial surfaces were developed through unbalanced magnetron sputtering was producing titanium nitride and silver surfaces (Ag content = 15.03 % 25.45 %) which were to be assessed alongside surfaces commonly utilised in both medical and food industries; 3042R and 316L stainless steel, titanium, 316Ti, 316TiN. The surfaces were assessed for their physical and chemical properties through the use of SEM, EDX, goniometry, FTIR-DRIFTS, WLP and through comparisons with the pristine surfaces (control), assessment of what changes the addition of the bacterial species *Staphylococcus aureus* and *Escherichia coli*, 10 % bovine plasma conditioning film, or both the bacteria and conditioning film made to those parameters. The antimicrobial properties of the surfaces and their propensity to retain the bacterial species was also measured through ZOI, NTV and retention assays, as well as assessment of what effect the addition of the conditioning film upon the surfaces had upon bacterial retention. Results demonstrated the addition of the conditioning film made no significant difference to the physical properties of the surfaces but did affect the chemical properties. Assessment of the antimicrobial properties of the surfaces demonstrated that only the TiN/Ag surfaces were antimicrobial and that the bacterial response was species specific. Retention assays demonstrated that upon the pristine surfaces the *E. coli* was influenced by the physicochemistry of the surfaces, whilst the *S. aureus* was influenced by the surface topography. The conditioning film produced a significant reduction in the numbers retain bacteria to the surfaces. In conclusion, the bacteria were more significantly affected by the presence of the conditioning film than the physical parameters of the surfaces. This result was mediated by the affects that the conditioning film had upon the chemical parameters of the surfaces in conjunction with the molecular effects of protein binding to surface adhesins. This work demonstrates that whilst characterisation of the surface parameters is important, this must also be done in the presence of an appropriate conditioning film.

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Chapter 1

Introduction

1.1.1 Surface Fouling in the Food Industry

Biofouling, the process of surfaces being contaminated by organic foulants, is a critical problem that is currently being faced by many industries ranging from paper production, oil drilling, food storage and processing to health-related fields such as the medical and dental industries. The resulting issues associated with the biofouling results in vast economic costs worldwide (Whitehead and Verran, 2009) as well as ever increasing risks towards the health of populations.

In the food industries, possible problems associated with surface fouling and subsequent biofilm formation include potential risks to food quality, product spoilage, biodeterioration and blockages of mechanical components, and subsequent risks to the health of the consumer (Verran et al., 2010). Outbreaks of food poisoning pose a particular risk to vulnerable members of society, especially the very old, young or immunocompromised. In today's globalising markets with worldwide transportation of produce and goods, these risks are no longer limited locally to the source of contamination (Van Houdt and Michiels, 2010).

In the UK, the Food Standards Agency estimates that the annual cost of foodborne illness is around £1.5 billion, and in 2011 the Center for Disease Control in the USA estimated that around 48 million people became ill as a result of contaminated food sources leading to 3000 deaths annually (Food Standards Agency, 2011; Scallan et al., 2011). These figures combined with increasing numbers of antibiotic resistance seen amongst bacterial communities highlight the requirement for antimicrobial or antiadhesive surfaces for use as a potential strategy to reduce such bio transfer and risk of infection (An et al., 2000; Whitehead and Verran, 2007; Yasuyuki et al., 2010).

Open work surfaces within the food industry are considered a potential source of microbial and organic contamination with a high risk of bio transfer between contaminated and clean products (Taylor and Holah, 1996; Verran et al., 2010). The completed elimination of bacteria and pathogens from food processing facilities would be a difficult task, made more difficult by the ability of bacteria to form biofilms on surfaces which enable them to resist cleaning protocols and survive

after disinfection (Brooks and Flint, 2008; Yang et al., 2012), and so contamination, or re-contamination, of food produce can occur at any stage of the process (Srey et al., 2013). Pathogens most commonly associated with food industry contamination are largely dependent upon what raw materials are entering the plant. Bacterial species most prevalent in the dairy industry are *Enterobacter, lactobacillus, streptococcus, staphylococcus, bacillus,* and *pseudomonas* species whilst those most associated with meat processing facilities are *Staphylococcus aureus, Salmonella* spp *campylobacter* spp, *Listeria monocytogenes* and enterohemorrhagic *Escherichia coli* (Gutiérrez et al., 2012).

Ideally, surfaces within the food industry should be resistant to soiling with good cleanability to ensure their hygienic status. Surfaces within the food industries are generally composed from stainless steel, particularly austenitic grades 304 and 316 with a 2B (standard finish) or 2R (Bright annealed) finish which are favoured for their cleanability, ease of manufacture, and resistance to corrosion (Shi and Zhu, 2009; Schlisselberg and Yaron, 2013; Whitehead and Verran, 2015). Austenitic grades describe the composition of the metal, grade 316 has similar mechanical properties to 304 but with a slightly higher resistance to corrosion due to the addition of molybdenum (Van Houdt and Michiels, 2010). Surfaces should also conform to accepted roughness parameters, with surfaces not exceeding an R_a value (arithmetic mean roughness) of over 0.8 μm to be considered hygienic. Those with higher R_a values could be more difficult to clean and susceptible to bacterial deposition (Flint et al., 2000; Milledge, 2010; Hsu et al., 2013; Whitehead and Verran, 2015).

The cleaning and sanitation of food processing surfaces is central to the reduction of bacterial contaminants and biofilm maturation. However, the attachment of microbes to food processing surfaces is a quick process and due to practicalities, it is not always possible to clean equipment frequently enough to avoid bacterial attachment (Van Houdt and Michiels, 2010). Material choice, product design to reduce areas of static product, and surface modifications can all influence the hygienic status of food surfaces and may prove effective in limiting or preventing biofilm formation (Lindsay et al., 2005).

1.1.2 Surface Fouling in the Medical Industry

Healthcare-associated infections (HCAIs) are classified as an infection that a patient has acquired due to interactions with healthcare systems and can be acquired in care homes, the patient's home or a hospital environment (van Kleef et al., 2013). The Health Protection Agency reported a prevalence in infection rates of 6.4 % with over 1,000,000 cases reported per annum in England alone (HPA, 2011). Infections due to the use of medical devices pose a huge economic burden to healthcare services and pose risks to patient health, increasing risks to patient morbidity and mortality (Donlan, 2008). Some of the most commonly reported HCAIs involve the use of ventilation equipment causing pneumonia or lower respiratory tract infections (22.8 %), catheter associated urinary tract infections (17.2 %) and surgical site infections (15.7 %) (HPA, 2011; Percival et al., 2015).

The use of biomaterials has increased considerably over the past 50 years, with huge in increases in the diversity and range of their application (Simchi et al., 2011). Biomaterials traditionally fall into one of three categories; metallic, polymeric or ceramic, the choice of which would depend upon the location and required functionality of the biomaterial. The use of metallic biomaterials is predominantly utilised for orthopaedic and orthodontic implants due to their excellent durability and mechanical properties (Su et al., 2018). However, there are still issues regarding poor biocompatibility of the implanted metals with problems arising from a lack of blood compatibility for blood contacting surfaces, poor osseointegration, and poor corrosion resistance for high-wear locations such as joint replacement (Kirmanidou et al., 2016; Su et al., 2018). Due to these issues, research into the use of different metal alloys and novel surface treatments for use as biomaterials could help reduce the rate of implant failure (Hussein et al., 2015; van Hove et al., 2015; Kirmanidou et al., 2016; Uwais et al., 2017).

The most common causative agents of HCAIs and biomaterials are *Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Klebsiella pneumoniae, pseudomonas aeruginosa* and *Candida* species (Donlan, 2001; Donlan and Costerton, 2002). A common pathogen affecting biomaterials and pin tract sites in the greatest prevalence is *Staphylococcus aureus* (Inzana et al., 2016) which is

difficult to treat due to its ability to form a biofilm and is often implicated in cases of biomaterial mediated osteomyelitis (Donlan and Costerton, 2002). Treatment of the infection often results in the removal of the implant and a lengthy treatment with antibiotics before further works can commence to fix the fracture (Harris and Richards, 2006; Trampuz and Widmer, 2006). Other bacteria associated with implant infections are *Staphylococcus epidermidis, Escherichia coli* and *Pseudomonas aeruginosa* (Wassall et al., 1997; Skovager et al., 2013).

The use of externally fixating bone pins, or Kirschner wires (K-wires) is a widely utilised method of temporarily immobilising and stabilising severely fractured limbs, allowing the patient to weight bear faster, and for the bones to heal quicker (Wormald et al., 2017). Rather than a permanent and invasive orthopaedic implant, K-wires are passed through the skin, transversally into the fractured bone, and then out the other side before being attached to an external frame. This protrusion from the skin acts as an open wound, allowing potential infection from opportunistic bacteria to migrate along the metal structure of the pin, and can lead to serious infections and osteomyelitis, requiring further surgery and risk to the patient (Gulati et al., 2011; Iobst, 2017; Wormald et al., 2017). The prevalence of eternal fixation infection has been reported from as low as 3 % to over 80 % (Collinge et al., 1994; Parameswaran, A. Dushi; Roberts, Craig S.; Seligson, David; Voor, 2003).

Despite many studies and reviews assessing the prevalence and severity of pin tract infections, there are problems encountered when attempting to compare data due to the lack of standardisation of cleaning protocols utilised in the studies (Jennison et al., 2014), especially if the patient becomes responsible for the wound care symptom monitoring, and through differences in individual clinicians accepted notion of what degree of inflammation, tenderness or erythema is demonstrated by the patient (Santy, 2010). These encountered problems can often lead to conflicting data regarding the prevalence of infections and the cause of the infections.

1.1.3 *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive, non-motile bacterium, with a diameter of 0.5 – 1.5 µm and characterised by individual cocci, which divide and form bunches like grapes. *Staphylococcus aureus* is a common commensal bacterium living on the skin and inside the nose of healthy people and is thought to persistently colonise around 20 % of the population at any one time (Foster et al., 2014). Whilst *S. aureus* is able to live superficially on the skin without causing illness to the host, it is also opportunistic readily colonising cuts and abrasions causing potentially life threatening infections, sepsis and abscesses when in deeper tissues (Foster et al., 2014). It is a major pathogen of increasing importance due to its ability to acquire resistance to methicillin-based antibiotics (MRSA). The cell wall of *S. aureus* is predominantly made of peptidoglycan (~50 %) and phosphate-containing polymer such as teichoic acid (~40 %), the remainder of the cell wall is up from surface proteins, exoproteins, and peptidoglycan hydrolases (Harris et al., 2002; Harris and Richards, 2006). These surface proteins, or adhesins, allow the bacteria to adhere to surfaces and host tissues, evade host immune responses and form biofilms, and have individual binding receptors for different host proteins such as fibronectin, fibrinogen and collagen, which play a role in their pathogenicity. Through structural and functional analysis of these surface proteins, four distinct classes of adhesin have been identified, the largest being Microbial Surface Component Recognising Adhesive Matrix Molecules (MSCRAMMs) and their expression is unique to each strain of *S. aureus.* Despite being one of the most heavily researched bacterial strains, the role of these MSCRAMMs is still being investigated (Patti et al., 1994; Moriarty et al., 2012).

Staphylococcus aureus is frequently associated with pin site infections, causing localised cellulitis and osteitis around the pin (Veerachamy et al., 2014; Ktistakis et al., 2015) and was discovered to be the causative agent 33 % of paediatric pin site infections (Schalamon et al., 2007). *Staphylococcus aureus* is also one of the leading causative agents in cases of food poisoning due to its possession of virulence genes which produce an assortment of enterotoxins which can cause cramps, vomiting and diarrhoea, and occasionally leading to hospitalisation in severe cases (Peacock

et al., 2002; Puah et al., 2016). The ability of *S. aureus* to form biofilms makes it a difficult pathogen to remove from food processing plants and from wounds and pin sites once infection has developed.

1.1.4 Escherichia coli

Escherichia coli is a Gram-negative, motile, rod shaped bacteria typically 0.25 – 1.5 x 2.0 µm in size, and is a facultative anaerobe most commonly found as a resident in the gut. Infection is generally from faecal contamination and has been responsible for food poisoning outbreaks from contaminated vegetables that had not been correctly washed prior to consumption (Franz and van Bruggen, 2008; Sharpov et al., 2016), and has been found to be a causative agent in up to 8 % of orthopaedic implant infections (Arciola et al., 2005). The cell wall of the *E. coli* is coated by an outer membrane of lipopolysaccharide, which gives the cell extra protection from the hostile environment inside of a mammalian gut. *Escherichia coli* cells also possess both flagella and fimbriae, which both play an important role in the adhesion to surfaces and host tissues. The flagella are predominantly used for the motility of the cell playing an important role in overcoming repulsive surface charges to allow attachment and in its virulence. The most common form of fimbriae are Type 1 which are an important factor in the formation of biofilms and increases virulence of the cell (Van Houdt and Michiels, 2005). Type 1 fimbriae are also known to mediate structural changes in the cell membrane once it is adhered to a surface, and is also known to bind to fibronectin allowing greater virulence in host tissues (Van Houdt and Michiels, 2005; Silhavy et al., 2010).

1.1.5 Antibiotic Resistance

The recent increased prevalence in antibiotic resistance has been described as a global crisis (Bell, 2014) which if left unchallenged could lead to simple bacterial infections once again becoming a threat to patient health and possible increased mortality (Spellberg and Gilbert, 2014; Ventola, 2015). It was reported by the Center for Disease Control (CDC) that in the US alone, antibiotic resistant bacteria was responsible for at least two million infections and 23,000 deaths per annum (Frieden and CDC, 2013). Bacterial resistance to antibiotics is not however a recent phenomenon and was first identified in the 1940s in strains of *Staphylococcus* spp

against penicillin (Ventola, 2015). Antibiotic resistance continued to be identified but was not seen as such a great cause for concern due to the development of new antibiotics inspiring confidence that the problem of resistance was manageable (Sengupta et al., 2013). It was not until the discovery of the first case of methicillin-resistant *staphylococcus aureus* (MRSA) in 1962 in the UK that concerns started to grow that we may begin to run out of new drugs to combat this problem (Spellberg and Gilbert, 2014). Since then resistance has now been identified against nearly all antibiotics currently available (Ventola, 2015; Ragheb et al., 2019) and with few new antibiotics currently being brought to market due to economic and regulatory obstacles (Bartlett et al., 2013) the risks to public health due to bacterial infections are once again of great concern.

Resistance in bacteria towards antibiotics can be acquired through inheritance, or from horizontal gene transfer (HGT) which is the process in which genetic data regarding resistance can be passed between bacteria via the plasmid and can occur between bacteria of the same or different species. Resistance can also occur due to spontaneous mutations in species, often when in the presence of low doses of antibiotic as a result of selective pressure (Read and Woods, 2014). Many epidemiological studies have demonstrated a direct correlation between the increased consumption of antibiotics in populations and the increased emergence of antibiotic resistant strains of bacteria (‘The Antibiotic Alarm,’ 2013). When a resistant strain is present in a population of bacteria, the antibiotics will remove the competition of the non-resistant strains, leaving only the resistant population behind to reproduce (Read and Woods, 2014).

It is thought that the main causes for the increased occurrence of antibiotic resistance are overuse, however, despite warnings, antibiotics are still overprescribed worldwide and are still available over the counter in some countries (Bell, 2014). Inappropriate prescribing is also a contributing factor, with inappropriate drug selection and duration of therapy often being demonstrated in 30 % to 50 % of all cases (Frieden and CDC, 2013; Luyt et al., 2014). Antibiotics are also often used extensively in the agriculture industry and are a common additive in growth supplements provided to animals as preventative treatments rather than to

cure illness once acquired (‘The Antibiotic Alarm,’ 2013; Bartlett et al., 2013; Frieden and CDC, 2013). This common overuse of antibiotics is thought to be driven by economic factors and common misbelief amongst farmers that the provision of antimicrobials to their stock ensures a higher yield (Michael et al., 2014). The antibiotics consumed by the animals are then passed through the food chain to humans, and resistant bacteria can also be passed directly to human through contact with farm animals (Ventola, 2015).

In previous decades a lot of effort was placed into the discovery and production of new antibiotics but more recently this development has halted. New antibiotic development is not through to be a wise investment by pharmaceutical companies who could invest the research costs into more profitable diseases that are not so curative and self-limiting (Bartlett et al., 2013; Golkar et al., 2014; Ventola, 2015). It is also thought that due to the restrictive development of antibiotics, when a new one does come to market, clinicians are reluctant to prescribe it, keeping it as a ‘last line of defence’ for serious illness which diminishes the financial return of the drug (Golkar et al., 2014).

Alternative approaches to combatting bacterial infections are now increasing in popularity with research being conducted into bacteriophage therapies (Donlan, 2008; Golkar et al., 2014), vaccines (Jansen et al., 2018) and immunotherapeutic strategies (Dunn, 1987; Ohlsen and Lorenz, 2010). However, whilst results are mostly promising, a lot more research must be conducted to ensure efficacy and compliance to regulatory bodies. Development of modified anti-adhesive or antimicrobial surfaces which could act towards prevention rather than cure of bacterial infections would aid in the reduced requirement of antibiotic therapies and provide better patient outcomes (Baveja et al., 2004; Zhao et al., 2011; Romanò et al., 2015).

1.1.6 Factors Influencing Biofouling

The attachment and adhesion of microbes to solid surfaces is an important step in the infection process, having implications to the development of foodborne infections (Driessen et al., 1984; Cox et al., 1989) and infection of biomaterials and surgical implants (Campoccia et al., 2013b; Ktistakis et al., 2015; Iobst, 2017).

Initial bacterial attachment to a surface is governed by the properties of both the substratum and the bacterial cells, such as hydrophobicity, surface roughness, surface charge, the presence of bacterial surface appendages / adhesins, and the potential to produce extracellular polymeric substances (EPS) (Donlan and Costerton, 2002; Zeraik and Nitschke, 2012). Environmental factors such as pH, temperature, humidity and nutrient source are also important to consider; for example, bacterial cells in an aqueous environment (solid-liquid interface) can attach to a surface rapidly, often within minutes (Whitehead and Verran, 2009), whilst this period could be extended on a dry surface (solid-air interface). There have been many studies into these individual parameters and their effects upon microbial retention and biofouling, but it seems that more in-depth studies into the effects of all these parameters in combination has not been investigated.

Bacterial cells comprise of differing topographies (on the micro and nano-scale) and chemical species, and some species specific variables such as the presence of fimbriae or flagella, which can lead to “islands” of different chemical and physicochemical properties being exhibited by the same cell (Donlan, 2002; Whitehead and Verran, 2009; Schaer et al., 2012; Zeraik and Nitschke, 2012).

Determination of these parameters is of importance to many research areas but is considered essential for the determination of microbial retention and the hygienic status of surfaces (Boonaert and Rouxhet, 2000; Briandet et al., 2001; Faille et al., 2002; Whitehead and Verran, 2009; Simões et al., 2010). Furthermore, if these parameters could be defined and controlled, then bacterial attachment and biofilm formation could also be more easily managed (Whitehead and Verran, 2009).

1.1.7 Biofilms

Bacteria are generally considered to exist in two main forms; planktonic cells which are free floating singular cells, or as part of a biofilm. A biofilm is defined as an "aggregate of microorganisms in which cells that are frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS) adhere to each other and / or to a surface" (Vert et al., 2012) and can be consisted of either single or mixed species. There are many advantages for bacteria to live within biofilms due added protection from external and environmental stressors such as water currents or desiccation as well as from antimicrobial agents and are considered the most abundant form of life for microorganisms on earth (Stoodley et al., 2002; Flemming, 2009; Flemming et al., 2016).

The formation of a biofilm (Figure 1) begins with the initial attachment of the planktonic cells to a surface which is mediated through interactions with the physicochemical forces of the surface (Brooks and Flint, 2008) and is aided by cellular appendages such as flagella, pili, fimbriae, and adhesins (Percival et al., 2015). For example, Staphylococcal species bacteria display cell surface proteins (SSP-1 and SSP-2) at the distal end of fimbriae like appendages which are able to adhere to surfaces (Eiff et al., 1999). The initial attachment of a cell is not permanent, and the cell can release from the surface to find another preferred location. However, if the conditions are appropriate, the cell will becomes permanently adhered to the surface which mediates modifications to gene expressions allowing for the potential production of EPS and / or quorum sensing (communication) in place of motility (Peacock et al., 2002; Van Houdt and Michiels, 2005; Tuson and Weibel, 2013). This process can take several hours and involves van der Waals interactions between the surface and the hydrophobic regions of the outer cell wall as well as protein mediated changes allowing for the permanent attachment, for example, in *E. coli,* lipopolysaccharides and pili are able to increase the rate of attachment and transition to permanent adhesion (Chao and Zhang, 2011; Tuson and Weibel, 2013). The production of EPS also facilitates the permanent adhesion of cells to surfaces, anchoring them to the substrate and providing protection (Tuson and Weibel, 2013).

After the adhesion of the pioneer cells, cells are able to proliferate through cell to cell adhesion and propagation, forming microcolonies. The architecture of these microcolonies is mediated by both the physicochemistry of the surface and the cells ability to produce EPS (Tuson and Weibel, 2013; Flemming et al., 2016). The role of EPS within a biofilm is multifaceted and mediated by the species contained within the biofilm. Molecules of EPS are able to fill the spaces between the bacterial cells, providing protection from external stress, nutrients, as well as structural and mechanical stability (Persat et al., 2015; Flemming et al., 2016).

Bacteria within the mature biofilm are able to detect cell density through assessment of the availability of nutrients and through quorum sensing; a mode of communication augmented through the release of chemicals (Donlan, 2002; Kim et al., 2008; Flemming et al., 2016). Cells and aggregations within a mature biofilm are then able to detach and disperse allowing the bacteria to actively colonise new niches before nutrients and space become limited (Hall-Stoodley and Stoodley, 2005). This behaviour of detachment and relocation has been considered a passive behaviour mediated by fluid sheer stress or starvation (Sauer et al., 2004) but it has also been noted that this 'shedding' of microcolonies could provide an infective dose if it was ingested or inhaled (Hall-Stoodley and Stoodley, 2005).

Whilst the formation of a biofilm is not considered to necessarily be a virulence factor due to the fact that many non-pathogenic bacterial species also produce biofilms (Kim et al., 2018), it is however a factor involved facilitating the survival of some species through adding protection from sheer force and mechanical cleaning as well as reduced susceptibility to biocides and antibiotics (Lomander et al., 2004; Ordax et al., 2010; Jennison et al., 2014). The added physical protection that growth within a biofilm provides makes their breakdown and removal more challenging than with planktonic bacteria. For example, Smith and Hunter (2008) assessed the efficacy of three commonly used hospital biocides (Chlorhexidine gluconate 4 %, triclosan 1 % and benzalkonium chloride 1 %) against clinical isolated of Methicillin resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa.* Results demonstrated that the recommended concentrations of the biocides were effective against planktonic bacteria but ineffective against biofilms of the same isolates. This

added protection makes it incredibly difficult to treat wound infections and remove biofilms from surfaces and equipment in industrial settings.

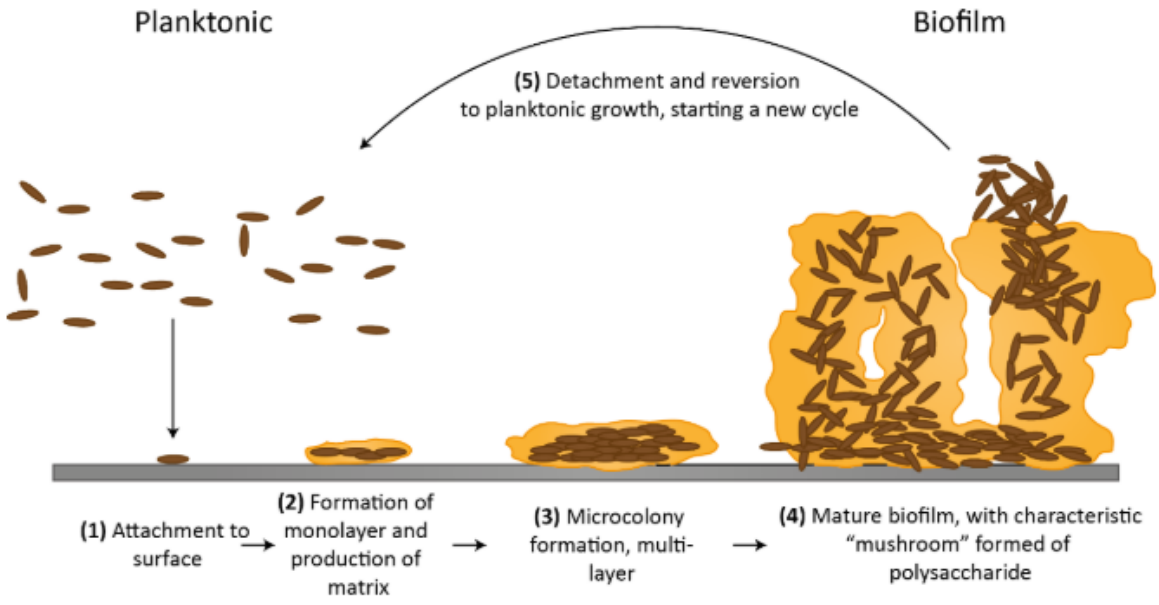


Figure 1: Simplified schematic representation of the formation of biofilms (Vasudevan, 2014).

1.1.8 Surface Physicochemistry

The correlation between the hydrophobic / hydrophilic parameters of bacteria cells and surfaces has been described in some works as a primary factor in driving the adhesion of microbes to surfaces (Zita and Hermansson, 1997; Sinde and Carballo, 2000; Faille et al., 2002; Whitehead and Verran, 2015). Combined with the interactions with other surface parameters such as the Lewis acid-base and van der Waals forces, hydrophobicity can result in preferential bacterial attachment and adhesion to surfaces. Some researchers argue that bacteria that have a negative charge overall will preferentially adhere to surfaces that have an overall negative charge (Zita and Hermansson, 1997; Sinde and Carballo, 2000; Zeraik and Nitschke, 2012). However, there are conflicts of opinion within the scientific community regarding this topic. Variations in results obtained in this area are most likely due to lack of standardisation in the assays being performed and variations in the substrates tested (Goulter et al., 2009). For example, assessments performed upon glass surfaces would not comparable to assessments performed upon metal or

polystyrene, and due to the large number of variabilities in metal surfaces such as composition and topography, assays performed upon different metals are also not comparable. Variabilities in environmental conditions such as humidity could also affect adhesion of bacteria to surfaces (Faille et al., 2002; Chae et al., 2006; Whitehead and Verran, 2015).

It is thought that modifications to the physicochemistry of a surface could be utilised as a method of reducing bacterial adhesion to surfaces through increases to the hydrophilicity (Whitehead and Verran, 2009; Bekmurzayeva et al., 2018). However, the modifications are unlikely to be long lasting as the adsorption of a conditioning film could further modify the physicochemistry of a surface (Briandet et al., 2001; Matsumura et al., 2003) and assessments of the feasibility and cost effectiveness of upscaling these technologies still requires further evaluation (Otter et al., 2015).

1.1.9 Surface Topography

Surface topography and roughness are important parameters to consider when investigating microbial retention to surfaces (Campoccia et al., 2013a). Previous investigations into the correlation between surface roughness and bacterial adhesion have proposed that there is a relationship between greater surface roughness and increases in the numbers of retained bacteria, and researchers hypothesize that this may be due to the protection that topographical features provide to adherent microbes (Jullien et al., 2003; Mitik-Dineva et al., 2009; Whitehead, Li. H., et al., 2011; Lüdecke et al., 2016). Similarly, topographical structures of a similar size to bacterial cells may also provide greater surface contact area between the surface and the microbe allowing for greater adhesion (Scardino et al., 2006; Braem et al., 2013). However, other studies have found no relationship between bacterial adhesion and roughness parameters, suggesting that bacterial adhesion to be a multifaceted issue of which topography is just one aspect, and that surface adhesion of bacteria is a problem without a ‘one size fits all’ answer (Medilanski et al., 2002; Hilbert et al., 2003; Whitehead et al., 2005; Milledge, 2010).

Attempts to produce nanostructured surfaces or imitate structures seen in nature that repel bacteria such as lotus leaves and shark scales (biomimetics) have gained in interest (Scardino et al., 2006; Mitik-Dineva et al., 2008; Lüdecke et al., 2016). So far these surfaces have produced varying results; Singh *et al*., (2011) observed a significant increase in the adhesion of *E. coli* and *P. aeruginosa* to titanium produced by supersonic cluster beam deposition with incremental increases in surface roughness, whilst Ivanova *et al*., (2010) observed a significant reduction of *S. aureus* to magnetron sputter coated titanium surfaces with incremental increases in roughness parameters. The authors of both studies proposed changes to the wettability produced by the increases in surface roughness to be responsible for the differences in adhesion behaviour.

Current methods of description of the surfaces may also be responsible for the differences in reported adhesion to parameters with some suggestion that they are lacking in descriptive power (Zhao et al., 2008; Wickens et al., 2014). Currently surface topographies are measured through vertical roughness parameters of the flat surfaces, and do not consider spatial / horizontal distribution of peaks and valleys (Lüdecke et al., 2016). It is possible that these parameters in conjunction with the shape of the cell adhering could lead to differences in the adhesion of bacteria and may not be detected by measurement of the roughness parameter alone.

1.1.10 The use of Antifouling Metals

It has been long established that some metals have antimicrobial properties and benefits which have been successfully exploited throughout history (Alexander, 2009). Whilst it is documented that specific metal ions are essential components of cell membranes and deoxyribonucleic acid (DNA), precipitating key cellular processes such as catalysis and electron transfer, excessive quantities of these same metals are also lethal to cells (Andreini et al., 2004; Waldron and Robinson, 2009). Furthermore, the presence of certain non-essential metals such as silver or mercury would be poisonous to most bacterial species, even in low quantities (Harrison et al., 2004). Due to these innate antimicrobial properties, metal coatings incorporating some of these elements are being used with increasing popularity

with applications in the medical industry and agriculture with the possibility of reducing the requirement for antibiotic use (Lemire et al., 2013).

It is thought that antimicrobial activity is produced due to the selective disruption of processes required for cell growth, influenced by chemical and physical properties of the atoms in the metals and the accessibility of donor ligands in intercellular biomolecules. This abolished enzyme activities, causes disruption and impairment to cell membrane functions and damages to DNA (Lemire et al., 2013). As this antimicrobial action occurs without the need for chemical biocides or antibiotics which can then propagate into the environment eliciting negative effects upon natural biodiversity of microbiomes in nature, the use of surfaces with antimicrobial elements would be better for the environment. However, metal pollutants can also be devastating for natural microbial diversity, with the ability to eliminate 99.9 % of natural microbial diversity in pristine soil (Gans et al., 2005). Furthermore, due to the ever increasing presence of these metals in consumer products, the potential for inadvertent damage to the ecosystem also increases, potentially trading one form of ecosystem damaging pollution for another (Lemire et al., 2013).

Copper is one of the most commonly used metals with known antimicrobial properties; its first recorded medical use dates back between 2600 and 2200 B.C (Grass et al., 2011). The use of medicinal copper continued, only waning in popularity upon the advent of antibiotics. However, with the rise in antibiotic resistant bacteria it is now being used in increasing frequencies in hospitals, food processing plants and industrial animal rearing facilities (Grass et al., 2011; Santo et al., 2012).

Silver and silver based compounds are also well known for their antimicrobial activity and have been incorporated into many antimicrobial applications and dressings (Godoy-Gallardo et al., 2015). Silver is readily mixed into other base metals such as titanium when in the presence of nitrogen, creating a matrix of silver particles in the metal surface whilst maintaining the mechanical and tribological attributes of the base metal. A disadvantage of the use of silver as an antimicrobial agent is the necessary presence of liquid to enable the transport of the silver ions

into the bacterial cells, as silver is otherwise relatively inert and poorly absorbed. However, with the presence of liquids in wounds or in high contact areas of meat processing plants, silver incorporated into metallic coatings could be a highly effective antimicrobial surface (Godoy-Gallardo et al., 2015).

1.1.11 Conditioning Films

Once a surface has been soiled, it can never return to its pristine condition again (Verran et al., 2010). After its first use a surface will have acquired a layer of fouling, known as a conditioning film, which has the ability to change the physicochemistry of a surface and therefore, either increase or decrease the ability of microbes to adhere to it (Jones et al., 2001). Conditioning films within the food industry are a known factor for fouling, allowing further organic materials, cells, cleaning deposits and other debris to adhere, further affecting the hygienic status of the surface (Whitehead et al., 2008, 2015; Whitehead and Verran, 2015). Despite the importance of conditioning films and their effect upon microbial retentions within the food and medical industry, the subject has received insufficient attention and study.

The absorption and binding of proteins onto a surface is thought to happen within seconds (Neoh et al., 2012), mediated by the surfaces' topographical and physicochemical parameters (Anselme et al., 2010; Ouberai et al., 2014). This layer of organic fouling then serves to mediate the subsequent attachment and retention of cells and microbes which would then compete in the proclaimed 'race for the surface' (Busscher et al., 2012). This layer of organic fouling could potentially increase or reduce the attachment of bacteria to a surface due to receptor/ligand interactions with the proteins, although this would be largely species dependent due to the differences in cell surface adhesins (An et al., 2000; Arciola et al., 2012).

Investigations into the application of conditioning films for the control of microbial attachment has had some positive results. Early works by Fletcher, (1976) discovered that the addition of albumin (from bovine serum albumin) was effective at impairing the attachment of marine Pseudomonad to polystyrene Petri dishes due to absorption of the albumin conditioning layer (Fletcher, 1976; An et al., 2000;

Eroshenko et al., 2015). However, other works have investigated the role of fibronectin and found it to have a stimulatory effect towards bacterial adhesion (An and Friedman, 1997; An et al., 2000; Eroshenko et al., 2015). Further investigations into the use of surface pre-conditioning have shown positive results; for example, the use of cod protein extract has been found to demonstrate promising reductions in bacterial adhesion to surfaces (Bernbom et al., 2006; Pillai et al., 2009; Whitehead and Verran, 2015). It is important to investigate the effects that conditioning films have on bacterial retention and adhesion, but also to assess the role of proteins from full protein sources, such as whole plasma or serum, rather than individual protein sources which do not offer a 'real world' demonstration of results.

1.2.1 Aims

The aim of this study was to characterise the chemical and physical properties of a range of antimicrobial and biologically inert surfaces which could be used in either the medical industries for external fixation, or within the food industries. Assessments of these parameters, performed upon the pristine surfaces, surfaces with bacteria, surfaces with conditioning film, and surfaces with conditioning film and bacteria would allow for assessment of the primary driving factors influencing the retention of *Staphylococcus aureus* and *Escherichia coli* to the surfaces.

1.2.2 Objectives

- To develop and produce an antimicrobial surface via magnetron sputtering.
- To characterise the physical properties of the surfaces, assessing what effects the addition of bacteria, conditioning film, or both had upon the surface parameters.
- To characterise the chemical properties of the surfaces, assessing what effects the addition of bacteria, conditioning film or both had upon the surface parameters.
- To investigate the different surfaces propensity to retain bacteria and assess what affect the addition of conditioning film had upon the bacterial retention.
- To assess the parameters in conjunction to one another with the aim of elucidating the primary driving factors affecting bacterial retention upon these surfaces.

Chapter 2

A Preliminary Study into the Production and Characterisation of Antimicrobial Surfaces and Stainless Steel

2.1 Introduction

2.1.1 Titanium-Nitride Silver Surfaces

The choice of a substrate material is largely dependent upon its intended purpose and the desired properties. The ability to mix metals or 'dope' surfaces with quantities of other metals that contain more favourable features allows for greater flexibility and choice for the material selection.

Silver and its compounds have long been known to produce broad spectrum antimicrobial effects against both Gram-positive and Gram-negative bacteria, yeasts, virus' and fungi (Alexander, 2009; Kelly et al., 2009; Godoy-Gallardo et al., 2015), as well as producing anti-inflammatory responses in tissue (Wong et al., 2009). However, pure silver is an expensive and limited metal with poor strength and tribological properties which would not make it suitable to use in either the food or medical industries alone. The incorporation of silver into an alloy of titanium-nitride would however provide the strong tribological properties of titanium-nitride, which is often used in surgical equipment due to its strength and durability and corrosion resistance (Whitehead, Li. H., et al., 2011), whilst reducing the costs incurred with its production and use.

The method of deposition of the silver to the titanium nitride is important because the arrangement of the silver nanoparticles in the matrix of the metal alloy regulates its antibacterial properties (Kelly et al., 2011). The deposition of metals utilising Ion Beam Assisted Deposition (IBAD) has been investigated and found to be a useful tool for the production of high density thick coats with good adhesion to the base substrate (Hirvonen, 1991). However, Ahearn *et al*., 1995, discovered that IBAD produced silver alloys that enhanced the adherence of bacterial cells to the surface , in comparison to ones produced by magnetron sputtering. Additionally, these surfaces did not produce a zone of inhibition in agar diffusion tests. Surfaces produced through magnetron sputtering or physical vapour deposition (PVD) have however been found to demonstrate good tribological properties and strength, as well as antimicrobial properties (Kelly et al., 2009, 2011; Whitehead, Li. H., et al., 2011).

Previous studies in our laboratories have successfully demonstrated the antimicrobial effects of silver alloys produced through PVD upon a range of bacteria. In a study by Whitehead *et al*., (2011), the antimicrobial effect of different percentage titanium-nitride silver surfaces was assessed against samples of *S. aureus* and *Pseudomonas aeruginosa*. The results demonstrated that there was a species-specific response to the different content of the silver in the surfaces, with the *P. aeruginosa* cells retained in greater numbers to the surfaces which contained greater amounts of silver (16.7 %) but cells were easily removed when force was applied with the atomic force microscope. Whilst the *S. aureus* cells retained to the surfaces in increased numbers to the surfaces containing less silver (4.6 % and 10.8 %) but with a greater adhesive force. This work demonstrated that a wide range of tests are necessary when assessing novel surfaces and that often species-specific responses can be elicited from the results.

2.1.2 Bacterial Attachment and Adhesion to Surfaces

There are many advantages for planktonic bacteria to adhere to surfaces and eventually form biofilms as they provide greater protection from cleaning products and antibiotics, protection for mechanical threats such as sheer force, and greater opportunities for nutrient capture (Flemming et al., 2016). The first stage of this process is the reversable attachment of a bacterial cell to a surface, which is largely governed by either electrostatic forces and surface charge or cell motility via cellular appendages such as a flagellum. This stage of the process happens quickly (in the order of 0 – 1 minute) (Tuson and Weibel, 2013) and is not permanent, bacterial cells can detach from a surface if the conditions such as the surface physicochemistry and cellular alignment to surface features are not satisfactory. Once detached from the surface, the cells will continue the process of sensing the surface for a more favourable location.

If the conditions are suitable, the second stage of the process will occur in which the attached cells become irreversibly attached to the surface causing a switch in gene activation and expression, producing phenotypical changes to the cell surface (Blair et al., 2008; Krasteva et al., 2010) aiding the binding process. For example, in some motile bacterial species, the attachment of a cell to a surface produces a

switch in gene activation, causing the flagella to be turned off by the same transcription factor that turns on the production of the extracellular matrix rendering the cell unable to detach from the surface even if it wished to (Vlamakis et al., 2008; Tuson and Weibel, 2013).

Other species specific extracellular appendages which can mediate the irreversible attachment of bacteria to surfaces include pili (or fimbriae) and curli fibres, all of which are often terminated in proteins (adhesins) which bind to specific surface molecules (Neu, 1996; Tuson and Weibel, 2013). The unique characteristics of the adhesins presented by the different bacterial species explains why bacterial species interact with surfaces in different ways, occupying different niches and exploiting surface features in their own way (Patti et al., 1994; Tuson and Weibel, 2013; Souza et al., 2015), as well as exploiting the proteins presented in different conditioning films and biological agents which may be on the surfaces (Müller et al., 2009; Campoccia et al., 2013a). From the attachment of initial bacterial cells, other bacteria are then able to adhere to the surface, using the previously adhered cells as further anchor points, creating aggregations of bacteria and micro colonies. It is from these initial interactions of the bacterial cells to the surfaces which act as precursors to the future formation of biofilms and is therefore an important area of study.

2.1.3 Retention Assays

Measurement of the number of bacteria retained to a surface through a standardised retention assay provides a ‘snap-shot’ demonstrating how a bacterial species interreacts with a specific surface over a defined period. It does not however provide information about bacterial cell attachment, adherence or further biofilm formation and proliferation, or information regarding adhesion behaviour under flow conditions. Utilisation of a standardised assay allows for comparisons to be made between different surfaces, assessing their performance for anti-adhesive qualities, antimicrobial assessments (Live / dead assays) and the interactions of different bacterial species to surfaces through analysis of cellular dispersal patterns (Wickens et al., 2014).

The assay requires that the surfaces are submerged in 25 ml of a standardised microbial broth for one hour at a set temperature before a standardised rinse step is completed and the surfaces are dried. This repeatable method reduces the numbers of variables which could affect the results, such as bacterial concentration in the broth which would alter the numbers adhered to the surfaces, as well as variables from external factors such as humidity and temperature which would otherwise affect bacterial adhesion.

2.1.4 Magnetron Sputtering

The production of titanium-nitride silver surfaces though magnetron sputtering(a physical vapour deposition process) is a widely used and versatile method for the deposition of thin-films and metallic coatings (Kelly and Arnell, 2000; Whitehead et al., 2004; Waite et al., 2010). The basic principle of magnetron sputtering is that a target (cathode) is bombarded by energetic ions that are generated in a plasma situated in front of the target, dislodging ions which are deposited onto the substrate as a thin film (Kelly and Arnell, 2000). The introduction of "unbalanced" magnetron sputtering in the 1980's and subsequently its development into a "closed-field" rig system, has allowed the technique to become one of the most popular and versatile applications used for the deposition of well-adhered and high quality / hard wearing surfaces, and co-sputtered surfaces (Kelly and Arnell, 2000; Waite et al., 2010). The ability to co-sputter onto a substrate from two separate targets allows for the production of metallic nanoparticles which can be visualised under a high magnification. The use of nanoparticle technology for antimicrobial surfaces is particularly advantageous as they can confer their antimicrobial activity whilst retaining the original hardwearing nature of the surface (Kelly et al., 2011). The titanium-nitride silver surfaces in this study were developed from the investigations of other antimicrobial surfaces such as zirconium-nitride silver and chromium-nitride silver thin films which were deposited utilising the same methods (Kelly et al., 2009, 2010; Skovager et al., 2013; Wickens et al., 2014).

2.1.5 Scanning Electron Microscopy (SEM)

Scanning electron microscopy is the gold standard method of obtaining high magnification images of both inert solid surfaces and of biological samples such as

tissues and cells, allowing for the collection of data regarding their morphology and organisation (de Souza and Attias, 2018). The images are obtained as the surfaces are scanned with a focused beam of electrons that the atoms of the samples react to, and release signals that allow for the data on the image to be collected. Scanning electron microscopy utilises a vacuum chamber to reduce interference from the atoms in the passive air between the beam and the sample and are able to obtain images at a resolution better than 1 nm. High resolution images of biological samples is possible, but first the samples must be desiccated to prevent damage due to the effects of the vacuum on the fluids within the cells (Greif et al., 2010; de Souza and Attias, 2018).

2.1.6 Energy-Dispersive X-Ray Spectroscopy (EDX)

Energy dispersive X-ray spectroscopy is an analytical technique utilised to assess the elemental composition of a sample, allowing elucidation of its chemical composition. This data is obtained through the excitation of the atoms in a sample with x-rays, the sample then releases energy providing data on its atomic structure. This data is collected and plotted into a spectral graph, where each peak relates to an atomic percentage (at.%) of the sample. This technique allows for the collection of data regarding the chemistry of a surface as well as its heterogeneity/homogeneity of elemental structure and composition which can help with the analysis of bacterial adhesion.

2.1.7 White Light Interferometry

White light interferometry is a method of interference microscopy, which allows for the analysis of a surface's 3D topography, providing high-resolution quantitative and qualitative data through non-contact methods. This optical technique utilises a source of light, as conventional microscopy, but adds a beam splitter and reference mirror, and measures the phase shift between the light source and the light that is reflected back from the sample. From the image obtained, the interference fringes are then used to reconstruct the topographical data from the surface (Baryshev et al., 2012; Spencer et al., 2013). The advantage of white light interferometry is that it is able to process surfaces with speed and accuracy to the nano-meter and has a wide field of view. In addition, as it is a non-contact method of surface analysis, so

there is little risk of damaging a surface with a stylus or tip. A disadvantage of white light interferometry is its limitations when attempting to analyse thin translucent films (Salvi et al., 2010; Baryshev et al., 2012).

The measurement of average roughness produced by a white light interferometry is S_a. This measurement is comparable to the often-cited R_a measurement but is taken from an area of the surface and the R_a measurement is over one line, often produced by a stylus. S_a is typically used for machined surfaces and is suitable for this application. The '*S*' parameters are defined by ISO25178-2 (*BSOL British Standards Online*, n.d.).

2.1.8 Measurement of Physicochemistry - The Sessile Drop Technique

The sessile drop technique is a static method of analysing the physicochemistry of a surface through the measurement of contact angles from droplets of liquid; the choice of the liquids would be dependent upon the type of surface being assessed. The liquid is dropped onto the surface with a syringe, and the image of the droplet is captured on a camera. The angles of the droplet in contact with the surface are measured by software. This is the simplest method of measuring the surfaces physicochemistry, and providing that the surface is large enough, the surface can be tested for homogeneity through multiple drops of liquid (Janssen et al., 2006). A limitation of this process is the size of field over which the measurements are taken due to the relative size of the liquid droplets in comparison to the size of bacterial cells. It could be argued that measurements taken do not provide accurate descriptions of the physicochemical parameters that a bacterial cell adhered to a surface would encounter. However, it should be noted that until such time that a more advanced method of measurement is discovered, this is the best option available.

2.1.9 Chapter Aims

In this preliminary chapter, two surfaces were initially characterised and assessed for their antimicrobial properties and performance, the results of which would inform the decision upon which surfaces were to be used for an extended study incorporating the use of a conditioning film. Utilisation of a preliminary study would

also allow assessment of the methodological approaches, allowing for any necessary improvements or alterations to the techniques used prior to the extended study.

2.2 Methods

2.2.1 Substrates for Sputter Coating

The 304 grade stainless steel coupons (20 mm x 20 mm, 1.2 mm thick) with a 2R finish (Aalco, UK) were used as the substrate for experimental sputter coatings and as the control samples left untreated for microbiological testing. Surfaces were cleaned using methanol (Sigma Aldrich, UK) and a lint free cloth (Buehler, USA) prior to sputter coating.

2.2.2 Coupon Cleaning

The coupons (20 mm x 20 mm) were placed in sequence into glass Petri dishes containing either 30 mL acetone (Sigma Aldrich, UK), methanol, ethanol (Sigma Aldrich, UK) or sterile purified water. The coupons were soaked sequentially for 10 minutes per liquid. Coupons were then dried with an autoclaved fibre free cloth (Buehler, USA) and further dried in a class II airflow cabinet for one hour.

2.2.3 Magnetron Sputtering

The coatings for the study were produced following a method from a previously published article (Whitehead, Li. H., et al., 2011) which gave favourable % content of silver in the TiNAg surfaces which produced antimicrobial results from agar diffusion tests. The titanium-nitride silver (TiNAg) surfaces were produced by closed field unbalanced magnetron sputtering in a Teer Coatings UDP350 rig (Worcester, UK). The chamber was evacuated down to a pressure below 2.0×10^{-6} Pa before argon gas (99.99 % purity) was introduced to the chamber by use of a mass flow controller (MKS instruments, UK) set to a flow rate of 19.00 sccm to give an operating pressure of 0.24 Pa. The titanium and silver magnetrons were driven in pulsed DC mode at 1500 W, 20 kHz pulse frequency (90 % duty), and 100 W, 20 kHz pulse frequency (90 % duty), respectively. The targets were sputter cleaned for 5 minutes prior to the deposition process to ensure the purity of the coating, and a

guard was placed inside the chamber to reduce the amounts of material coating onto opposing targets (Figure 2). The substrates (20 mm x 20 mm, 304-grade stainless steel with a 2R finish) were placed upright into the chamber on a cylindrical holder, facing away from the titanium and silver targets during the sputter cleaning process. To ensure good coating adhesion to the substrates, a full metal titanium and silver interlayer was sputter coated onto the substrates for five minutes before nitrogen was introduced into the sputtering process. Nitrogen was delivered into the chamber with use of a reactive sputter controller (Megatech, UK) with the monochromator set to 503 – 504 nm, which delivered the nitrogen in relation to the optical emission signal from the titanium ions in the plasma. The controller was set at 60 % of the full metal signal. The deposition time was a constant one hour per run and cooling times of three hours were used prior to venting of the chamber to minimise thermal stresses on the coatings.

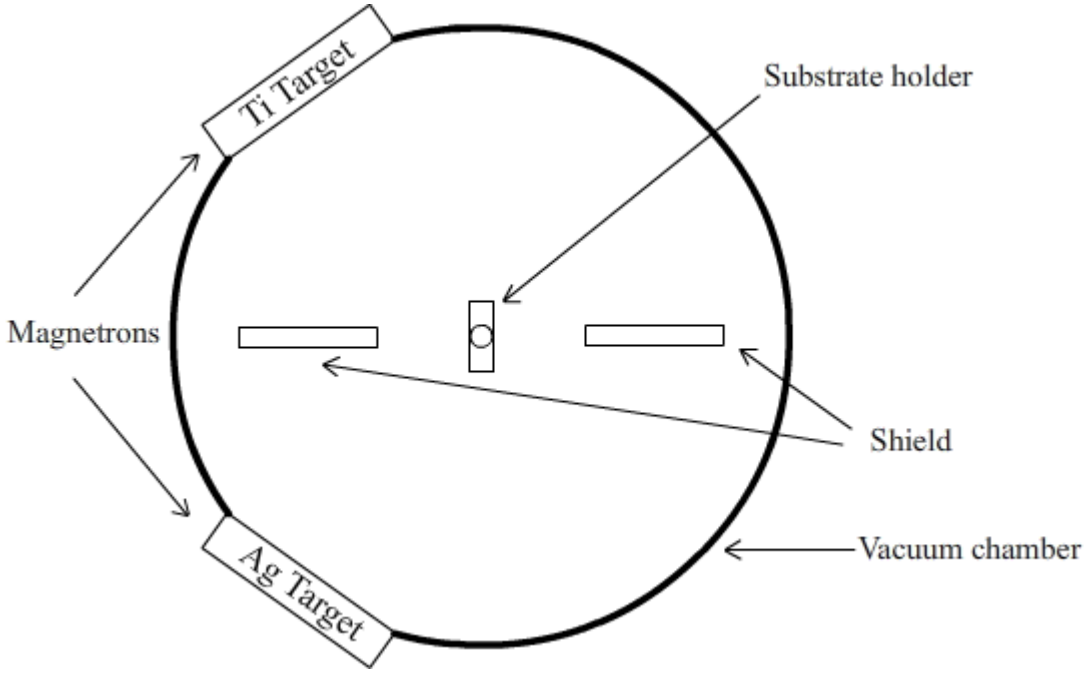


Figure 2: Schematic diagram the magnetron chamber as viewed from above.

2.2.4 Scanning Electron Microscopy (SEM)

Each of the surfaces were analysed with a Zeiss, Supra VP40 (Carl Zeiss LTD, UK) field emission gun scanning electron microscope (SEM) with SmartSEM software (Carl Zeiss LTD, UK) which was used to allow visualisation of their morphology and structure. Images of the surfaces were taken at x 5000 and x 20,000 magnifications to allow visual descriptions of the surfaces.

2.2.5 Energy-Dispersive X-Ray Spectroscopy (EDX)

Analysis of the elemental content of the surfaces was performed on three areas of three replicates (n = 9) by use of a INCA X-ray microanalysis system with INCA x-sight Si(Li) EDS detector probe (Oxford Instruments LTD, UK; model 7388) with INCA Suite software (version 3.04). Sampling was conducted at 15 mm working distance with 20 Kv acceleration volts. Sampling depth was between 1 – 2 µm. The composition of the substrates was calculated as an atomic percentage, giving the percentage content of an element relative to the number of total atoms scanned.

2.2.6 White Light Profilometry

The topography of the surfaces was measured using white light profilometry. Surface roughness was defined qualitatively through images (n = 9), and quantitatively through measurement of S_a values using a Zemetrics, Zegage 3D optical profiler (Zygo, USA). The replicates were analysed at x 50 magnification. The image analysis software used was Zemaps (version 1.14.38) and SPIP (version 6.3.5). Peak heights and widths were measured manually using the Zemaps software and averaged to provide data regarding the largest and smallest peak and width measurements ± standard deviation (n = 3).

2.2.7 Physicochemistry

Contact angle measurements for the pristine surfaces (30 x 50 mm) were determined at room temperature using the sessile drop technique. Five microliters of HPLC grade water (BDH, UK), or formamide (Sigma Aldrich, UK) or 1-bromonaphthalene (Sigma Aldrich, USA) were deposited onto the horizontal surfaces using a 1 mL syringe. Contact angle measurements were determined using a goniometer (KRÜSS GMBH, Germany). Three measurements were taken from

each surface for each liquid, and different newly prepared samples were used for each test liquid in order to eliminate absorbed liquid conditioning the surfaces and affecting the contact angles produced. Physicochemical parameters were calculated through the surface tension parameters of the polar and apolar liquids (Van Oss and Giese, 1995).

Equations from Van Oss (1995) were used to calculate physicochemistry:

Free energy interactions between molecules (*i*) immersed in water (*w*) can be expressed as ΔG_{iwi}:

$$\Delta G_{iwi} = -2\Upsilon_{iw} \quad [1]$$

The surface free energy of a liquid is or a solid γ_i, is the sum of its polar acid-base interactions γ_i^{AB} and apolar Lifshitz Van der Waals γ_i^{LW}:

$$\Upsilon_{i=} \Upsilon_i^{LW} + \Upsilon_i^{AB} \quad [2]$$

Where γ_i^{AB} comprises of two parameters, the electron donor γ_i^- and acceptor γ_i^+ can be calculated as:

$$\Upsilon_i^{AB} = 2\sqrt{\Upsilon_i^{\oplus} \Upsilon_i^{\ominus}} \quad [3]$$

There are three unknowns within these parameters that were used in the calculation. Using contact angle measurements (Θ) of three different liquids (two polar, one a polar) in conjunction with Young's equation (4), the, γ_i^{LW}, γ_i^+ and γ_i^- can be calculated:

$$(1 + cos\theta)\, \Upsilon_L = 2\left(\sqrt{\Upsilon_S^{LW}\Upsilon_L^{LW}} + \sqrt{\Upsilon_S^{\oplus}\, \Upsilon_L^{\ominus}} + \sqrt{\Upsilon_S^{\ominus}}\, \Upsilon_L^{\oplus}\right) \quad [4]$$

2.2.8 Maintenance of Microbiological Cultures

The microorganisms S*taphylococcus aureus* NCTC 10788 and *Escherichia coli* NCTC 10418 were used for microbiological assays. Stock cultures were stored at -80 °C. Cultures were thawed as required and inoculated onto brain heart agar (Oxoid, UK) media and incubated overnight at 37 °C. Stock cultures were re-frozen following use. To maintain cell physiology, inoculated plates were replaced every 4 weeks and stored (4 °C) in a fridge.

2.2.9 Preparation of Cultures for Microbiological Assay

Colonies of *S. aureus* and *E. coli* were inoculated into 10 mL of sterile brain heart infusion (Oxoid, UK) and incubated overnight at 37 °C in an orbital incubator at 150 revolutions per minute (RPM) for 18 hours. Following incubation, the inoculated broths were centrifuged at 3600 RPM for 12 minutes and the supernatant removed. The cells were re-suspended in sterile reverse osmosis membrane filtered water (Milipore ELIX, USA). The cell solution was diluted to an optical density (OD) of 1.0 ± 0.05 at 540 nm, the latter was measured using a spectrophotometer (Jenway 6305, Bibby Scientific, UK), with sterile purified water as a blank to calibrate. Cell numbers were calculated from the determination of colony forming units / mL^{-1} (CFU/mL) using serial dilutions. Diluted cell suspensions (100 µL) were spread onto brain heart infusion agar (Oxoid, UK) plates in triplicate (n = 3) and incubated at 37 °C overnight and the colonies then counted. This process was repeated twice (n = 6) with new overnight bacterial cultures to ensure continuity.

2.2.10 Microbial Adhesion to Hydrocarbons (MATH) Assay.

The MATH assay methodology was carried out using an adapted method from Bellon-Fontaine et al., (1996). Cultures were prepared by inoculating 100 mL of brain heart infusion (Oxoid, UK) with the bacterial species and incubated overnight at 37 °C. Cells were harvested by centrifuge (3000 RPM) and washed in PUM buffer (Table 1) three times before being re-suspended to an optical density of 1.0 at 400 nm. A volume of 1.2 mL of the cell suspension was added to round bottomed glass test tubes (15 mm) before 200 µL of either chloroform (Sigma Aldrich, USA), ethyl acetate (Sigma Aldrich, USA), hexadecane (Sigma Aldrich, USA) or decane (Sigma

Aldrich, USA) was added. Each sample was mixed using a vortex mixer for 2 minutes and was left to stand at room temperature for 15 minutes to seperate of the two phases. The lower aqueous phase of the mixture was removed, and the optical density recorded. The calculation used to determine affinity to hydrocarbons was from Rosenberg et al., (1980);

$$adhesion = \left(1 - \frac{A}{A_0}\right)\ x\ 100 \qquad [5]$$

Where A_0 is the optical density measured at 400 nm prior to mixing and *A* is the absorbance after mixing.

Table 1: Ingredients required to make one litre of PUM buffer [pH 7.1]

Chemical	**Quantity (g)**	**Source**
Potassium phosphate trihydrate	22.2	BDH, UK
Monobasic potassium phosphate	7.26	BDH, UK
Urea	1.8	Sigma, UK
Magnesium sulphate heptahydrate	0.2	BDH, UK

2.2.11 Zones of Inhibition

Coupons which had been previous cleaned (as described in the previous Method 2.2.2) were aseptically adhered to the bottom of sterile Petri dishes with double sided sticky tape (Guilbert Niceday, UK). Cell suspensions were prepared (Method 2.2.9) (between 10^5 and 10^6 CFU/mL) and 1.25 mL of the latter was added to 23.75 mL of molten brain heart infusion agar. The suspension of cells and agar was agitated gently to disperse the cells evenly before being gently poured over the coupons and left for the agar to set. The samples were then incubated at 37 °C overnight before the zones of inhibition were measured using digital Vernier callipers (Mitutoyo CD- 6" CP, Japan). Three coupons were tested per run and the tests performed in duplicate (n = 4).

2.2.12 Cell Viability (Nitro Tetrazolium Violet) Assay

The method was modified from Wickens., (2014) and Barnes *et al.,* (1996). Cultures were prepared (Method 2.2.9) and diluted to give a suspension containing between 10^4 and 10^5 CFU/mL. The diluted cell suspension (10 µl) was pipetted onto the individual coupons, spread aseptically with the pipette tip and dried in a class II airflow cabinet for 1 hour. Once dry, molten (50 °C) brain heart infusion (25 mL) (Oxoid, UK) was poured gently over the coupons and allowed to set fully before incubating at 37 °C overnight. Following incubation, 0.01 % filter sterilised Nitro Tetrazolium Violet (2 mL) (Sigma-Aldrich, USA) was flooded onto the top of the agar and incubated at room temperature for 6 hours. Viable colonies were visible as dark violet colonies which allowed counting for quantitative data and photographs were taken from qualitative data. Two coupons were tested per run and the tests performed in duplicate (n = 4).

2.2.13 Retention Assay

Microorganisms were prepared as per Method 2.2.9. The coupons were cleaned as per method 2.2.5 and dried for 15 minutes in a class II airflow cabinet before being stuck to the bottom of a glass Petri dish using double sided sticky tape (Guilbert Niceday, UK). The prepared cell suspension (30 mL) was poured over the coupons and lids placed on top of the Petri dishes. Samples were incubated for one hour at 37 °C without agitation. Following incubation, the coupons were removed with sterile forceps and each washed gently with 5 cm^3 of sterile purified water from a wash bottle at a 45° angle with a 3 mm nozzle. Coupons were then air-dried in a class II airflow cabinet for 1 hour (n = 4).

To allow for visualisation with an epifluorescence microscope, the samples were stained using 0.03 % acridine orange (w/v) (Sigma, USA) in 2 % glacial acetic acid (w/v) (BDH, UK). The stain solution was flooded onto the samples and left for 2 minutes, rinsed with 5 mL of sterile purified water, and dried once more in a class II airflow cabinet in the dark for 30 minutes. The cells retained on the surface were visualised using epifluorescence microscopy (Nikon Eclipse E600 epifluorescence microscope, Tokyo, Japan) at 502 – 526 nm wavelength. Cell-F software was used to visualise and capture the images (n = 40) and then place a counting rid over the

images to allow for manual counting of the cells. False colouring was added to the black and white images utilising Cell-F.

2.2.14 Statistical Analysis

Replicate samples were tested in duplicate or in triplicate, and experiments repeated with the mean values plotted. Error bars on the graphs represent the standard error of the mean data. Data was tested for normality before statistical analysis was performed using IBM SPSS (version 21) in order to calculate ANOVA with post-hoc Tukey test, and t-test values. The statistical confidence interval was considered significant when $p < 0.05$.

2.3 Results

2.3.1 Scanning Electron Microscopy (SEM)

SEM images of the 304 stainless steel with a 2R finish (Figure 3, a, and b) and TiN/25.65at.%Ag coupons (Figure 3, c and d) were taken at different magnifications (x 5000 and x 20,000) in order to demonstrate the topographical difference of the surfaces before and after sputtering with TiN/25.65at.%Ag at the macro and micro level. The lower magnification images confirmed that the micron sized features on the substrates prior to sputter coating (Figure 3 c) were still visible after sputter coating (Figure 3 a). Following sputter coating, a fine granular topography was evident for the TiN/25.65at.%Ag surfaces.

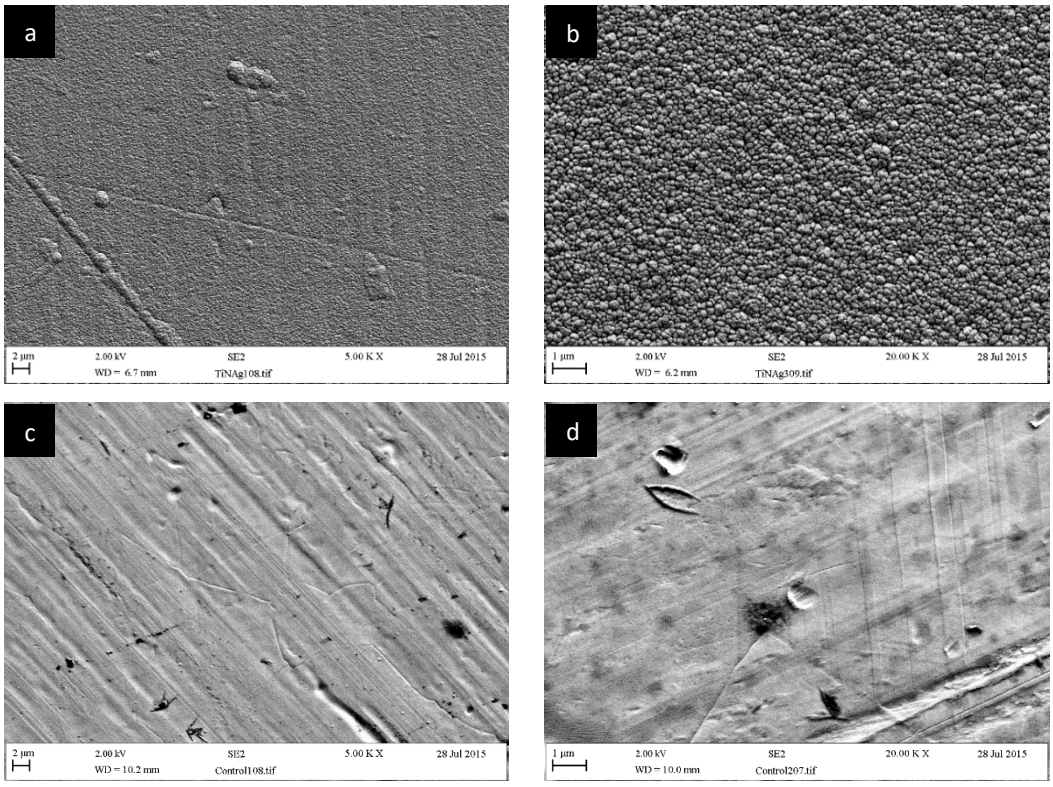


Figure 3: SEM images of a) TiN/25.65at.%Ag surface x 5,000 magnification b) x 20,000 magnification, c) 304-2R stainless steel surface x 5,000 magnification), d) x 20,000 magnification demonstrating the macro and micro topography of the different substrata.

2.3.2 Chemical Composition (Energy Dispersive X-ray Spectroscopy Analysis)

EDX analysis was performed on both the 304-2R stainless steel control and the TiN/25.65at.%Ag coated 304-2R stainless steel coupons in order to determine the percentage elemental content for each metal. The data obtained (Table 2) indicated that the TiN/25.65at.%Ag surfaces contained 25.7 % ± 0.3 silver. The low standard error demonstrated that the coatings were homogeneously sputtered. As the penetration depth of the EDX beam was between 1 µm – 2 µm, the results demonstrated that the thickness of the TiN/25.65at.%Ag coating exceeded the penetration depth as the elemental content of the underlying substrate was not apparent.

Table 2: Mean chemical composition (atomic %) of the test coupons ± standard error (n = 3).

Element	304-2R	TiN/25.65at.%Ag
Chromium	20.82 (± 0.09)	0
Iron	70.42 (± 0.35)	0.21 (± 0.09)
Nickel	7.46 (± 0.16)	0
Silicon	0.16 (± 0.16)	0
Manganese	1.14 (± 0.40)	0
Nitrogen	0	41.07 (± 0.93)
Silver	0	25.65 (± 0.32)
Carbon	0	2.30 (± 0.60)
Titanium	0	30.77 (± 0.96)

2.3.3 White Light Profilometry

White light profilometry was performed on both the 304-2R stainless steel control, and the TiN/25.65at.%Ag coated coupons in order to provide improved resolution of the surface topography and to quantify their roughness over 160 µm x 160 µm. The scans performed (Figure 4) allowed for visual assessment of the surfaces which demonstrated linear striations and pits on both the 304-2R stainless steel and TiN/25.65at.%Ag. Determination of the S_a value (Figure 5) demonstrated no significant difference between the test replicates p = 0.575 (304-2R stainless steel = 25.27 nm, TiN/25.65at.%Ag = 29.06 nm). Line profiles (Figure 6 and Table 3) performed on the surfaces demonstrated that the 304-2R stainless steel substrates possessed wider valleys (3.21 nm ± 0.5 nm) than the TiN/25.65at.%Ag (1.9 nm ± 0.5 nm) but the differences between the smaller valleys were similar between the two surfaces (0.56 nm ± 0.04 nm and 0.62 nm ± 0.03 nm respectively). The average peak height for both the 304-2R stainless steel and TiN/25.65at.%Ag substrates were similar in size, the TiN/25.65at.%Ag being slightly bigger (23.2 nm ± 4.8 nm and 29.3 nm ± 5.9 nm respectively). The peak to valley ratio (Figure 7) of the 304-2R stainless steel was lower than the TiN/25.65at.%Ag surfaces (186.7 nm and 283.3 nm respectively) demonstrating that the TiN/25.65at.%Ag surfaces had larger peak averages than the 304-2R stainless steel.

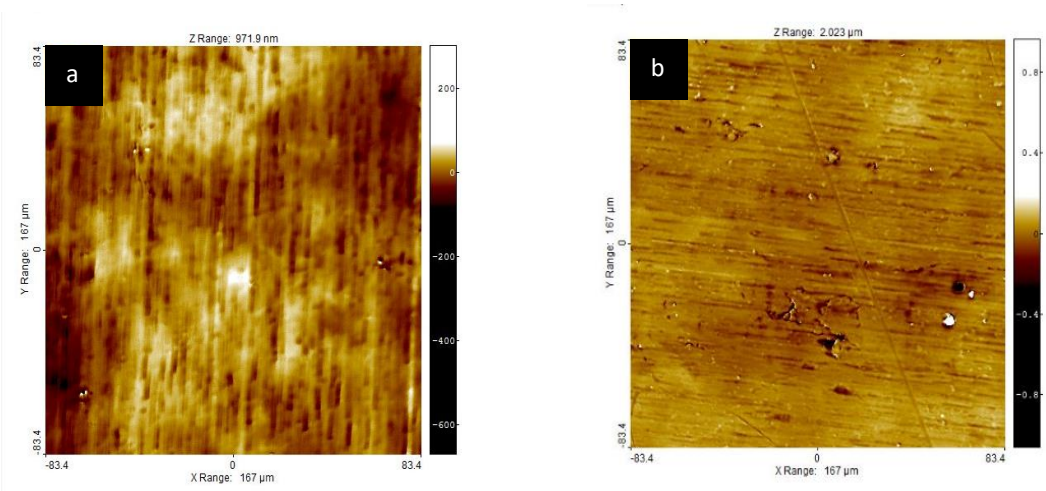


Figure 4: White light profilometry pictures demonstrating the surface topography of a) 304-2R stainless steel and b) TiN/25.65at.%Ag coupons.

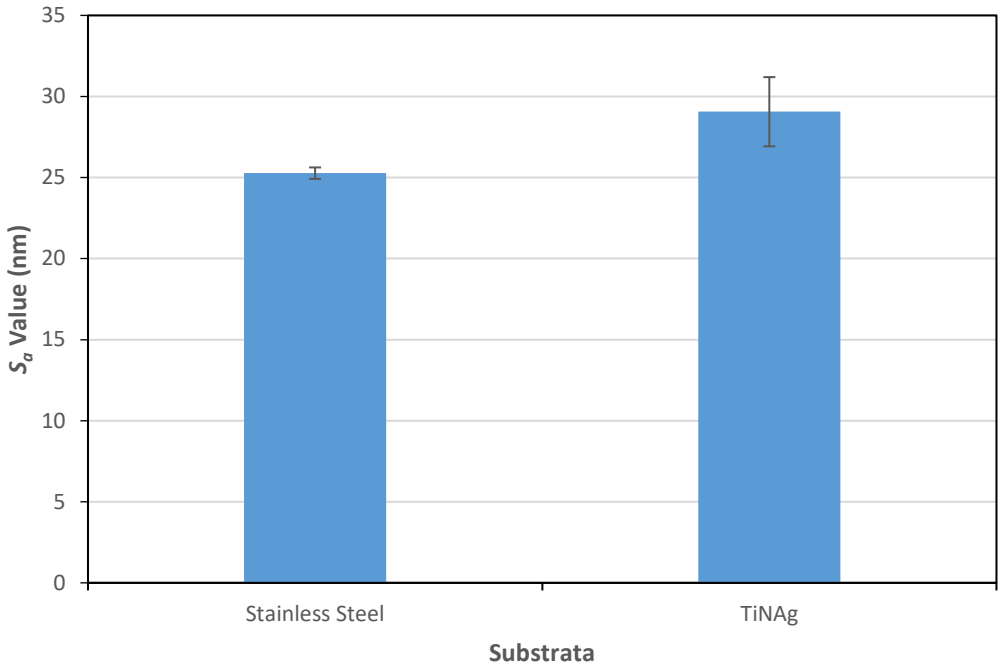


Figure 5: S_a values obtained by white light profilometry on the two substrates, TiN/25.65at.%Ag and 304-2R stainless steel ± standard error (n = 9).

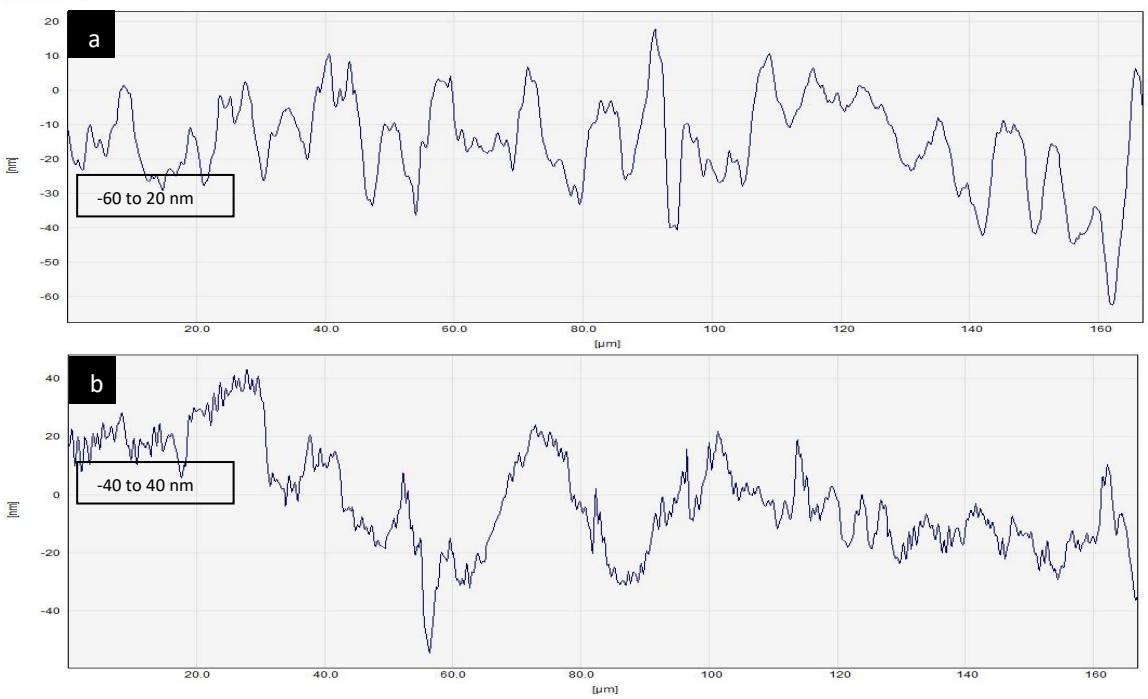


Figure 6: Line profiles from the WLP scans of a) 304-2R Stainless steel, b) TiN/25.65at.%Ag surfaces displaying a cross-sectional profile of the width and depth of the peaks and valleys on the surface over 160 µm (n = 3).

Table 3: Average values of peaks and valleys from line profiles (n = 3).

Substrate	Peak width (µm)	Peak height (nm)
Stainless Steel	0.56 - 3.21	5.95 - 40.38
TiN/25.65at.%Ag	0.62 - 1.90	10.43 - 48.19

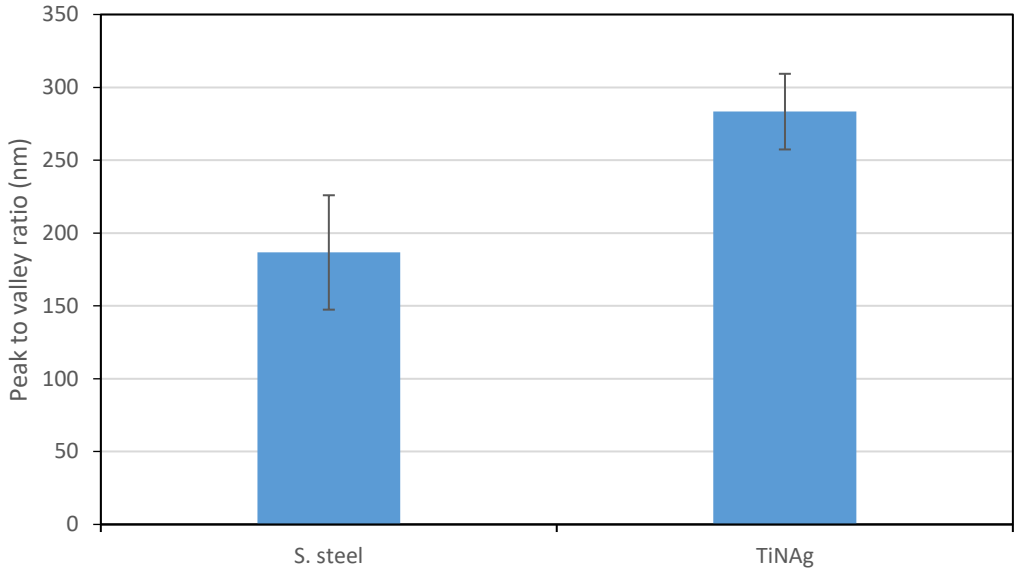


Figure 7: Peak to value ratio values obtained from line profiles upon 304-2R stainless steel and TiN/25.65at.%Ag surfaces ± standard error (n = 3).

2.3.4 Physicochemistry

Physicochemical analysis of the surfaces was carried out to determine how the different topographical properties and chemical characteristics of the 304-2R stainless steel were altered following TiN/25.65at.%Ag coating. These results were later used to determine if these factors had any effect on microbial retention to the substrata. Using the data obtained from the contact angle measurements, the hydrophobicity (ΔG_{iwi}) of the surfaces was determined and demonstrated that both substrates were hydrophobic in nature but also that the TiN/25.65at.%Ag surfaces was slightly more hydrophilic than the 304-2R stainless steel (Figure 8).

The surface free energy (γ_s) was calculated and used to determine if it affected bacterial adhesion to the surface. The results demonstrated that the TiN/25.65at.%Ag coating had significantly higher surface free energy (p = 0.01) than the 304-2R stainless steel (Figure 9). The results from the contact angle measurements were also used to calculate the Lifshift Van der Waals forces (Figure 10) the substrates. The results demonstrated that there was no significant difference between the TiN/25.65at.%Ag and 304-2R stainless steel substrates (p = 0.71). The acid-base interactions (γ_s^{AB}) of the surfaces demonstrated (Figure 11) that the TiN/25.65at.%Ag surfaces had a slightly higher γ_s^{AB} value than the 304-2R stainless steel surfaces. A higher γ_s^{AB} value could suggest potential changes to the surface charge of the substrates. However, statistical analysis of the γ^{AB} data revealed that the difference were not statistically different (p = 0.25).

Calculation of the electron accepting or donating potential of the surfaces revealed that the TiN/25.65at.%Ag surfaces were slightly more electron accepting than the 304-2R stainless steel and that there was little difference (5.9 mJ/m^2) between the electron donating potential of the surfaces (Figures 12 and 13). Statistical analysis of these results showed that there was no significant difference at the 95% confidence level.

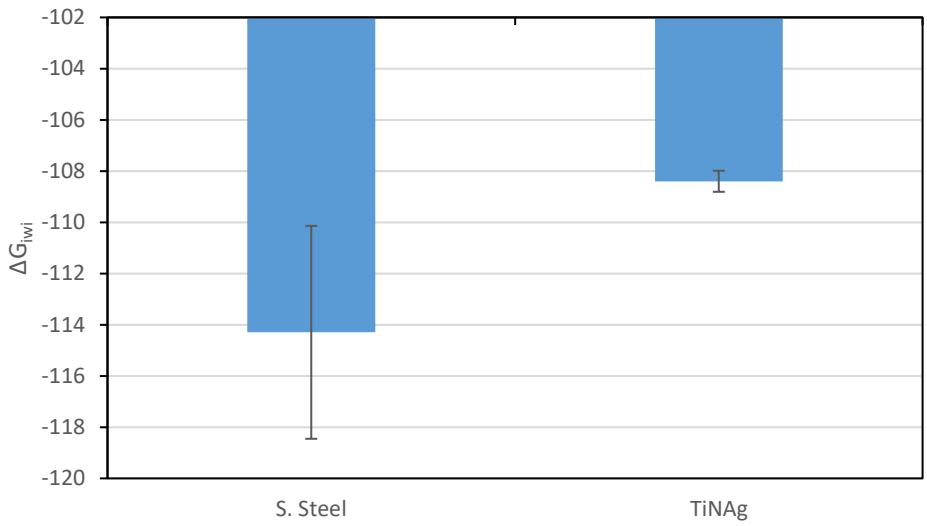


Figure 8: ΔG_{iwi} values for the two substrates demonstrating the quantitative measurement of hydrophobicity ± standard error (n = 3).

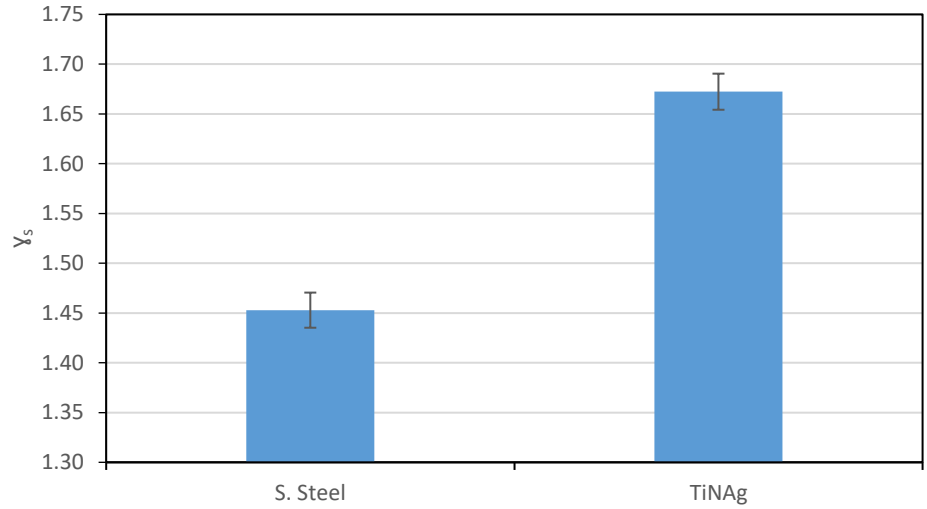


Figure 9: γ_s values of the surfaces indicate the measurement of surface free energy (SFE) ± standard error (n = 3).

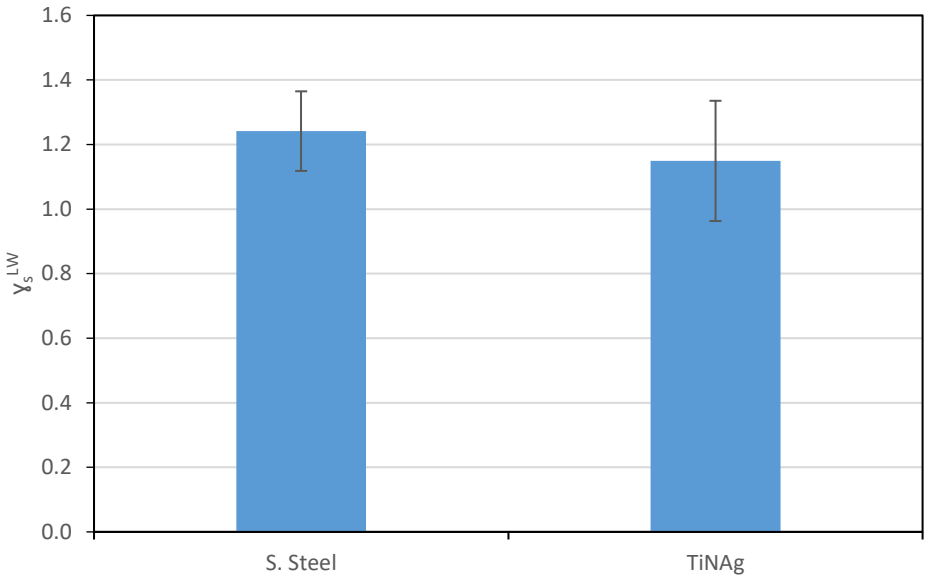


Figure 10: γ_s^{LW} values of the surfaces demonstrating the Lifshift Van der Waals forces acting upon the surfaces ± standard error (n = 3).

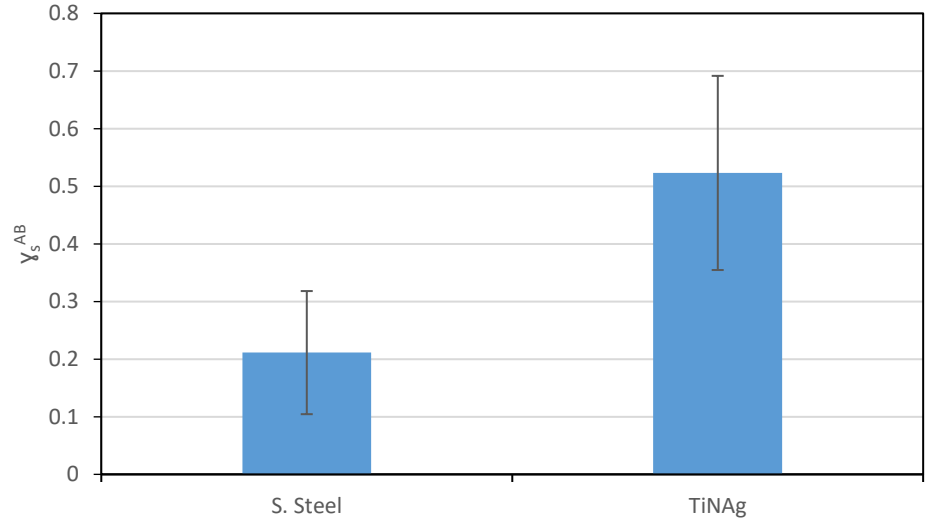


Figure 11: γs^{AB} values of the surfaces showing the Acid-Base interactions ± standard error (n = 3).

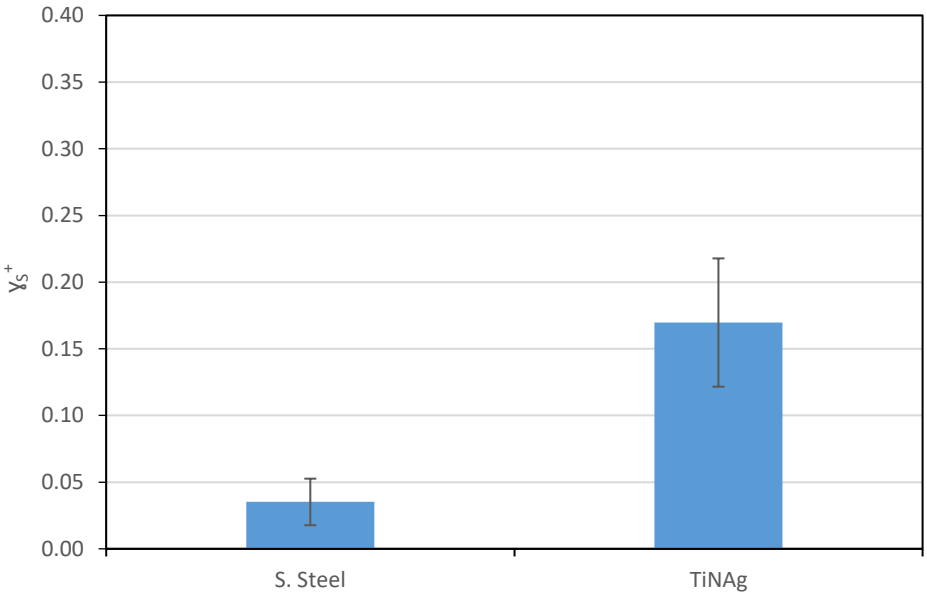


Figure 12: The γ_S^+ values of the surfaces demonstrated the electron accepting potential of the surfaces ± standard error (n = 3).

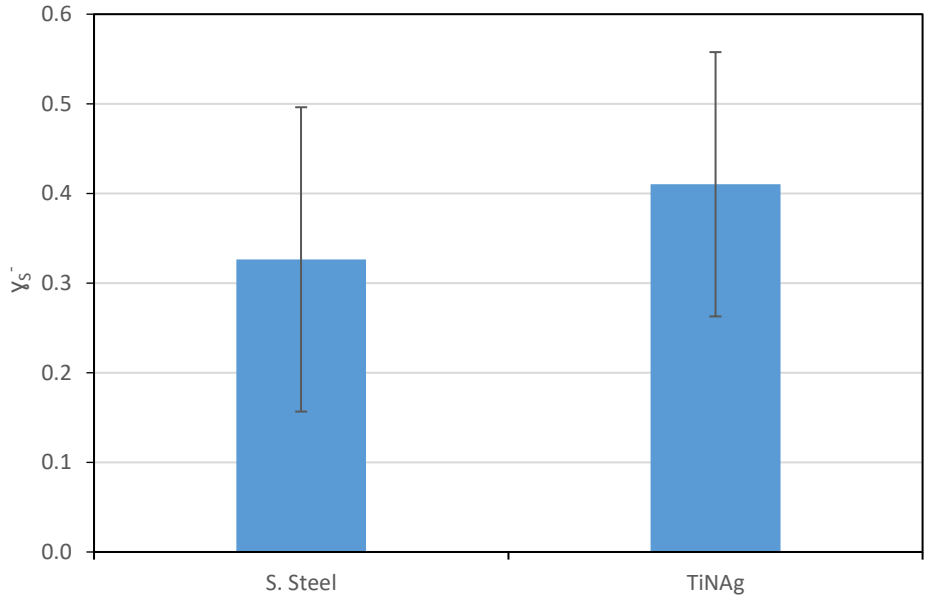


Figure 13: The γ_S^- values demonstrated the number of the electron donors at the surface ± standard error (n = 2).

2.3.5 Zones of Inhibition

Zone of inhibition testing of TiN/25.65at.%Ag and the control substrates was carried out in order to assess the antimicrobial action and leaching potential of the surfaces. Qualitative assessment (Figure 14) revealed that a clear zone of inhibition was produced by TiN/25.65at.%Ag against *E. coli* (Figure 14, b) but no zones were produced against *S. aureus* (Figure 14, c and d). Quantitative assessment of the zones of inhibition demonstrated that 304-2R stainless steel surfaces produced no zones of bacterial clearance against *E. coli* or *S. aureus* thus demonstrating that there was no antimicrobial efficacy (Table 4). TiN/25.65at.%Ag also produced no measurable zone of inhibition against *S. aureus*, however it did produce a statistically significant zone of inhibition against *E. coli* (p = 0.00) suggesting that when using this antimicrobial assay, *E. coli* was more susceptible towards the antimicrobial action of silver.

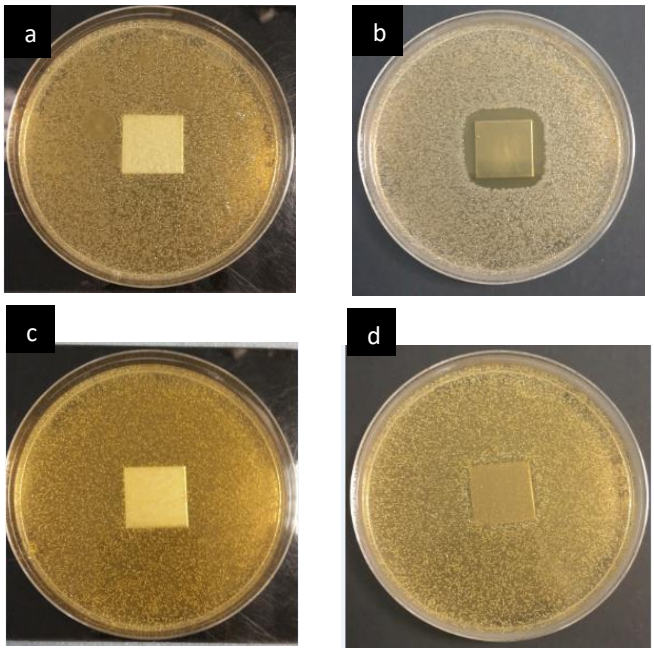


Figure 14: Zone of inhibition assays a) *E. coli* on 304-2R stainless steel, b) *E. coli* on TiN/25.65at.%Ag, c) *S. aureus* on 304-2R stainless steel, d) *S. aureus* on TiN/25.65at.%Ag (n = 4).

Table 4: Zones of inhibition (mm) produced by 304-2R stainless steel and the TiN/25.65at.%Ag substrates against *E. coli* and *S. aureus* (n = 4).

	304-2R stainless steel (mm)	TiN/25.65at.%Ag (mm)
E. coli	0	5.7
S aureus	0	0

2.3.6 Nitro Tetrazolium Violet Assay (NTV)

Nitro tetrazolium violet assays were performed using *E. coli* and *S. aureus* on the 304-2R stainless steel control and TiN/25.65at.%Ag surfaces. The assays demonstrated that both microorganisms were susceptible to the antimicrobial action of the TiN/25.65at.%Ag surfaces (Figure 15). For *S. aureus* this result was statistically significant (p = 0.002). The number of *E. coli* colonies on the control coupon were too numerous (TNTC) to count so statistical analysis was not possible.

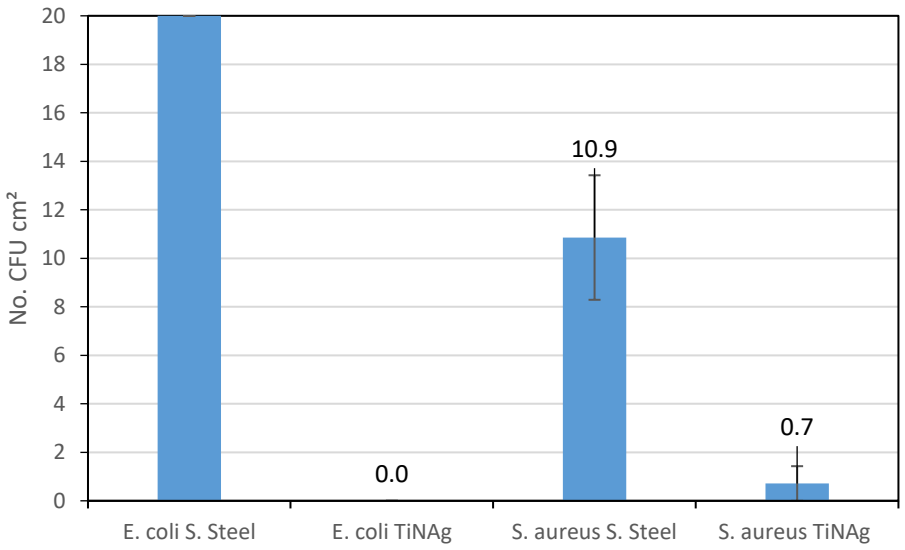


Figure 15: NTV assays demonstrating the reduction in colonies produced by the coupons containing TiN/25.65at.%Ag against *E. coli* and *S. aureus* ± standard error (n = 4).

2.3.7 Retention Assays

Retention assays were performed on both test substrata (TiN/25.65at.%Ag and 304-2R stainless steel) using *E. coli* and *S. aureus* (Figure 16). The number of retained cells observed using epifluorescence microscopy demonstrated that there was no significant difference in the number of cells retained on the different test substrates. *Staphylococcus aureus* showed slightly greater retention on 304-2R stainless steel (1793.25 ± 199.7) than TiN/25.65at.%Ag (1211.25 ± 209.5), whilst *E. coli* exhibited a slightly greater affinity for the TiN/25.65at.%Ag (1767 ± 572) than the 304-2R stainless steel (1440.1 ± 199.7). Statistical analysis of the data showed that there was no significant difference between the TiN/25.65at.%Ag and 304-2R stainless steel replicates for either bacterial species (p = 0.10 and p = 0.36, respectively), although a trend in preference was demonstrated. Both microorganisms showed a tendency to clump together arranging themselves heterogeneously whilst in contact with the 304-2R stainless steel coupons, yet whilst when in contact with the TiN/25.65at.%Ag coupons both microorganisms were observed to be arranged more homogenously and in fewer clumps, thus displaying single cells.

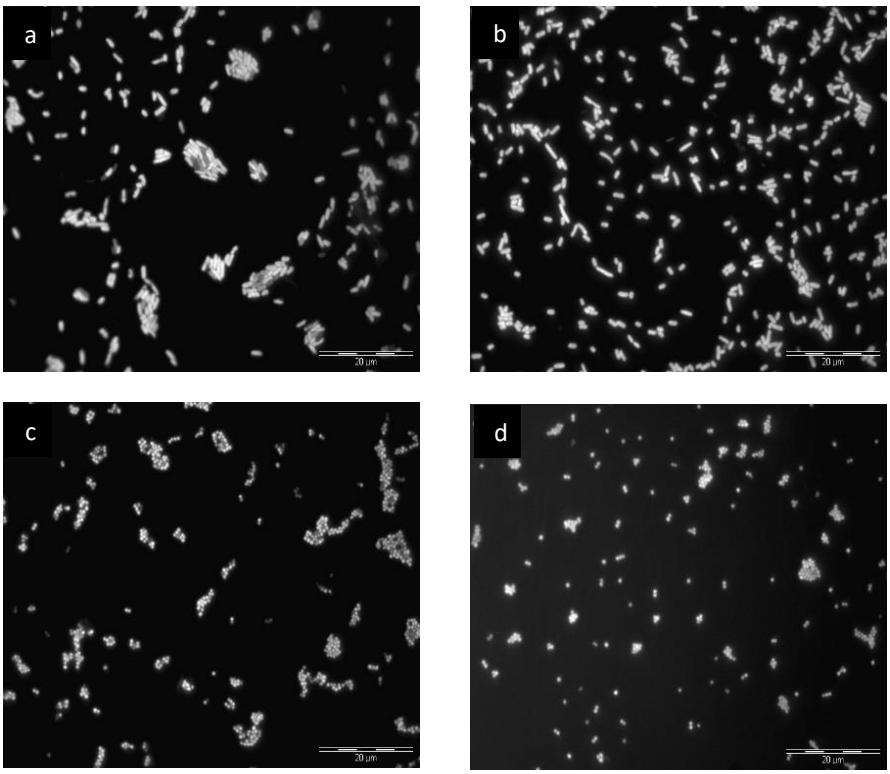


Figure 16: Epifluorescence images depicting the retention a) *E. coli* on 304-2R stainless steel, b) *E. coli* on TiN/25.65at.%Ag, c) *S. aureus* on 304-2R stainless steel, d) *S. aureus* on TiN/25.65at.%Ag coupons.

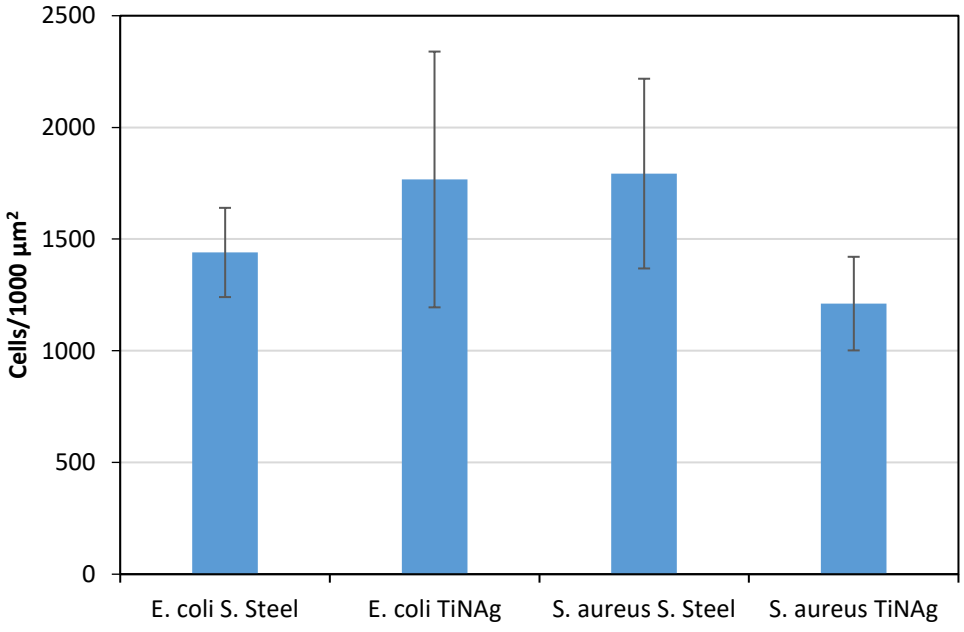


Figure 17: Retention assays performed on 304-2R stainless steel and TiN/25.65at.%Ag coupons using *S. aureus* and *E. coli* ± standard error. x 1000 magnification (n = 20).

2.3.8 Microbial Adhesion to Hydrocarbons Assay

Microbial adhesion to hydrocarbons (MATH) assays were performed upon both the *S. aureus* and *E. coli* to quantify their physicochemistries (Figure 18). Results showed that *S. aureus* demonstrated a stronger affinity to chloroform (99.9 %), hexadecane (81.0 %) and decane (81.5 %) than it did to ethyl acetate (41.7 %). *E. coli* also demonstrated a greater affinity to chloroform and ethyl acetate than to hexadecane and decane (64.6 %, 29.4 %, 4.4 %, and 0 % respectively). This demonstrated that both microbes were electron donors. *E. coli* demonstrated a very low affinity to hexadecane, which when combined together with its greater affinity towards chloroform and ethyl acetate showed that it was more hydrophilic than *S. aureus*. The latter demonstrated a high affinity towards the apolar solvents (hexadecane and decane) demonstrating that it was the more hydrophobic of the two bacteria tested.

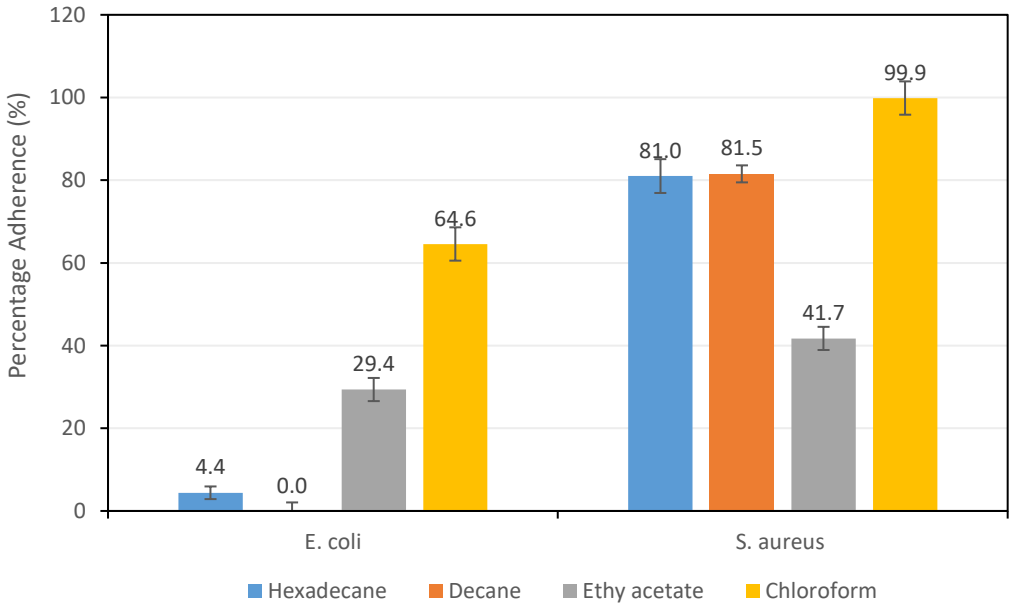


Figure 18: MATH assay displaying percentage affinity of the microorganisms toward the different solvents, demonstrating the surface chemistry of the different bacteria ± standard error (n = 3).

2.4 Discussion

The 304-2R stainless steel was coated with TiN/25.65at.%Ag to determine the effect of the surface properties (chemistry, topography and physicochemistry) on the antimicrobial activity and bacterial retention to the surface. 304-2R stainless steel and TiN/25.65at.%Ag surfaces were tested for potential antimicrobial action. Antimicrobial testing upon the surfaces demonstrated that the 304-2R stainless steel surfaces did not produce any zones of inhibition as predicted. The TiN/25.65at.%Ag surfaces did produce a clear zone of inhibition against *E. coli* but not against *S. aureus.* This could be due to the thinner peptidoglycan wall of Gram-negative bacteria (*E. coli*) in comparison to Gram-positive bacteria (*S. aureus*). Works by Ahearn et al., (1995) used ion beam assisted silver, silver oxide and silver chloride surfaces and although they demonstrated that the surfaces inhibited both bacterial growth and bacterial adherence, they also found that the silver surfaces did not produce zones of inhibition in agar diffusion tests as did McLean et al., (1993) using Ag/Cu surfaces. It was proposed that rather than an antimicrobial effect caused by ion leaching that the Ag operates by promoting a catalytic interaction with oxygen which then promoted bactericidal activity (Heinig, 1993).

The nitro-tetrazolium violet respiratory assay, which demonstrated metabolism of microbes whilst in contact with the surfaces, demonstrated that as expected, the 304-2R stainless steel produced no inhibition of the bacteria, whilst when in contact with the surfaces. The TiN/25.65at.%Ag surfaces did produce a significant reduction in the number of colonies against both bacterial species. This demonstrated that the TiN/25.65at.%Ag surfaces did have antimicrobial mechanisms against both *E. coli* and *S. aureus* when in contact with the surface. These results demonstrated that the method of testing, and thus the antimicrobial delivery to the bacterial species was important. Works by others has hypothesized upon the mechanisms of the antimicrobial action of silver, suggesting that the silver ions caused structural changes in the bacterial cell walls by disrupting the permeability and respiration of the cells. These disruptions would affect the interactions of thiol groups in proteins and enzymes, or create interruptions in DNA replication (Feng et al., 2000; Morones et al., 2005; Rai et al., 2009; Skovager et al., 2013).

The differences in bacterial cell wall morphology between the Gram-positive and the Gram-negative bacteria could explain why the mechanism for antimicrobial action and results produced by the TiN/25.65at.%Ag surfaces were different between the bacterial species used in this study, since *S. aureus* is Gram-positive whilst *E. coli* is Gram-negative. Similarly, a study by (Kelly et al., 2009) examined the effect of differing Ag concentrations of TiN/25.65at.%Ag surfaces upon *Pseudomonas aeruginosa* and *S. aureus,* and demonstrated that the surfaces that contained higher concentrations of silver had a greater bactericidal effect upon Gram-negative bacteria. Cell surface area and shape could also be factors contributing towards antimicrobial efficacy as a cell with a larger surface area, such as the rod shaped *E. coli*, could potentially be in contact with more silver ions from the TiN/25.65at.%Ag surfaces than the cocci shaped *S. aureus* (Guzman et al., 2012).

Analysis of the surfaces by white light profilometry demonstrated that there was no statistically significant difference in the surface roughness (S_a) between the two surfaces. Line profile traces of the surfaces did reveal differences in the size and distribution of the peaks that made up the surface topography. The 304-2R stainless steel surfaces possessed wider valleys than the TiN/25.65at.%Ag substrates which could provide larger sites for retention and adhesion of bacteria (Flint et al., 2000; Hilbert et al., 2003; Hsu et al., 2013). Differences in the surface chemistry and topography and roughness parameters can potentially change the physicochemical parameters of the surfaces for example, the electrostatic interactions at the cell: surface interface (Hsu et al., 2013) which therefore affects the retention of bacteria. Changes in the hydrophobicity of the surfaces are important when considering bacterial adhesion (Zeraik and Nitschke, 2012) since it may affect preferential binding to surfaces by different bacterial species (Whitehead and Verran, 2009). The TiN/25.65at.%Ag was found to be less hydrophobic than the 304-2R stainless steel surfaces. Sinde and Carballo (2000) reported that the presence of a hydrophobic substrata would favour bacterial adhesion, however, others have found the opposite (Chae et al., 2006), and have suggested that other factors must also be considered when looking at the physicochemistry of a surface and bacterial

adhesion such as electrostatic surface charge, roughness and the properties of the bacterial cell (Whitehead and Verran, 2009; Zeraik and Nitschke, 2012; Skovager et al., 2013).

Both the Lewis acid-base values and the Lifshift van der Waals values represent the sum of either the attractive or repulsive forces acting upon the substrates, but from either a polar or apolar perspective respectively. The Lifshift van der Waals, acid-base and electron accepting forces were found to be greater for the TiN/25.65at.%Ag surface than the 304-2R stainless steel whereas the electron donating forces were found to be greatest for the 304-2R stainless steel surface.

Assessment of the electron donating/accepting potential and hydrophobicity of the microorganisms has been suggested to be essential for assessing the hygienic status of substrates (Bellon-Fontaine et al., 1996) and for predicting patterns of microbial attachment. The microbial adhesion to hydrocarbons assay enabled calculation of the physicochemistry for the bacterial species used in this study. This demonstrated that whilst both microbes were electron donors, *S. aureus* was hydrophobic and *E. coli* was more hydrophilic in nature.

When analysing the bacterial retention data in conjunction with the physicochemistry of both the bacteria and substrata, trends emerged between the bacterial species. The hydrophobic *S. aureus* showed preference towards the more hydrophobic 304-2R stainless steel, whilst the hydrophilic *E. coli* showed a preference for the TiN/25.65at.%Ag which was less hydrophobic. These results are comparable with research by Whitehead *et al*., (2015) in which it was found that *E. coli* retention was most affected by the physicochemistry of the surfaces. Works by others have documented that surface hydrophobicity is one of the primary driving forces involved in the adhesion of pathogens to surfaces (Sinde and Carballo, 2000; Faille et al., 2002; Zeraik and Nitschke, 2012). However, other have found no correlation in physicochemical parameters between the surfaces and bacteria (Chae et al., 2006; Whitehead and Verran, 2009; Whitehead et al., 2015). Whitehead *et al*., (2015) also found that *S. aureus* retention was also influenced by the topography of the surfaces, which was not demonstrated within this study. The lack of correlation between topography and bacterial retention within this study may be

due to both surfaces being of low roughness, with little difference between their roughness parameters. The use of surfaces with a greater variety of roughness values would help to study this further.

The results of this chapter concluded that for the two species of bacteria used upon these surfaces, hydrophobicity was the main driving force that affected bacterial retention, and that within this instance, bacteria preferred to be retained to surfaces with similar hydrophobicity characteristics to their own. These surface properties affected the spread and clumping of the cells. Cell density was also affected by cell type. Thus, these surface properties affected the bacterial retention of the different species in specific ways. This work suggests that the type of surface influences specific species / surface interactions and therefore surfaces need to be tailored to specific requirements depending on the environment and microorganisms to be targeted. Further research into the effects of topography upon these bacterial species would be required to enable any conclusions to be drawn upon its effects on bacterial retention.

Chapter 3

Characterisation of the Physical Parameters of the Surfaces

3.1 Introduction

The measurements of the physical characteristics of a surface are often utilised and compared to the results obtained from bacterial retention assays to hypothesize correlation between the two. Many studies have made claims regarding bacterial preferences towards surfaces based upon the topography of a surface, suggesting that greater fouling occurs with rougher surfaces (Wirtanen et al., 1995; Flint et al., 2000; Anselme et al., 2010; Puckett et al., 2010; Lüdecke et al., 2016) and promoting the use of a standard measurements in the food industry to describe whether a surface is "hygienic" or not (Flint et al., 2000). However, many other studies, including one study by Hilbert *et al*., (2003) found no correlation between the roughness of stainless steel surfaces and bacterial adhesion, suggesting that bacterial retention to surfaces was affected by many factors and that surface roughness was just one contributing factor. Many of these studies can be contradictory or lack the depth of analysis to make full conclusions, and whilst they may be studying the same parameter of roughness, the lack of consistency in the results may be due to different finishes of metal or different grades of stainless steel as they would contain different contents of elements (Bekmurzayeva et al., 2018).

Another important consideration regarding the physical parameters of surfaces relates to those used as biomaterials as surface topography has a mediatory role in the osseointegration of bone to biomaterial surfaces, conferring better healing times and stability to the implant (Deligianni et al., 2001). Increases in the roughness of a surface can stimulate greater differentiation and attachment of osteocytes as well as promotion of mineralisation (Novaes Jr et al., 2010). This is of special importance for dental implants and indwelling biomaterials such as joint replacements where prolonged strain and pressure exertion is to be expected at the bone / surface interface. Therefor the parameters of materials chosen for use as a biomaterial are important and should take onto consideration the balancing of requirements for functionality, osseointegration, and risk of bacterial contamination.

3.1.1 The use of Titanium Alloys and Stainless Steel

Both titanium and stainless steel are the two most favoured surfaces in both the medical and food industries (Galanakos et al., 2009; Whitehead et al., 2014). Both of these surfaces have been traditionally chosen for these purposes due to their strength, relative ease of cleaning, corrosion resistance, and relative ease of fabrication (Whitehead et al., 2014) and both of these metals have been studied in depth (Galanakos et al., 2009) yet the results of these studies are often contradictory.

Advantages of using stainless steel are that is it an iron-based alloy which typically contains at least 12 % chromium providing the metal with rust resistant properties (Davis et al., 1994; Bekmurzayeva et al., 2018) making it ideal for orthopaedic and dental implants (Hermawan et al., 2011). However, titanium and its alloys have a higher level of biocompatibility (Niinomi, 2003; Bekmurzayeva et al., 2018) but is typically a softer material which does not perform well under stress (Subramanian et al., 2011). Studies investigating bacterial preference and bacteriostatic properties of both titanium and stainless steel have suggested that the properties of stainless steel could lead to higher infection rates than titanium (Cheng et al., 2007; Veerachamy et al., 2014) and that despite stringent cleaning, both surfaces can harbour persistent strains of bacteria which could affect wound healing and the hygiene of industrial food surfaces (Carpentier and Cerf, 2011; Whitehead et al., 2014).

To improve the wear-resistance and strength of titanium a coating of titanium nitride can be added to the surface of the titanium metal providing a hard coating with excellent mechanical properties, corrosion resistance and biocompatibility (Zhao et al., 2004; Moseke et al., 2011). Studies comparing bacterial attachment to titanium and titanium-nitride surfaces provided contrasting data with some studies demonstrating that bacterial adhesion to titanium increased with the addition of Nitrogen (Jeyachandran et al., 2006), and others demonstrating greater numbers of adhered bacteria to titanium surfaces and reduced metabolic activity in bacteria adhered to the TiN surfaces (Groessner-Schreiber et al., 2004). The difference in the results of these studies are likely, as with the studies into stainless steel, due to

differences in the finish or composition of the metals being studied which demonstrates the necessity to measure the surface properties to allow for more direct comparison of the data.

3.1.2 Blood Component Conditioning Films

Conditioning films are formed when organic or inorganic compounds found in aqueous environments are adsorbed to a surface, modifying the measurable parameters of the surface irreparably (Verran and Whitehead, 2006; Lorite et al., 2011; Berne et al., 2018). The nature of a conditioning film is dependent upon the environment in which it becomes soiled, for example; a surface from a marine environment would become differently conditioned from those in a meat processing facility, so it is important to correctly assess a surface utilising an appropriate conditioning agent that the surface would most likely encounter.

The most abundant proteins found in plasma are albumin, globulins and fibrinogen which due to their relatively smaller size in comparison to bacterial cells are able to adsorb onto surfaces a lot quicker than bacteria. The adsorption of these proteins and macromolecules to the surfaces is largely mediated by the surfaces' electrostatic forces, but also by external forces such as temperature and pH (Rabe et al., 2011; Ouberai et al., 2014). Investigations by Vroman *et al.,* (1969; 1980) into the competitive adsorption of plasma proteins to solid interfaces demonstrated that fibrinogen rapidly adsorbed to surfaces reaching full coverage and equilibrium after only a short amount of time before later bring replaced by proteins with a higher surface affinity. This demonstrates the necessity to assess full component plasma conditioning films as it is likely that surfaces that were pre-treated with singular component conditioning films would soon have that replaced with other proteins from the surrounding environment.

Some studies into the effect of singular plasma and serum proteins on titanium surfaces have been promising, demonstrating that the absorption of human serum albumin has the capacity to inhibit the adherence of *S. aureus, Pseudomonas aeruginosa,* and other pathogenic oral bacterial samples (Kinnari et al., 2005; Badihi Hauslich et al., 2013). Human salivary albumin has also been demonstrated to

significantly reduce adhesion of oral pathogenic bacterial strains (Steinberg et al., 1998). However, a study by Taylor *et al*., (1998) demonstrated that gradient increases in the concentration of albumin upon hydrogel contact lenses increased the numbers of adhered *P. aeruginosa* and *staphylococcus epidermidis* to the surface of the lens. Similarly, investigations into another blood plasma component, fibronectin, has also demonstrated conflicting data with some studies indicating a significant increase in the numbers of adhered coagulase negative *S. aureus* due to strain specific receptor interactions (Herrmann et al., 1988), and others indicting significant reductions in strains of *S. epidermidis* (Dunne and Burd, 1993; Eroshenko et al., 2015). This demonstrates that the effects of the conditioning film are still dependent upon the substrate underneath and the bacterial species being studied.

Whilst many studies have investigated the role that conditioning films of singular plasma proteins such as serum albumin (An et al., 1996; Kapalschinski et al., 2013) or fibrinogen (Safiullin et al., 2015) play upon bacterial adhesion to surfaces, few studies have fully investigated the interplay of all the plasma proteins and amino acids in conjunction, and how this may affect their absorption to surfaces and the retention of bacteria. In a study by Xu and Siedlecki, (2012) the effects of human plasma proteins ([25 %]) upon bacterial adhesion to submicron-textured polyurethane urea surfaces which are often used in catheters and other indwelling biomaterials were investigated. Their work tested biofilms of *S. aureus* and *S. epidermidis* at different tine points and under flow conditions and demonstrated a range of reductions in bacterial numbers between 37 % and 75 % dependent on species and time. Whilst this study was performed upon a very different formulation of surface, it demonstrates that this is an area of which further investigation is required.

3.1.3 Chapter Aims and Supporting Information

Following the results of the preliminary study, the issues that were encountered with the poor tribology of the TiN/25.65at.%Ag surfaces needed to be addressed prior to further assessment. Surfaces were made utilising the same method of magnetron sputtering, produced for previous studies (Kelly et al., 2010, 2011) in the same surface engineering and microbiology group but with a lower concentration

of silver that had been found to have greater strength and fewer lamination issues (Kelly et al., 2011). It was decided that those surfaces with a lower percentage of silver content should be used to ensure that the surfaces were durable enough for the extended studies. As those surfaces had been produced utilising a lower KV of silver in the sputter coating process they would likely have different surface characteristics and properties, therefore it was imperative to re-characterise the surfaces alongside the titanium, titanium-based alloys, and stainless steel surfaces that were chosen for the extended study.

The aims of this chapter were to measure the physical parameters of the five metal surfaces and assess how the addition of the bacterial species, conditioning film, and conditioning film with the bacterial species may affect those physical parameters.

3.2 Methods

3.2.1 Experimental Surfaces

The surfaces chosen for experiments were the sputter coated titanium nitride-silver (TiN/15.03at.%Ag), 316L stainless steel (316L), titanium (Ti), 316L-titanium (316 Ti), and 316L-titanium nitride (316 TiN). With exception of the TiN/15.03at.%Ag, all surfaces were purchased from Goodfellow Cambridge LTD, UK.

3.2.2 Conditioning Film

The conditioning film chosen for this study was bovine plasma in a 10 % concentration. The use of a bovine plasma conditioning film would be relevant for studies utilising metals for both bioimplants and for surfaces in meat packing/processing in the food industry as these surfaces would encounter plasma proteins in a 'real world' situation. The concentration of 10 % was chosen due to previous works within our laboratories which demonstrated that this concentration did not produce an excessively thick layer upon the metal surfaces (Whitehead et al., 2010).

3.2.3 Coupon Cleaning

The 10 mm x 10 mm coupons were cleaned as per Method 2.2.2.

3.2.4 Scanning Electron Microscopy (SEM)

Each of the surfaces were analysed as per Method 2.2.4 and scanned at x 5000, x 10,000, x 15,000 and x 20,000 magnification.

3.2.5 White Light Profilometry

The topography of the surfaces was measured using white light profilometry as per Method 2.2.5 (n = 18). Peak heights and widths were measured and calculated from the line profiles through use of a personally developed programme (Louise's Peak Finder, Brett Hewitt, 2016) (n = 3). The software automatically identifies the minimum and maximum points from the frame containing the line profile, standardising the technique and ensuring accuracy of the counts by removing human error. This data was then placed into box plots to allow visualisation of the distribution of the peaks and widths over the line trace.

3.3 Results

3.3.1 Scanning Electron Microscopy

SEM images of the five surfaces under the differing conditioning parameters were taken to visually analyse the surface structures, the morphology of the coating on the TiN/15.03at.%Ag surfaces and to assess if the addition of the surface conditioning agents affected the surfaces. The images would also allow for visual assessment of the bacterial interaction with the different surfaces.

Assessment of the pristine surfaces (Figure 19) demonstrated that the 316L stainless steel and the TiN/15.03at.%Ag surfaces had fewer surface features than the other surfaces, although the TiN/15.03at.%Ag surfaces continued to demonstrate the striations of the underlying 316L stainless steel. The TiN/15.03at.%Ag surface had a granular appearance due to the sputter coating process, however, this was less prominent than upon the TiN/25.65at.%Ag surfaces (Chapter 2). The titanium, 316Ti and 316TiN surfaces visually had greater surface features, with pits and columns evident.

SEM images of the surfaces after retention assays of *S. aureus* and *E. coli* were used to give a visual assessment of how the bacterial cells adhered to the surfaces and

interacted with the topographical features. The *S. aureus* cells (Figure 20) appeared to clump together on surfaces which had a reduced roughness and topographical features, such as 316L stainless steel and TiN/15.03at.%Ag surface. The *S. aureus* visible on the 316TiN at x 15,000 magnification (Figure 20 h), were clumped together in the valleys of the surface, which potentially provided a greater surface area for attachment, as well as refuge from potential sheer stress.

In contrast, The *E. coli* cells (Figure 21) did not appear to clump together on the 316L stainless steel and TiN/15.03at.%Ag surfaces as the *S. aureus* had, but appeared evenly spaced across the surface. The flagella were visible on the 316L stainless steel surface at x 15,000 magnification (Figure 21 b insert) which was possibly used to 'anchor' the cells to the surface in the absence of topographical features. However, when presented with surfaces with linear striations, the *E. coli* appeared to prefer to adhere within the valleys of the titanium and 316Ti surfaces, aligning themselves directionally with the striations, potentially to maximise surface contact with their rod-shaped cell structure.

SEM images of the five surfaces in the presence of the 10 % bovine plasma conditioning films demonstrated that the conditioning film did not produce any visual changes to the surfaces, which still appeared as pristine surfaces.

Images were also taken after retention assays of either *S. aureus* with 10 % bovine plasma or *E. coli* with 10 % bovine plasma had been performed to allow for a visual assessment of how the bacteria adhered to the surfaces and interacted with the topographical features in the presence of the conditioning film.

As previously demonstrated, the addition of the 10 % bovine plasma conditioning film made no visual difference to the surface of the metal samples. The *S. aureus* cells (Figure 23) appeared to clump together upon the less rough 316L stainless steel and TiN/15.03at.Ag surfaces, as previously seen when the bacteria were alone, but in much smaller numbers. Similarly to when the bacterial species were on their own on the surfaces, the *S. aureus* sat within the linear striations and pitted valleys of the titanium, 316Ti and 316TiN surfaces. A reduction in the numbers of bacterial cells was also noted as with the 316L stainless steel.

The addition of the 10 % bovine plasma to the *E. coli* (Figure 24) had produced a similar effect of reducing the numbers of *E. coli* cells found upon all the surfaces with the exception of the titanium surface. The *E. coli* cells still demonstrated a preference to sit within the topographical structures of the surfaces where possible, seemingly in places that would provide the greatest surface area contact for the cells to the surface. However, analysis of the *E. coli* attached to the 316L stainless steel in the presence of the conditioning film demonstrated that the flagella was no longer visible upon the less rough metal surfaces as it had been in when no conditioning film was present.

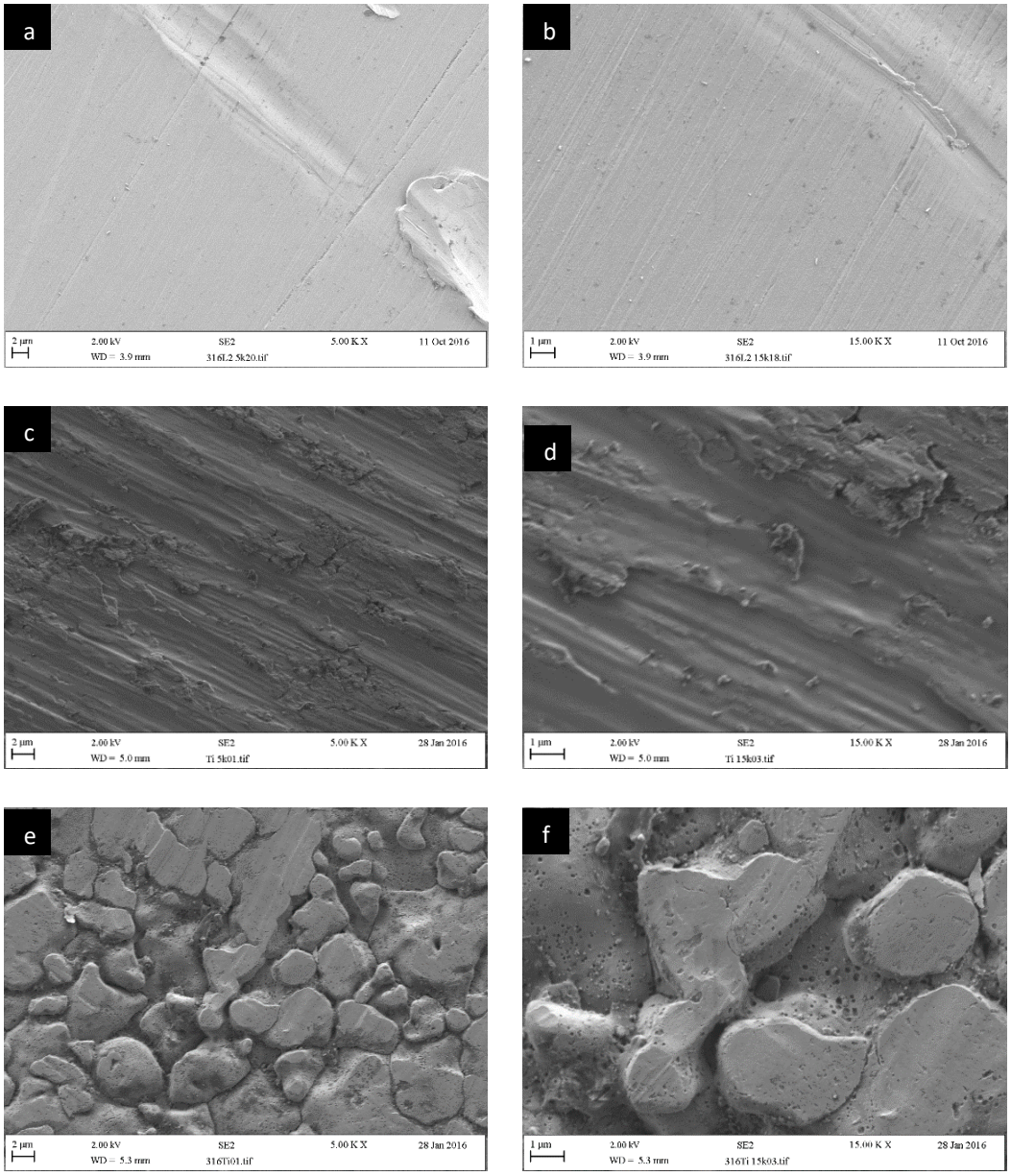


Figure 19: SEM images of a) 316L stainless steel at x 5,000 magnification, b) x 15,000 magnification, c) titanium at x 5,000 magnification, d) x 15,000 magnification, e) 316Ti at x 5,000 magnification, f) x 15,000 magnification.

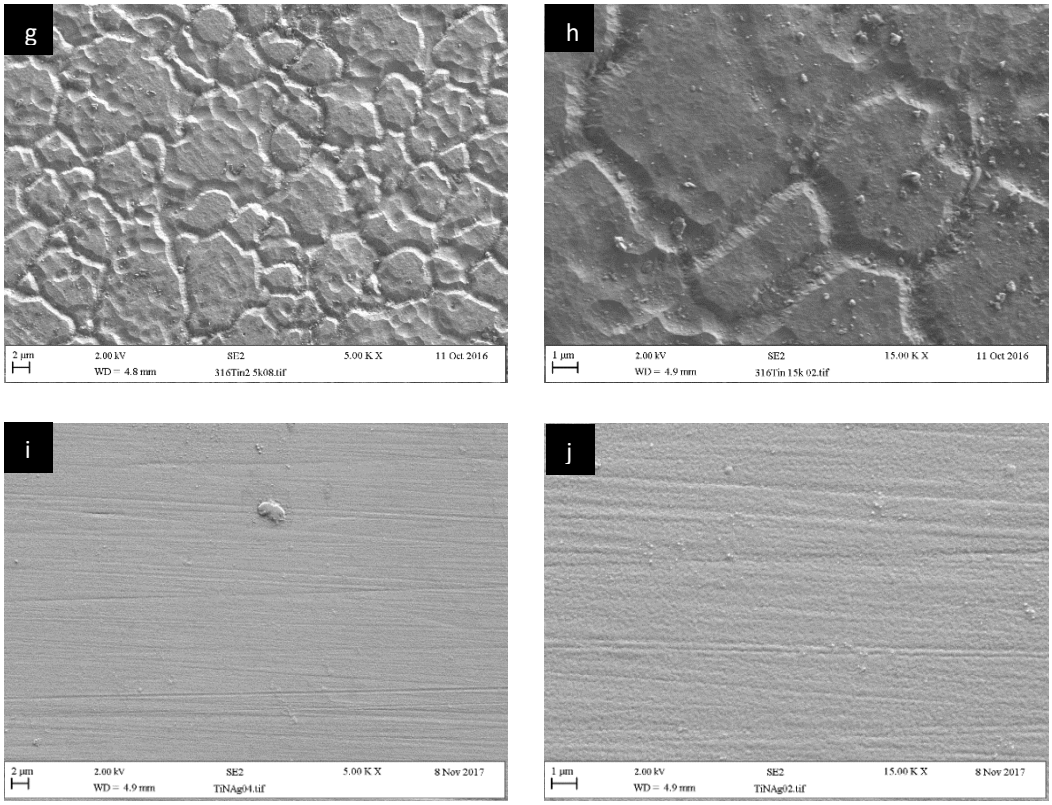


Figure 19 continued: g) 316TiN at x 5,000 magnification, h) x 15,000 magnification, i) TiN/15.03at.%Ag x 5,000 magnification, j) x 15,000. The images demonstrated the macro and micro topographies of the different substrates.

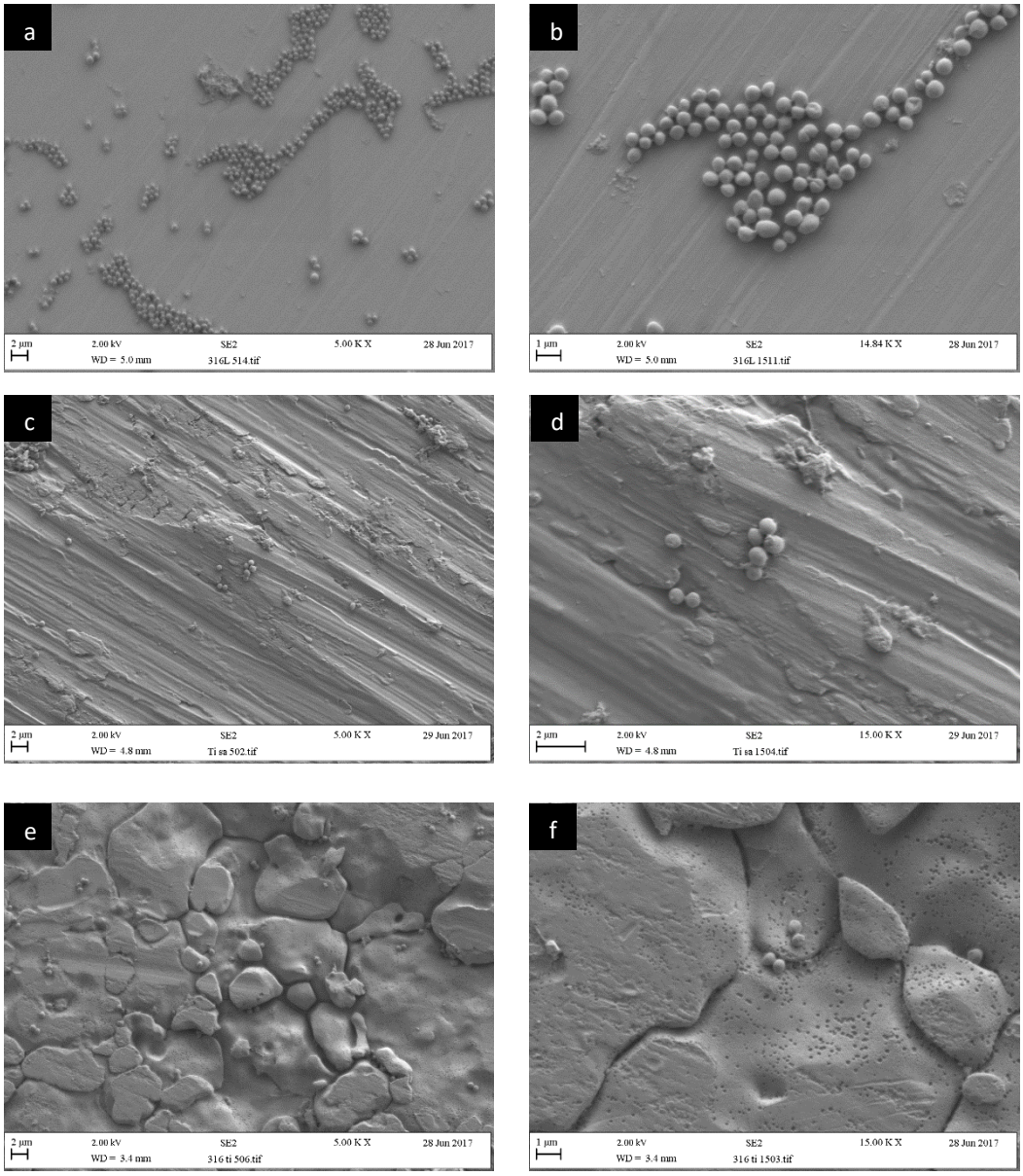


Figure 20: SEM images of a) 316L stainless steel plus *S. aureus* at x 5,000 magnification, b) x 15,000 magnification, c) titanium plus *S. aureus* at x 5,000 magnification, d) x 15,000 magnification, e) 316Ti stainless steel plus *S. aureus* at x 5,000 magnification, f) x 15,000 magnification.

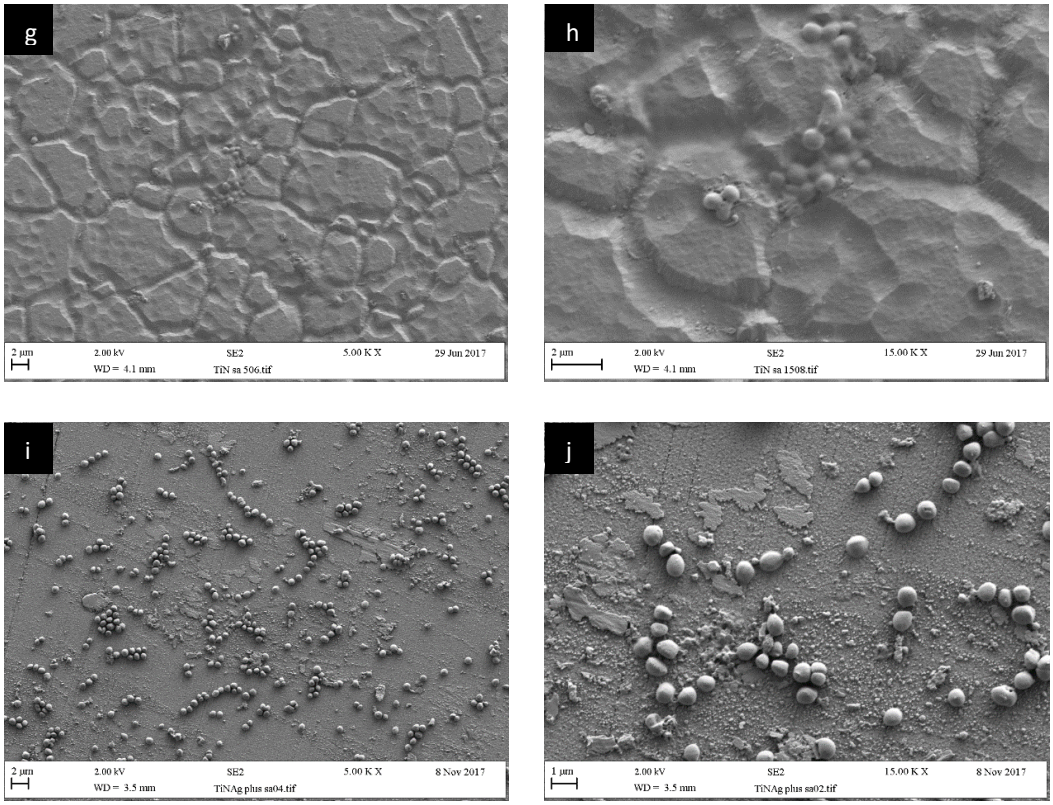


Figure 20 continued: g) 316TiN plus *S. aureus* at x 5,000 magnification, h) x 15,000 magnification, i) TiN/15.03at.%Ag plus *S. aureus* x 5,000 magnification, j) x 15,000. Images gave qualitative assessment of the interaction between the *S. aureus* cells and the macro and micro topographies of the different surfaces.

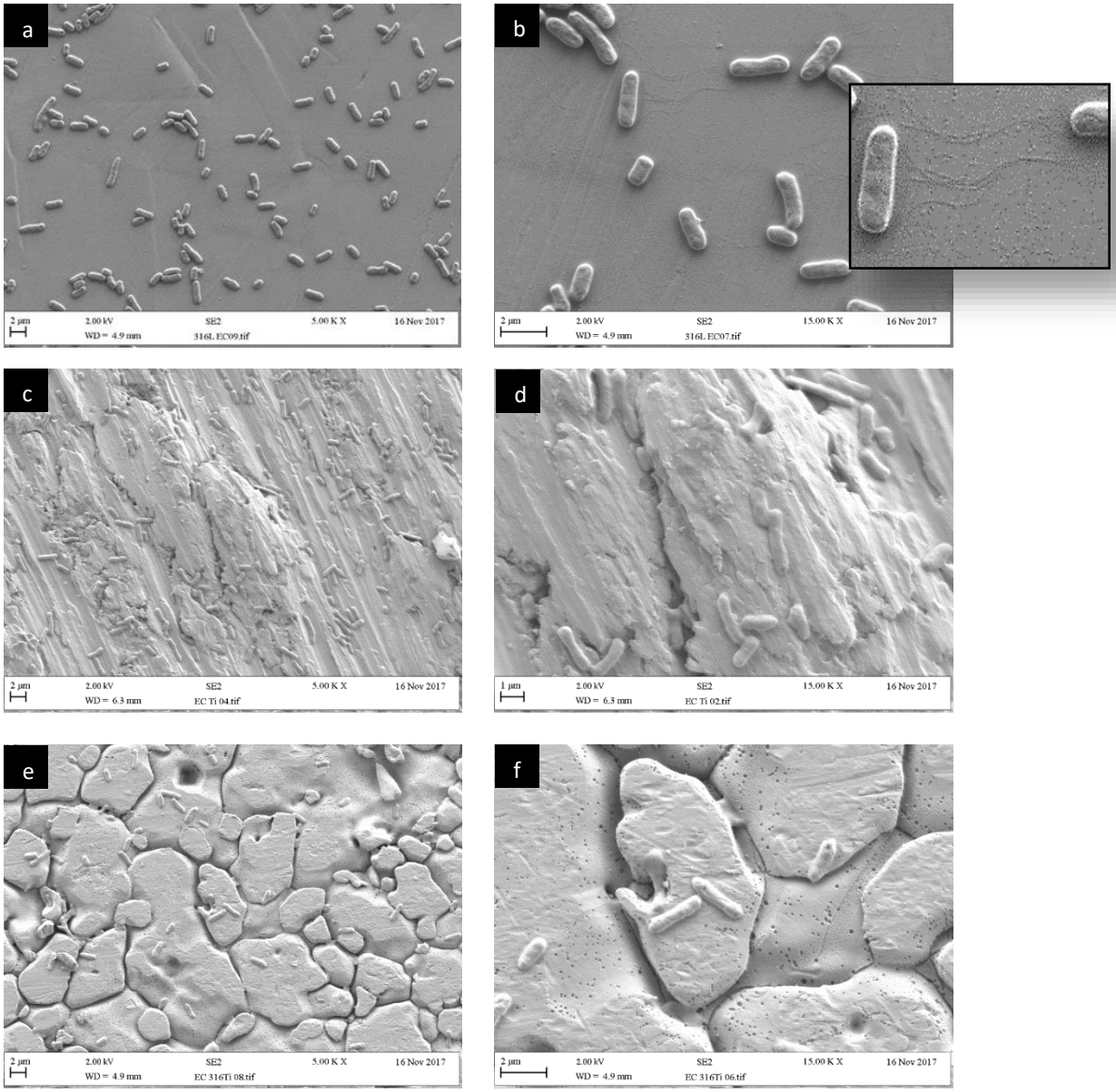


Figure 21: SEM images of a) 316L stainless steel plus *E. coli* at x 5,000 magnification, b) x 15,000 magnification with magnified cut out to demonstrate flagella, c) titanium plus *E. coli* at x 5,000 magnification, d) x 15,000 magnification, e) 316Ti plus *E. coli* at x 5,000 magnification, f) x 15,000 magnification.

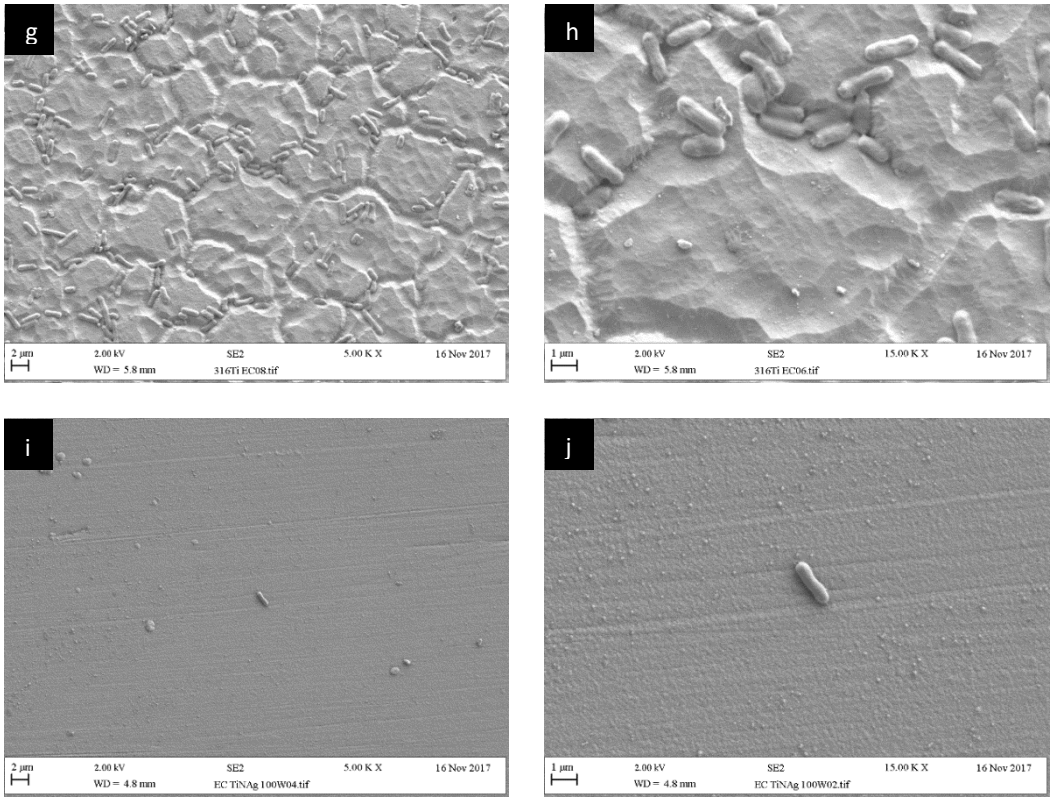


Figure 21 continued: g) 316TiN plus *E. coli* at x 5,000 magnification, h) x 15,000 magnification, i) TiN/15.03at.%Ag plus *E. coli* x 5,000 magnification, j) x 15,000. Images allowed for qualitative assessment of the macro and micro topography of the different substrates.

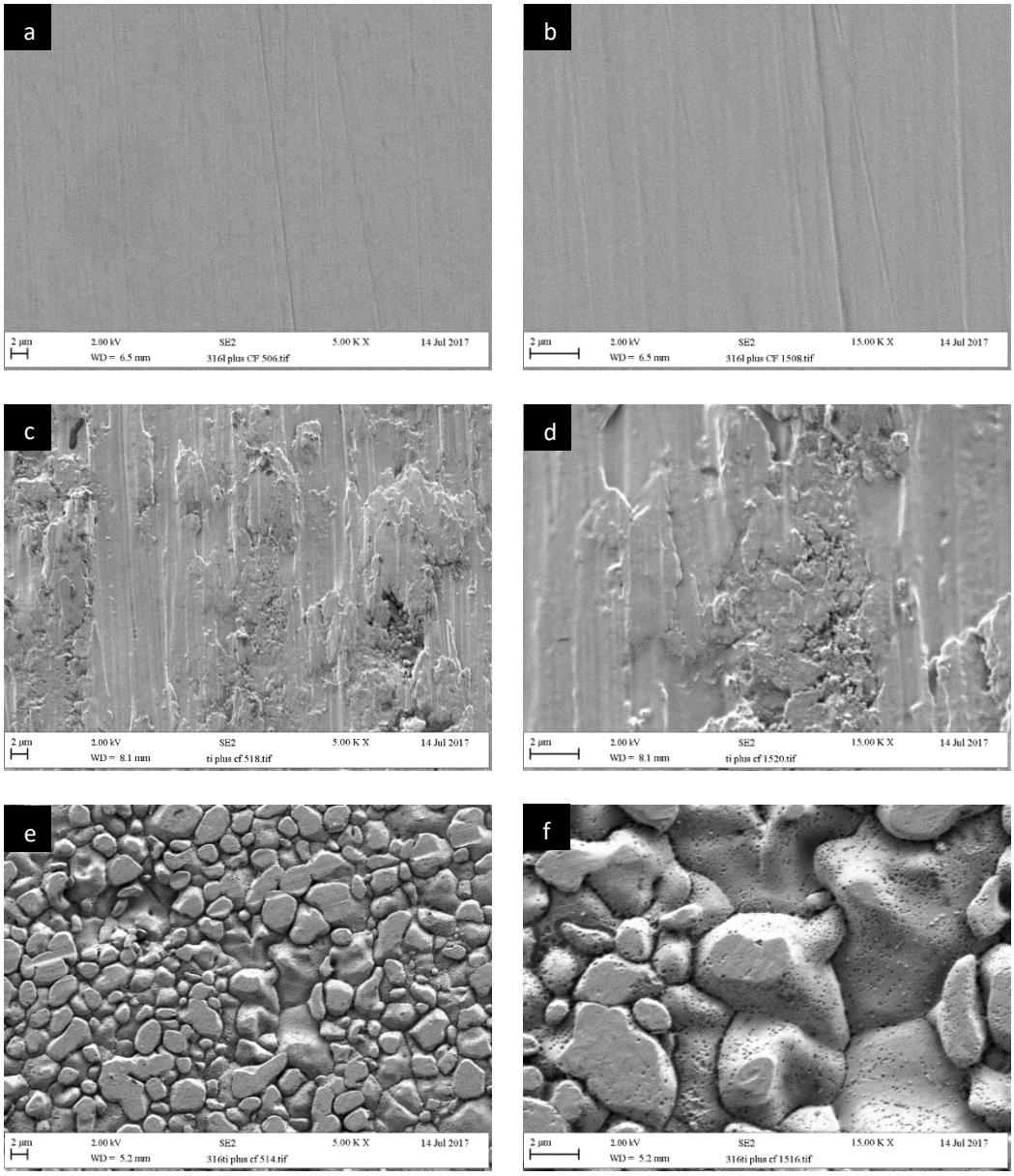


Figure 22: SEM images of the surfaces with 10 % bovine plasma retained; a) 316L stainless steel at x 5,000 magnification, b) x 15,000 magnification, c) titanium at x 5,000 magnification, d) x 15,000 magnification, e) 316Ti at x 5,000 magnification, f) x 15,000 magnification.

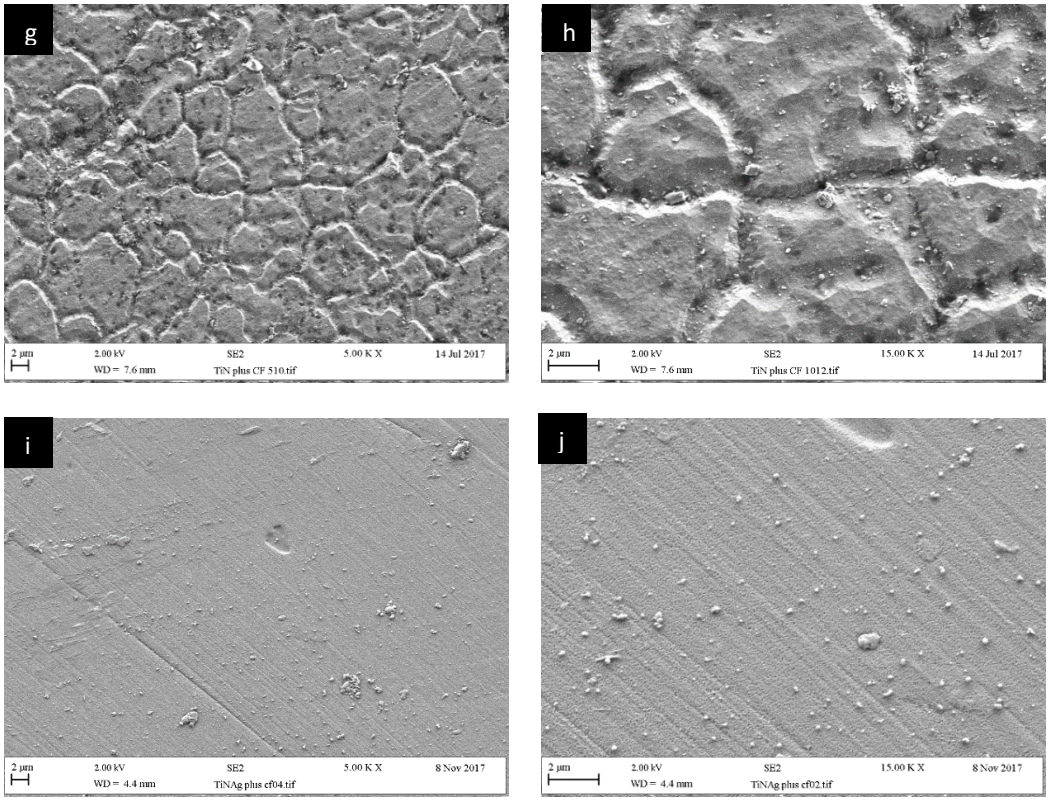


Figure 22 continued: g) 316TiN at x 5,000 magnification, h) x 15,000 magnification, i) TiN/15.03at.%Ag at x 5,000 magnification, j) x 15,000 magnification. Images allowed for qualitative assessment of the macro and micro topographies of the different substrates.

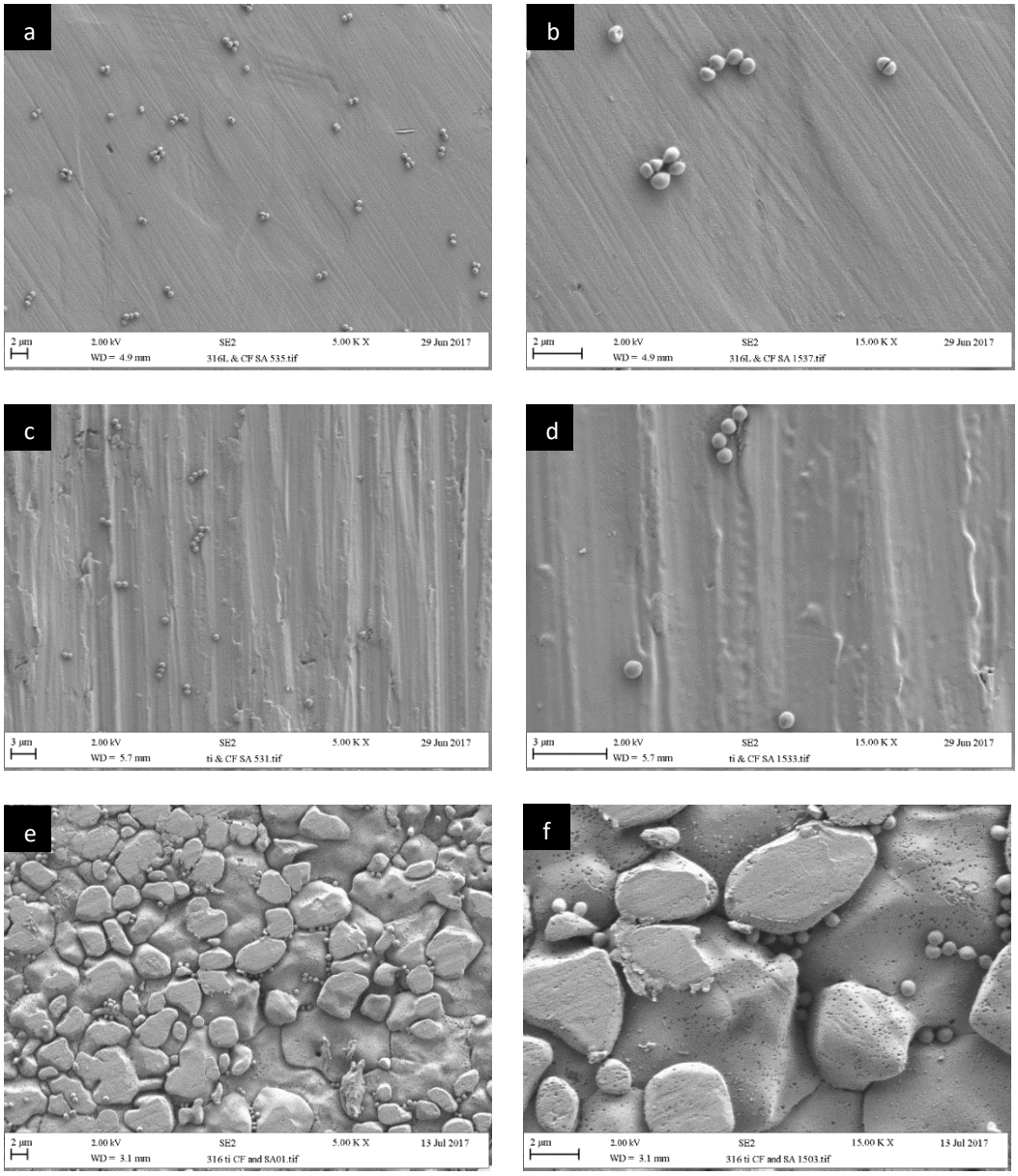


Figure 23: SEM images of the surfaces after retention assays of *S. aureus* in the presence of 10 % bovine plasma; a) 316L stainless steel x 5,000 magnification, b) x 15,000 magnification, c) titanium x 5,000 magnification, d) x 15,000 magnification, e) 316Ti stainless steel x 5,000 magnification, f) x 15,000 magnification.

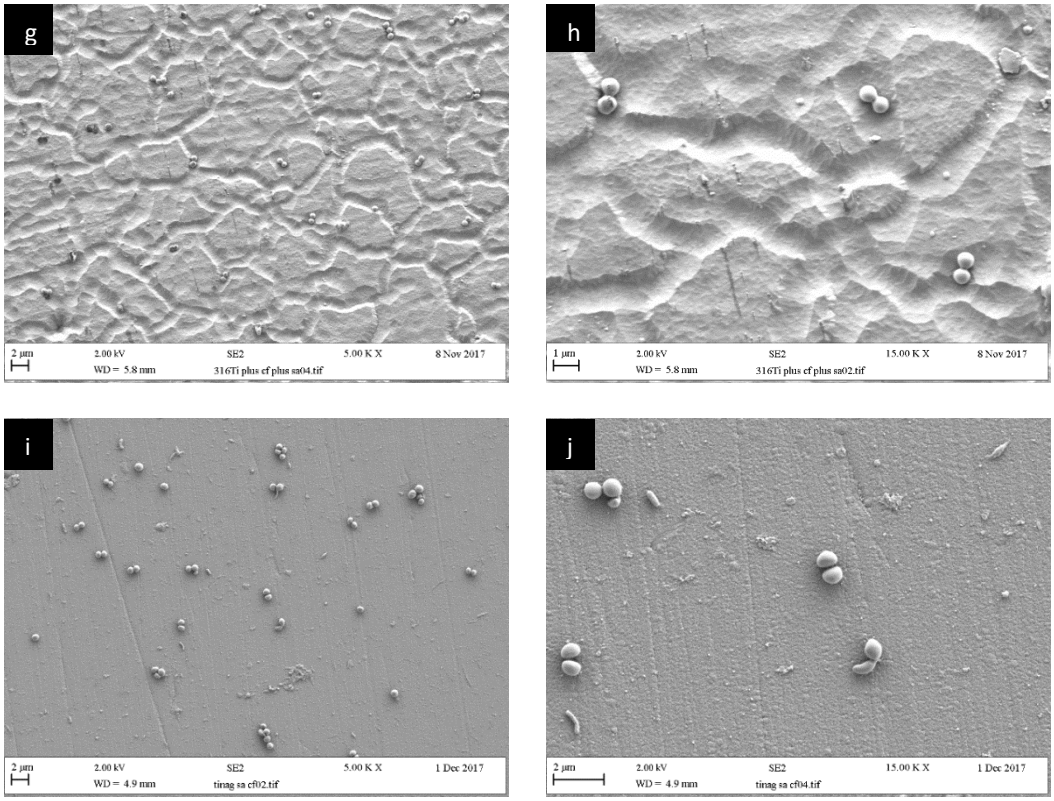


Figure 23 continued: g) 316TiN x 5,000 magnification, h) x 15,000 magnification, i) TiN/15.03at.%Ag x 5,000 magnification, j) x 15,000. Images allow qualitative assessment of the interaction between the *S. aureus* cells with the conditioning film and the macro and micro topographies of the different surfaces.

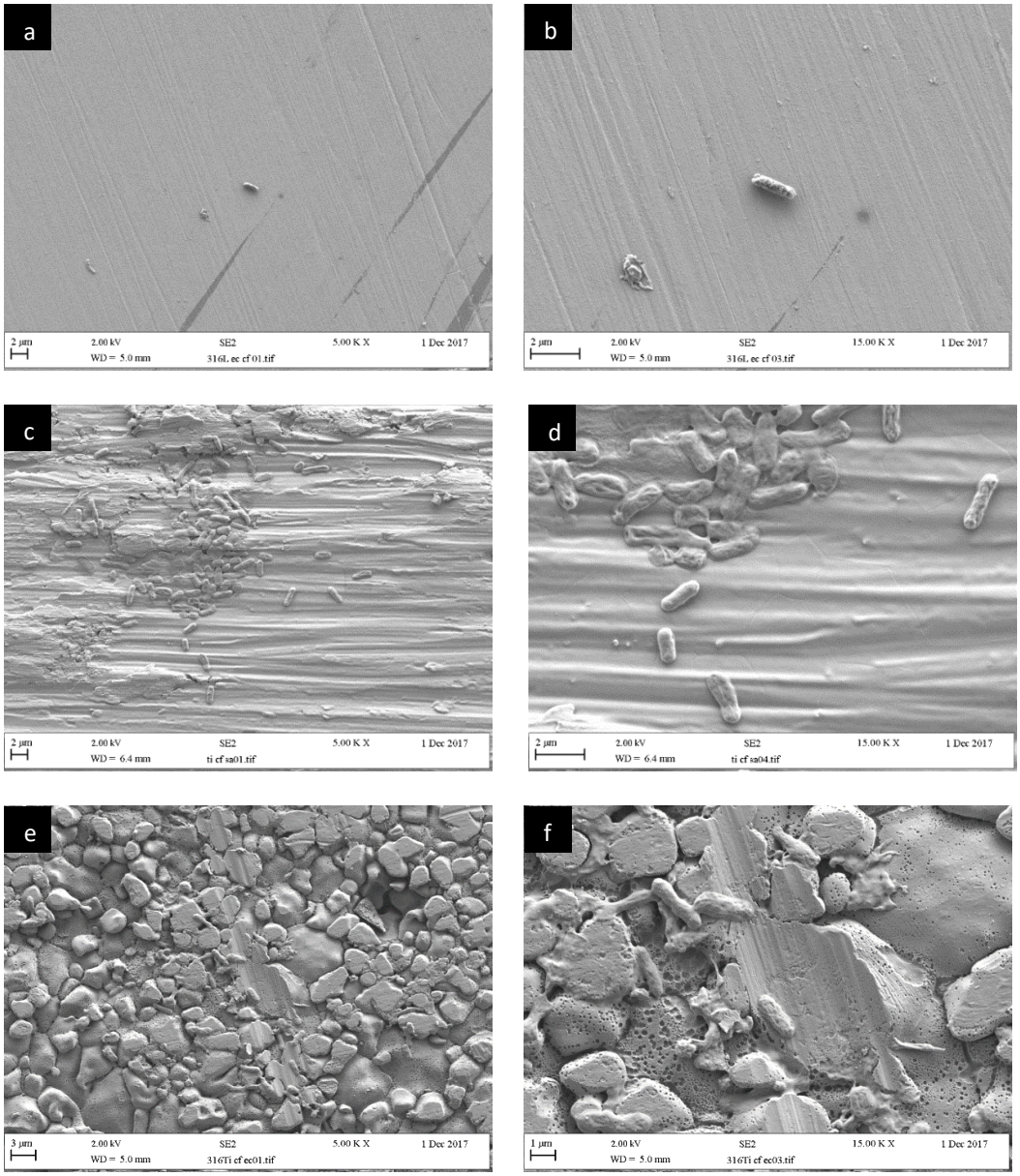


Figure 24: SEM images of the surfaces after retention assays of *E.coli* in the presence of 10 % bovine plasma; a) 316L stainless steel x 5,000 magnification, b) x 15,000 magnification, c) titanium x 5,000 magnification, d) x 15,000 magnification, e) 316Ti stainless steel x 5,000 magnification, f) x 15,000 magnification.

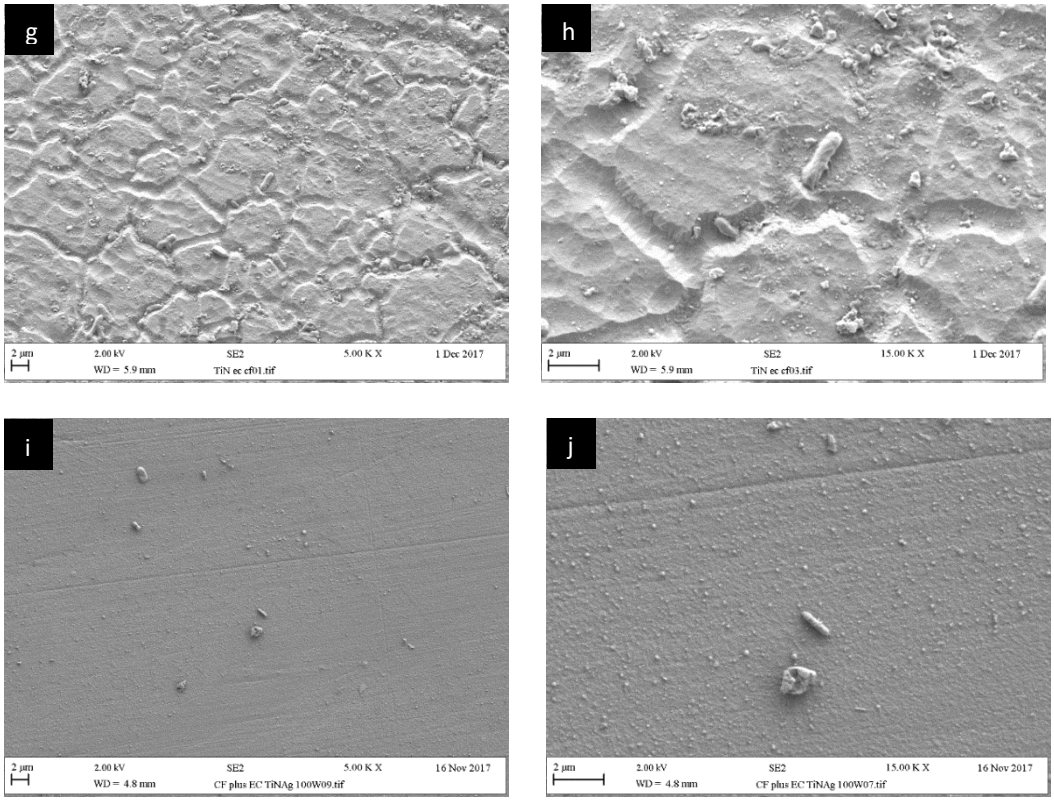


Figure 24 continued: g) 316TiN x 5,000 magnification, h) x 15,000 magnification, i) TiN/15.03at.%Ag x 5,000 magnification, j) x 15,000. Images allow qualitative assessment of the interaction between the *E. coli* cells with the conditioning film and the macro and micro topographies of the different surfaces.

3.3.2 White Light Profilometry

White light profilometry was performed upon the five metal surfaces under the different surface conditioning treatments (pristine, with bacteria, with conditioning film, and with both the bacteria and conditioning film) in order to provide an improved micro resolution of the surface topographies and to quantify their surface roughness over 160 µm x 160 µm.

The scans performed upon the pristine surfaces (Figure 25) allowed for a visual inspection of the metallic surfaces demonstrating the linear striations apparent on the 316L stainless steel, titanium, and TiN/15.03at.%Ag surfaces, and the columnar / pitted appearance of the 316Ti and 316TiN surfaces. The sputter coating process that produced the TiN/15.03ag.%Ag surfaces did not visually alter the topographical appearance of the underlying 316L stainless steel. Determination of the S_a values (Figure 31) demonstrated the differences in the roughness of each surfaces. The 316L stainless steel and the TiN/15.03at.%Ag surfaces were the least rough (13.98 nm ± 0.7 nm and 30.79 nm ± 3.3 nm respectively), whilst the 316Ti stainless steel and 316TiN surfaces were the roughest (410.63 nm ± 41.2 nm, and 377.97 nm ± 25.7 nm respectively). Statistical analysis of the surface roughness demonstrated that the least rough surfaces, 316L stainless steel and TiN/15.03at.%Ag, were significantly less rough than the titanium, 316Ti stainless steel, and 316TiN (all p = < 0.00) but not significantly different from one another (p = 0.870) demonstrating that the addition of the sputter coated TiN/15.03at.%Ag had not significantly altered the roughness of the metal. The roughest surface, titanium, was significantly rougher than 316L stainless steel, 316TiN and TiN/15.03at.Ag but not significantly rougher than 316Ti (p = 0.339). The roughness of 316TiN was significantly different to all the other metal surfaces.

Analysis of the surfaces in the presence of the bacterial species (Figure 26 and Figure 27) allowed the assessment of any affects that the addition of the bacteria had upon the roughness parameters of the metal surfaces. The scans demonstrated that the original features of the pristine surfaces had not been visually changed in the presence of either *S. aureus* or *E. coli.* The only visible differences were upon the 316L stainless steel and TiN/15.03at.%Ag surfaces where it was possible to see

the cells of *S. aureus* and *E. coli* retained upon the surfaces, possibly due to the reduced roughness and fewer surface features in comparison to the titanium and 316Ti surfaces.

Assessment of the roughness parameters of the surfaces post retention assays demonstrated that the surfaces followed the same trend in roughness as seen upon the pristine surface. The roughest surface continued to be the titanium (*S. aureus* S_a = 394.85 nm ± 12.10 nm, *E. coli* S_a = 441.11 nm ± 19.26 nm), followed by 316Ti stainless steel (S_a = 384.45 nm ± 11.45 nm and 359.31 nm ± 5.73 nm respectively). However, when assessing the statistical significance of the surface roughness between titanium and 316Ti stainless steel, it was noted that with the addition of *E. coli,* titanium became significantly rougher than the 316Ti (p = 0.01), but that with the addition of *S. aureus* titanium continued not to be significantly rougher than 316Ti (p = 0.961). The least rough metals continued to be the 316L stainless steel (S_a = 13.22 nm ± 0.66 nm, and 31.30 nm ± 3.73 nm) and TiN/15.03at.%Ag (S_a = 42.55 nm ± 2.37 nm, and 49.54 nm ± 5.35 nm). Statistical analysis of the differences between these two surfaces once retained with the bacterial species indicated that there was now a significance between the roughness of the surfaces (p = 0.00) that was not seen on the pristine surfaces.

Analysis of the effect that the different bacterial species had upon the individual metal surfaces demonstrated that the addition *E. coli* produced a significant increase in the roughness of 316L stainless steel (p = < 0.00) and the TiN/15.03at.%Ag surface (p = 0.010), but this was not demonstrated on the other surfaces. The addition of *S. aureus* increased the roughness of the 316TiN surface (p = 0.009) but no other significant changes were determined.

Determination of the roughness parameters produced by the differences between the bacterial species to the individual metal surfaces did produce statistically significant differences to the 316L stainless steel (p = < 0.01) and 316TiN (p = 0.021), but not for titanium (p = 0.127), 316Ti stainless steel (p = 0.488), or TiN/15.03at.%Ag (p = 0.596) where the variation produced by the different species was not high enough to be statistically significant.

Assessment of the surfaces in the presence of the 10 % bovine plasma conditioning film demonstrated that the conditioning film had not altered the surface features from those of the pristine surfaces. Determination of the roughness parameters (S_a) in the presence of the conditioning film demonstrated that the surfaces followed the same trend as seen upon the pristine surface. The roughest surface continued to be the titanium (S_a = 441.91 ± 0.84 nm), followed by the 316Ti (S_a = 369.90 ± 8.9 nm) and 316TiN (S_a = 178.55 ± 5.9 nm), and the least rough was the 316L stainless steel (S_a = 15.09 ± 0.84 nm) and the TiN/15.03at.%Ag (S_a = 43.84 ± 3.63 nm) surfaces. Statistical analysis demonstrated that all of the surfaces, except for the least rough 316L and TiN/15.03at.%Ag surfaces, were significantly different from one another but not from their original roughness parameters recorded from the pristine surfaces. This demonstrated that the addition of the 10 % bovine plasma conditioning film had not significantly altered the roughness parameters of the surfaces.

Assessment of the surfaces after retention assays with both the bacterial species with the 10 % bovine plasma conditioning film allowed assessment of how the addition of the retained bacteria with conditioning film had affected the surface roughness parameters (S_a). The scans performed demonstrated that when compared to the previous conditioning parameters, the visual appearance of the surface features had not changed with the addition of both the bacteria and conditioning film.

Measurement of the roughness parameters (S_a) of the surfaces after retention assays with the bacterial species and the conditioning film demonstrated that the surfaces continued to follow the same trends in roughness as seen on the pristine surfaces, the surfaces with bacteria, and the surfaces with conditioning film. The titanium surfaces were the roughest (*S. aureus* S_a = 461.61 nm ± 16.47 nm and *E. coli* S_a = 401.56 nm ± 13.88 nm), followed by the 316Ti surfaces (S_a = 382.02 nm ± 15.30 nm and 372.27 nm ± 11.35 nm) and 316TiN surfaces (S_a = 230.01 nm ± 13.19 nm and 137.96 nm ± 4.66 nm). The 316L stainless steel (S_a = 13.22 nm ± 0.65 nm and 31.30 nm ± 3.73 nm) and TiN/15.03at.%Ag (S_a = 42.55 nm ± 2.37 nm and 49.53 nm ± 5.35 nm) surfaces continued to be the least rough.

Analysis of the differences created by the bacterial species demonstrated that the surfaces with the *S. aureus* and 10 % bovine plasma retained were rougher than those with the *E. coli* and 10 % bovine plasma retained, significantly so for the titanium and 316TiN surfaces (p = 0.027 and p = < 0.00 respectively). This was in contrast to the surfaces with bacteria alone were the 316L stainless steel, titanium and TiN/15.03at.%Ag surfaces with the *E. coli* retained were rougher than when *S. aureus* was retained to them. Due to this change, statistical analysis of the roughness' between the surfaces demonstrated that the addition of the *S. aureus* with 10 % bovine plasma to the titanium made the surface significantly rougher than the 316Ti surfaces (p = < 0.00), whilst the addition of the *E. coli* with 10 % bovine plasma did not (p = 0.135). All of the other surfaces, no matter which bacterial species was added, were significantly different to one another (all under p = 0.001) with the exception of the 316L stainless steel and TiN/15.03at.%Ag surfaces (p = 0.174 and p = 0.587 respectively).

When the results were compared to those of the surfaces with bacteria, the surface roughness measurements demonstrated that the titanium surface became significantly less rough (p = 0.007) with the addition of the *S. aureus* with 10 % bovine plasma conditioning film. Also, the 316L stainless steel and 316TiN surfaces became significantly less rough with the addition of the *E. coli* with 10 % bovine plasma conditioning film (p = 0.029 and p = 0.016 respectively).

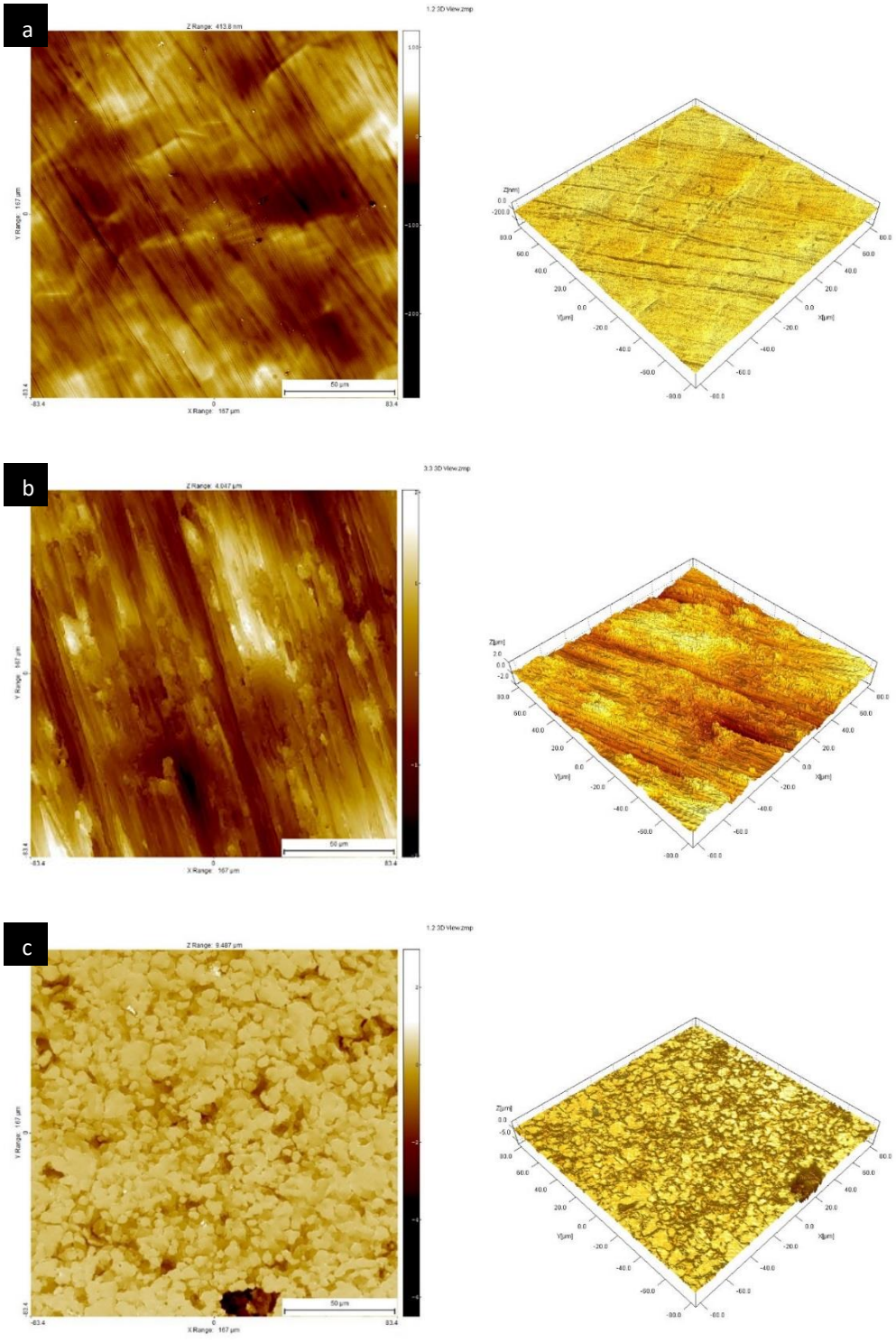


Figure 25: White light profilometry pictures demonstrating the surface topography in 2D and 3D of a) 316L stainless steel, b) titanium c) 316Ti stainless steel.

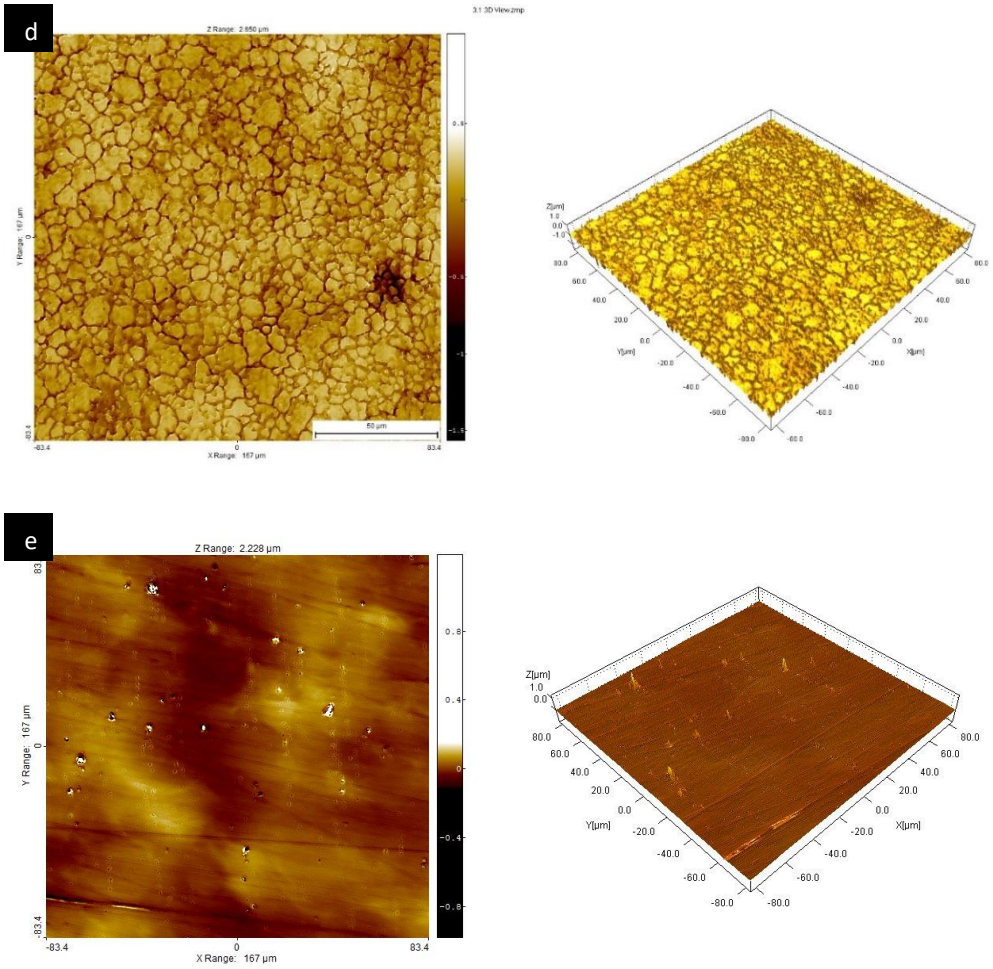


Figure 25 continued: White light profilometry pictures demonstrating the surface topography in 2D and 3D of d) 316TiN, and e) TiN/15.03at.%Ag surfaces. Note the sharp peaks visible on the TiN/15.03at.%Ag surfaces (Figure 19e) demonstrating the silver particles protruding from surface.

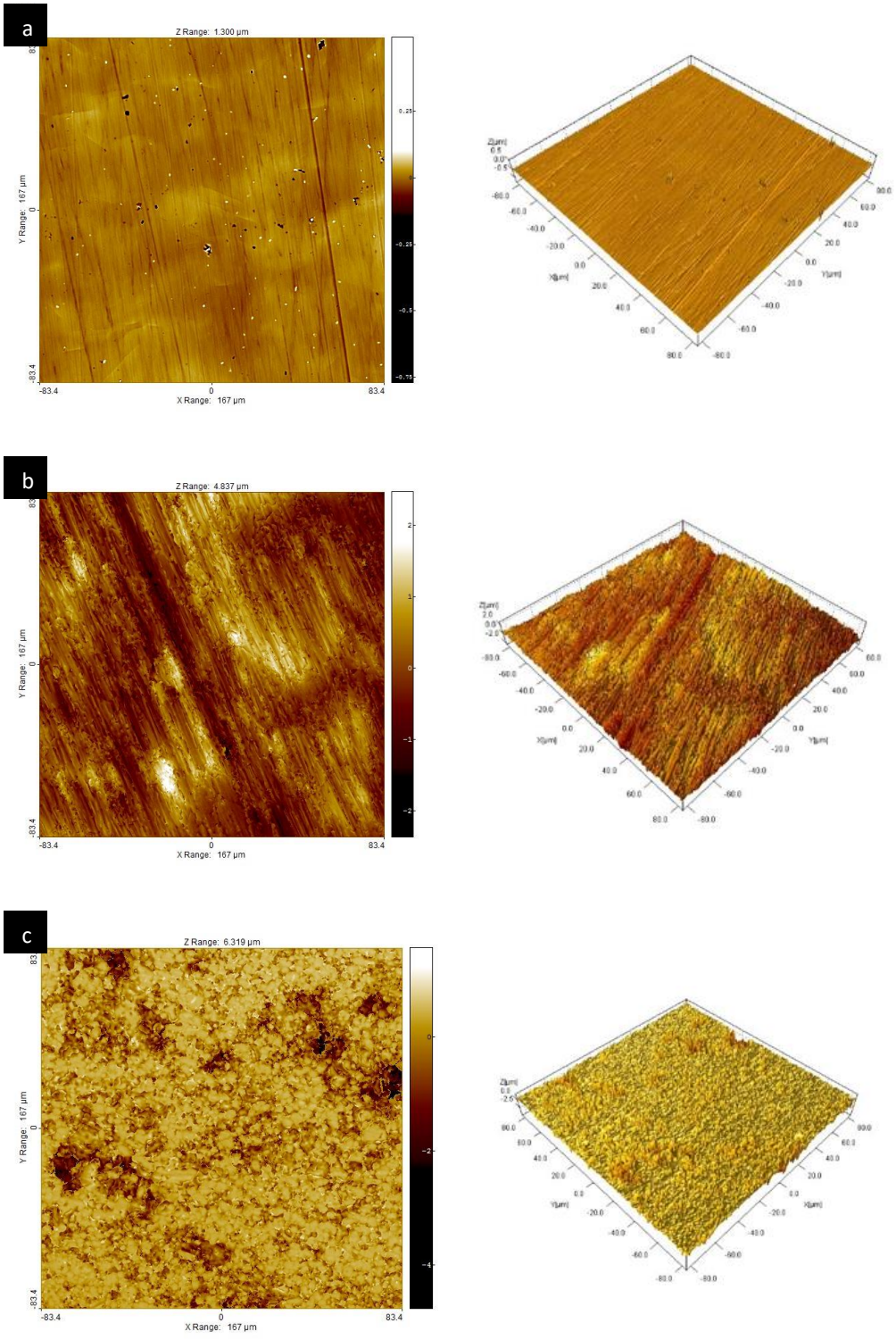


Figure 26: White light profilometry pictures demonstrating the surface topography of the surfaces in 2D and 3D after retention assays of *S. aureus* on a) 316L stainless steel, b) titanium, c) 316Ti.

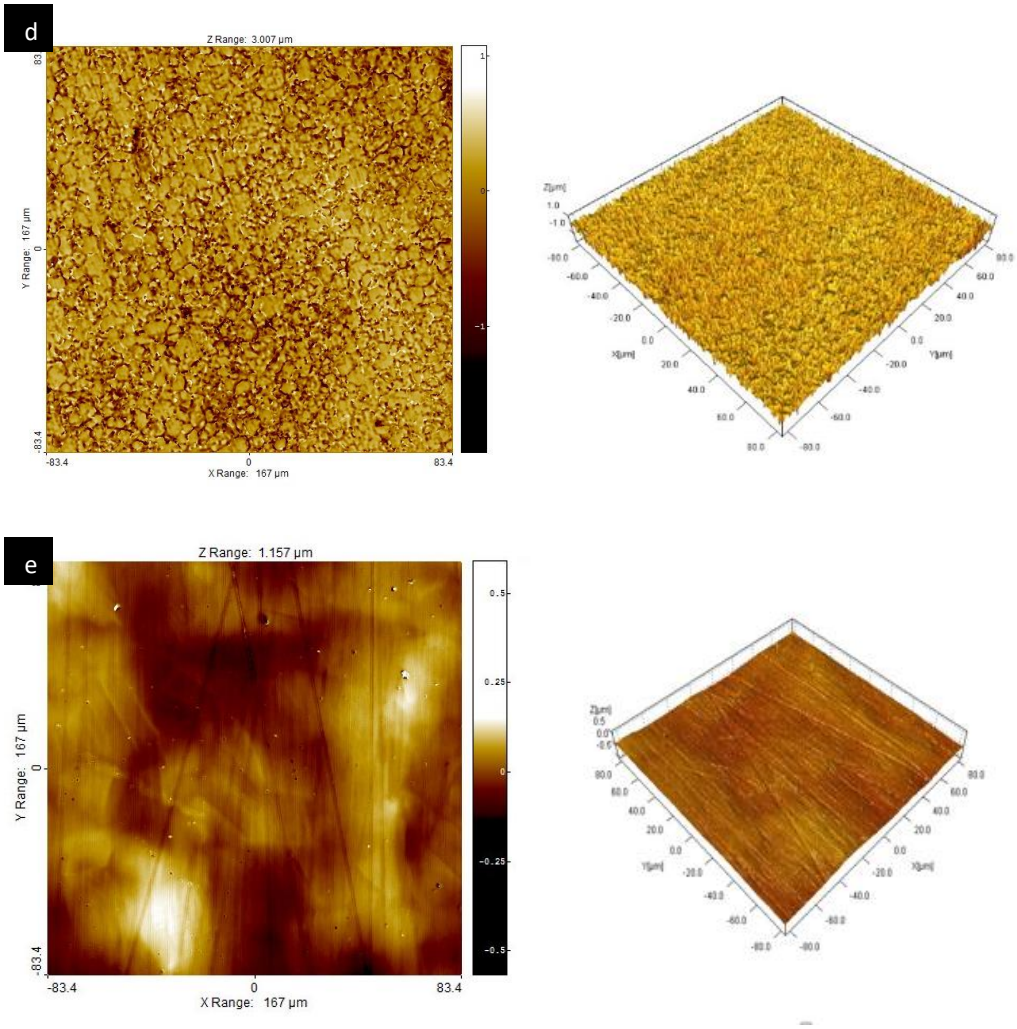


Figure 26 continued: White light profilometry pictures demonstrating the surface topography of the surfaces in 2D and 3D after retention assays of *S. aureus* on d) 316TiN, and e) TiN/15.03at.%Ag surfaces.

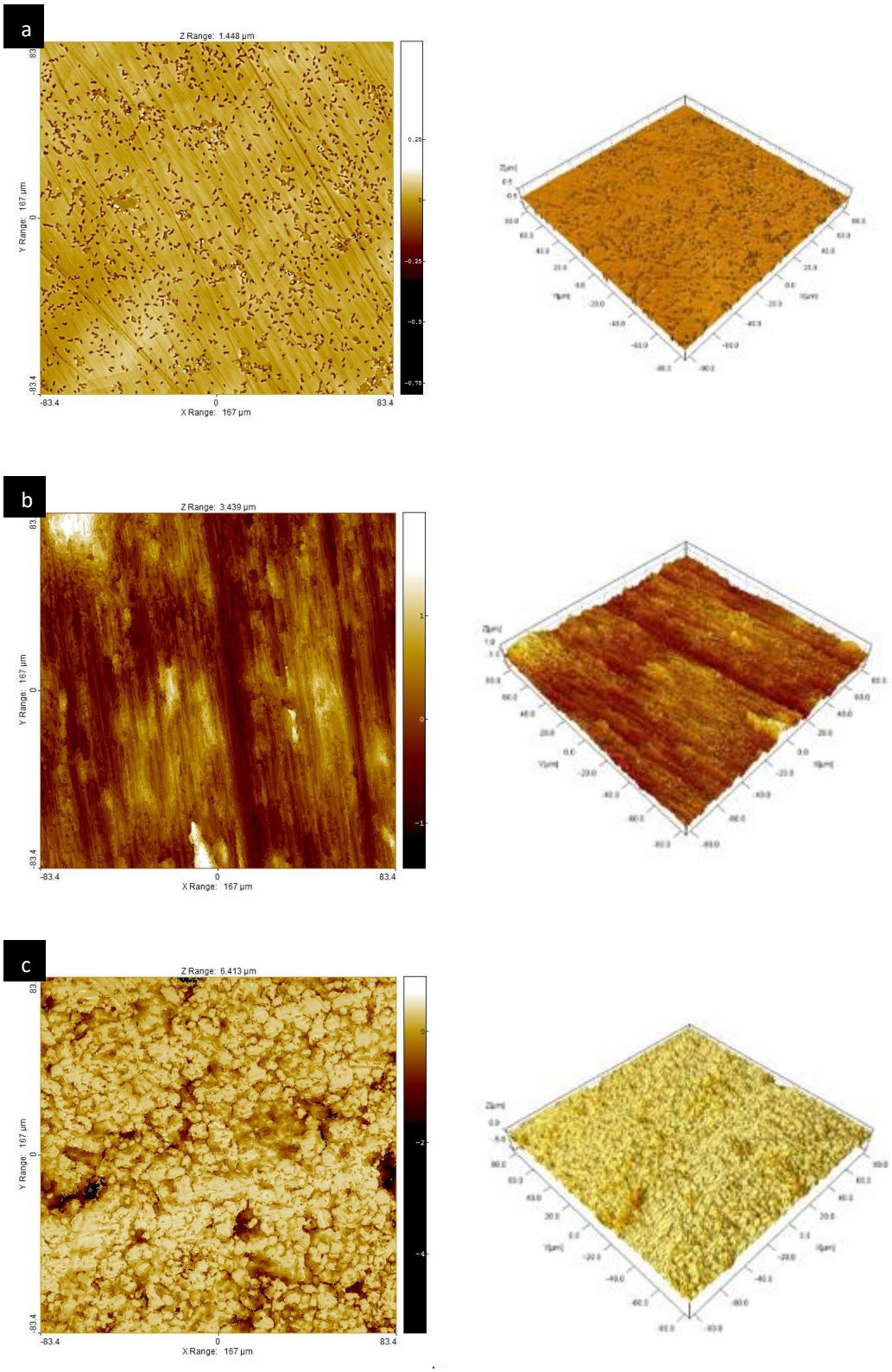


Figure 27: White light profilometry pictures demonstrating the surface topography of the surfaces in 2D and 3D after retention assays of *E. coli* on a) 316L stainless steel, b) Titanium, c) 316Ti.

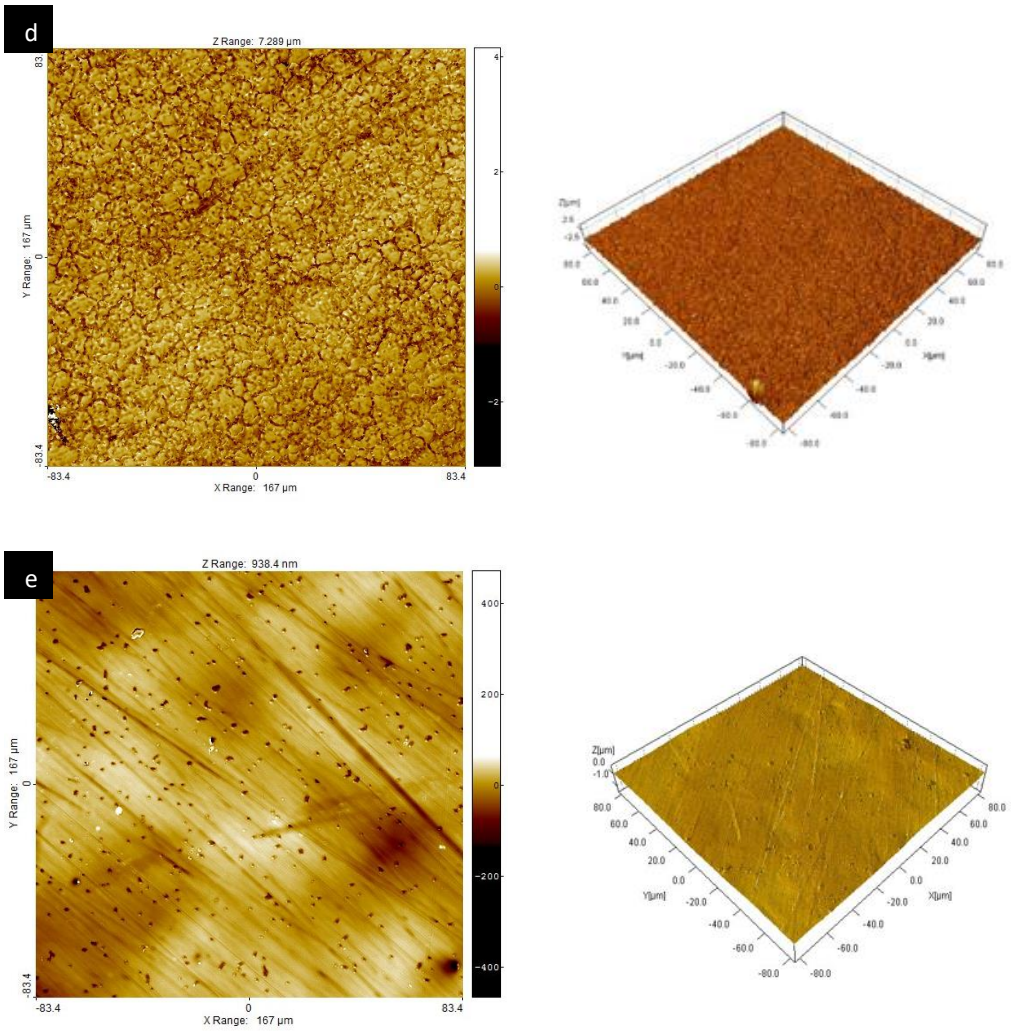


Figure 27 continued: White light profilometry pictures demonstrating the surface topography of the surfaces in 2D and 3D after retention assays of *E. coli* on d) 316TiN, and e) TiN/15.03at.%Ag surfaces.

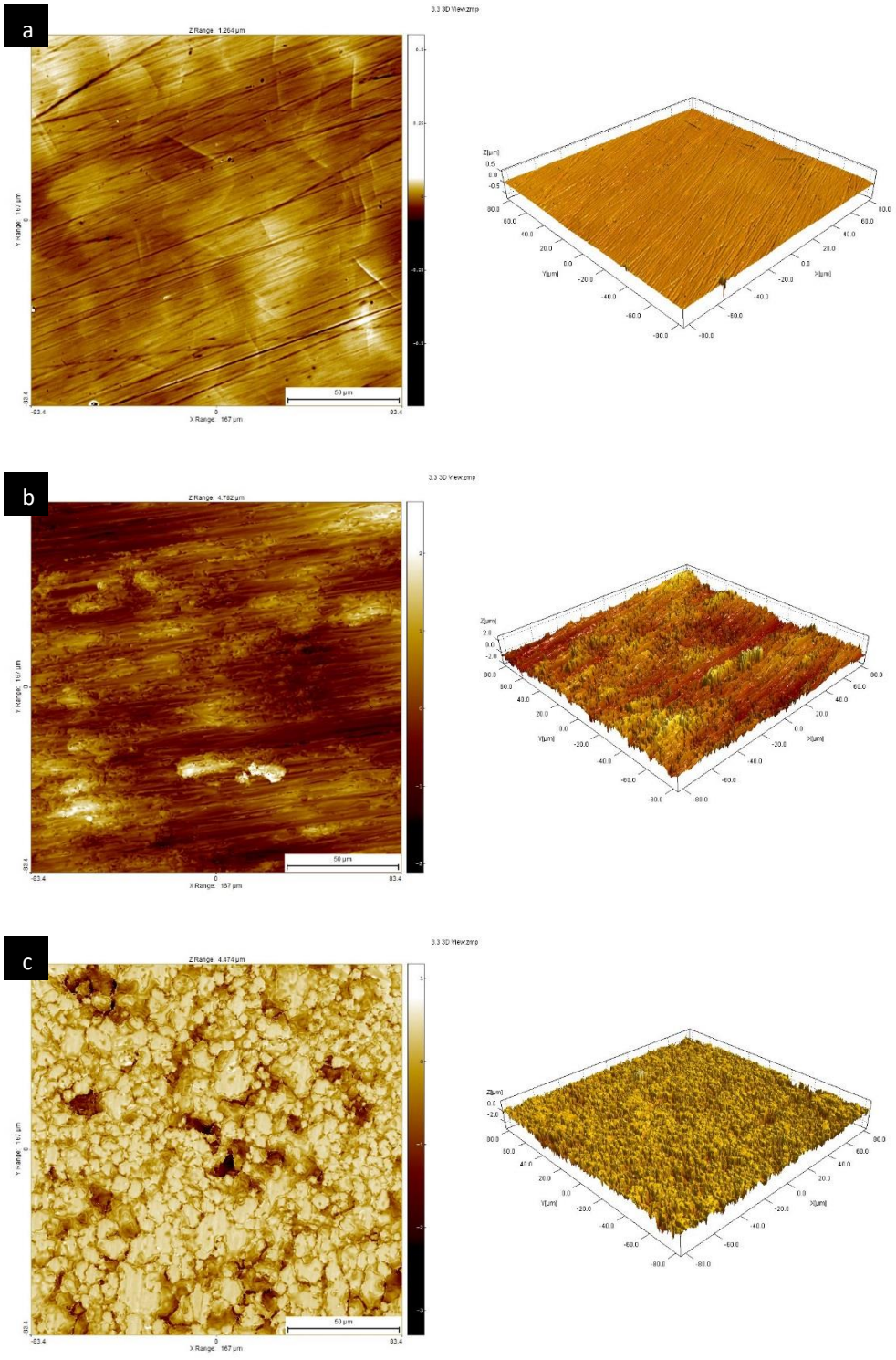


Figure 28: White light profilometry pictures demonstrating the surface topographies in 2D and 3D of the surfaces post retention assays of 10% bovine plasma: a) 316L stainless steel, b) titanium, c) 316Ti stainless steel.

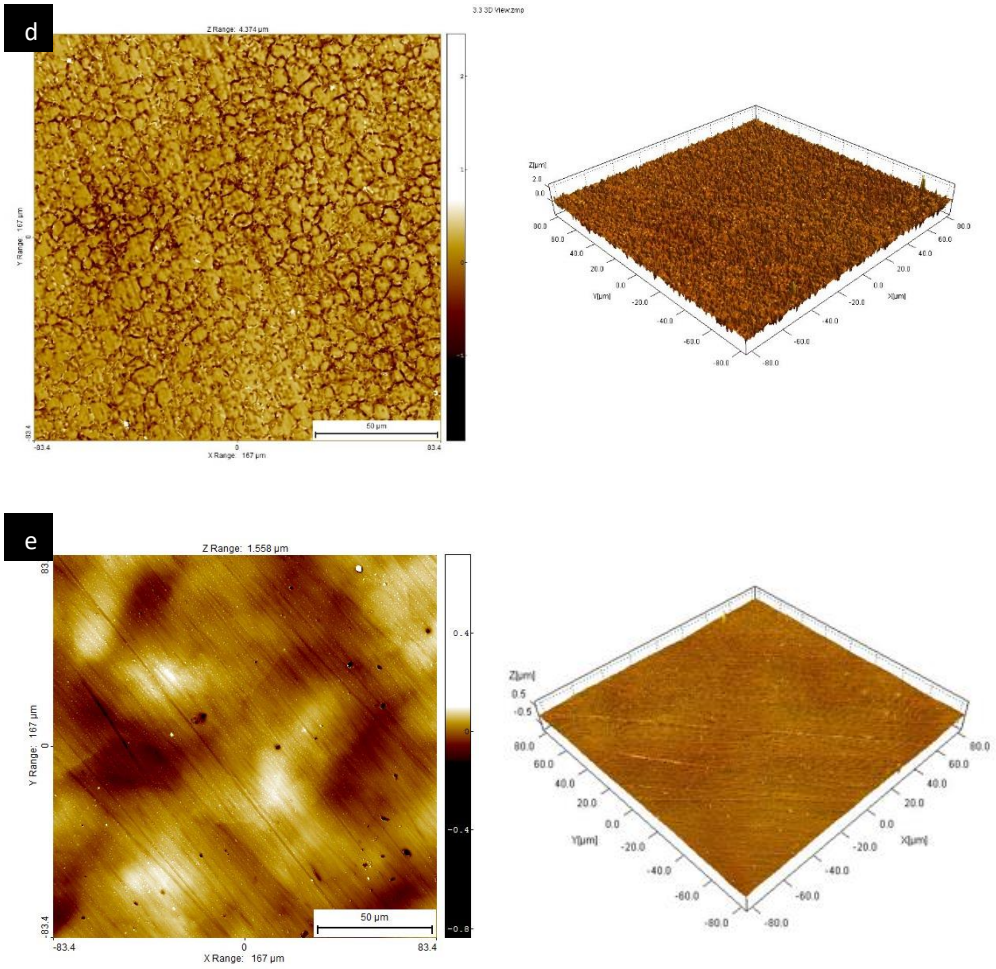


Figure 28 continued: White light profilometry pictures demonstrating the surface topographies in 2D and 3D of the surfaces post retention assays of 10% bovine plasma: d) 316TiN, and e) TiN/15.03at.%Ag surfaces.

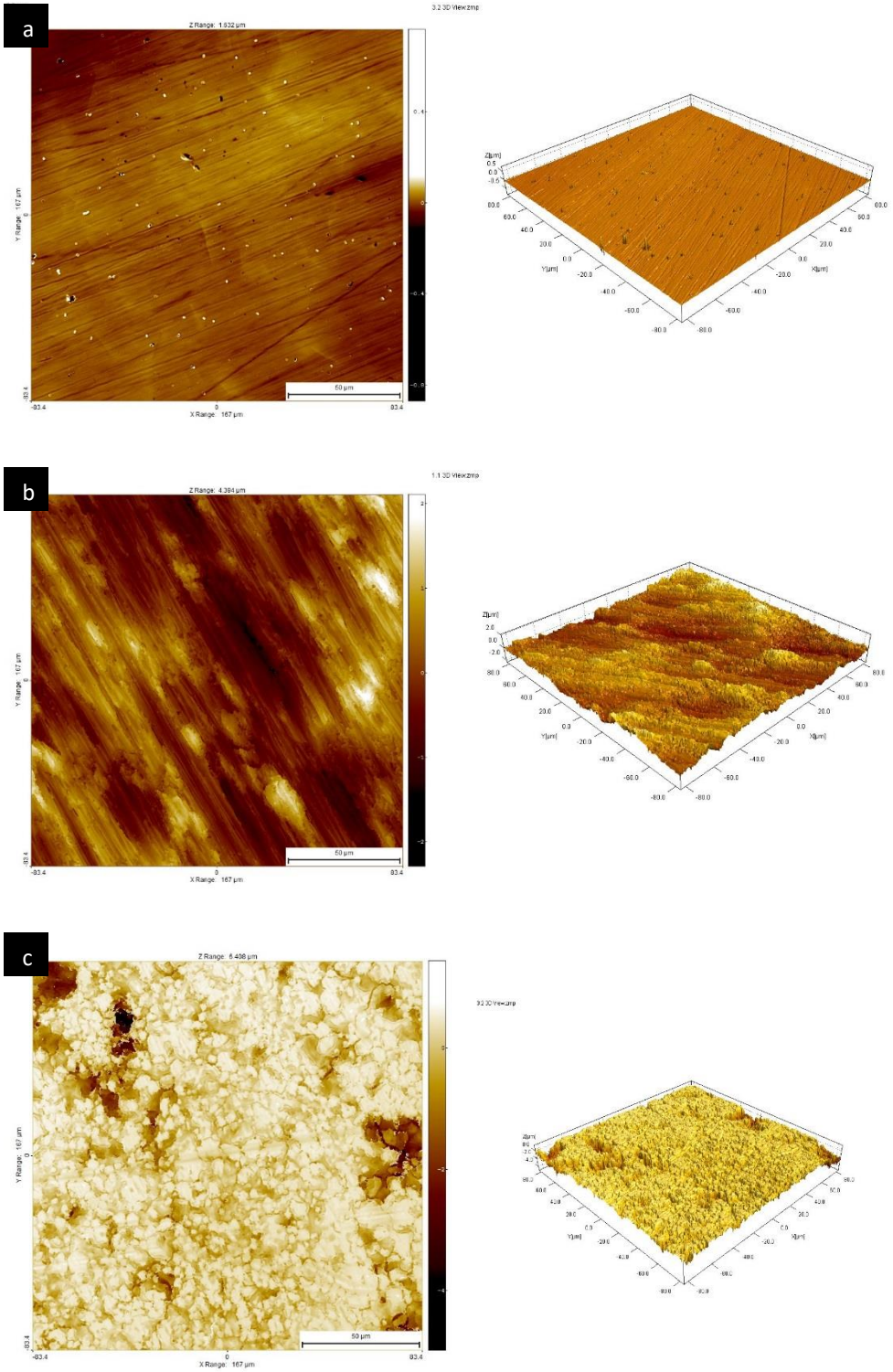


Figure 29: White light profilometry pictures demonstrating the topography of the surfaces in 2D and 3D after retention assays of *S. aureus* with 10 % bovine plasma on a) 316L stainless steel, b) titanium, c) 316Ti.

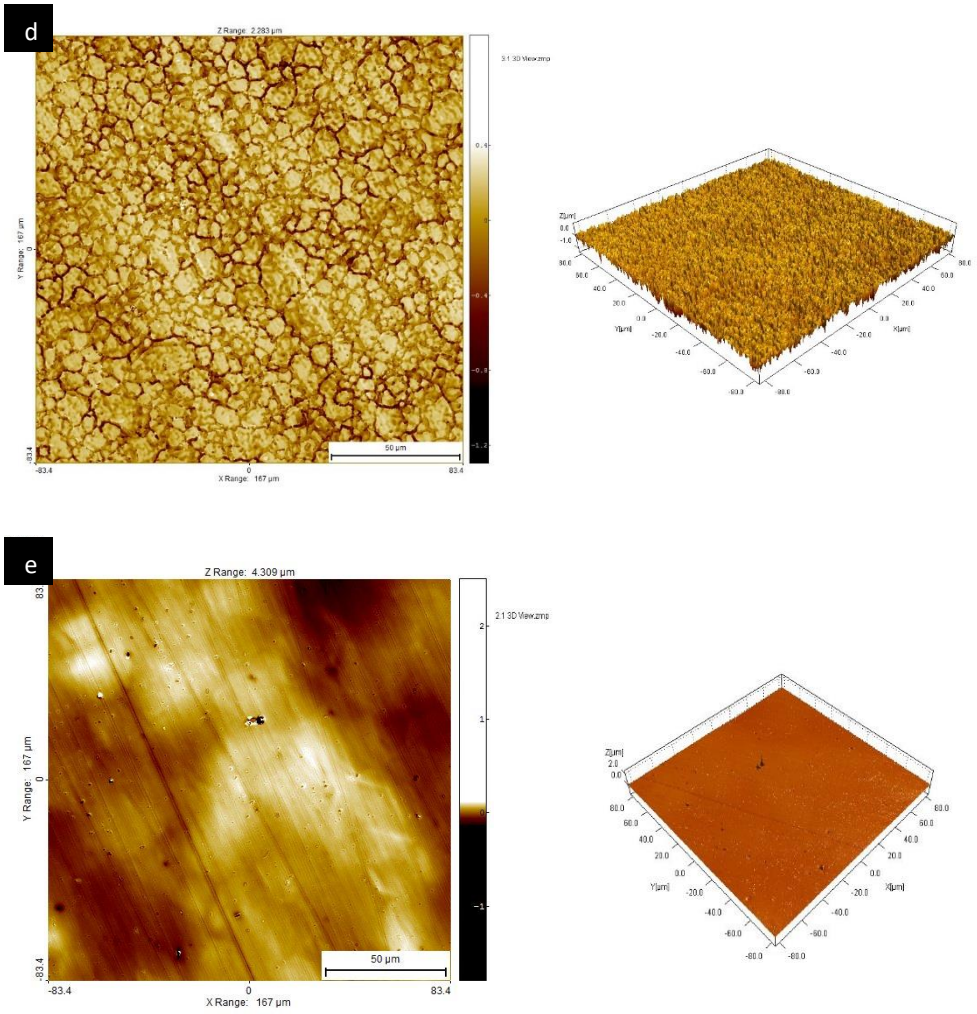


Figure 29 continued: White light profilometry pictures demonstrating the topography of the surfaces in 2D and 3D after retention assays of *S. aureus* with 10 % bovine plasma on d) 316TiN, and e) TiN/15.03at.%Ag surfaces.

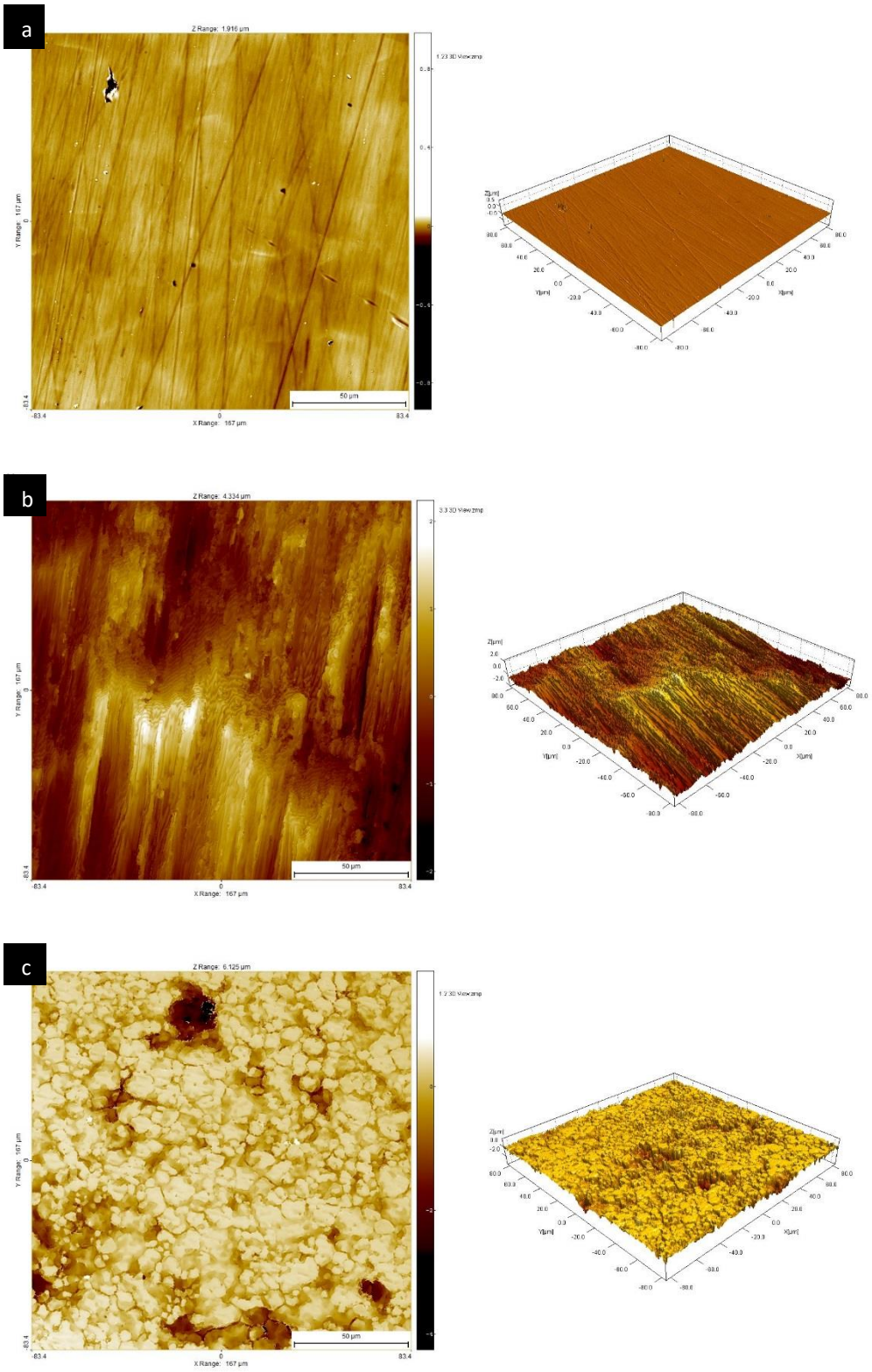


Figure 30: White light profilometry pictures demonstrating the topography of the surfaces in 2D and 3D after retention assays of *E. coli* with 10 % bovine plasma on a) 316L stainless steel, b) titanium, c) 316Ti.

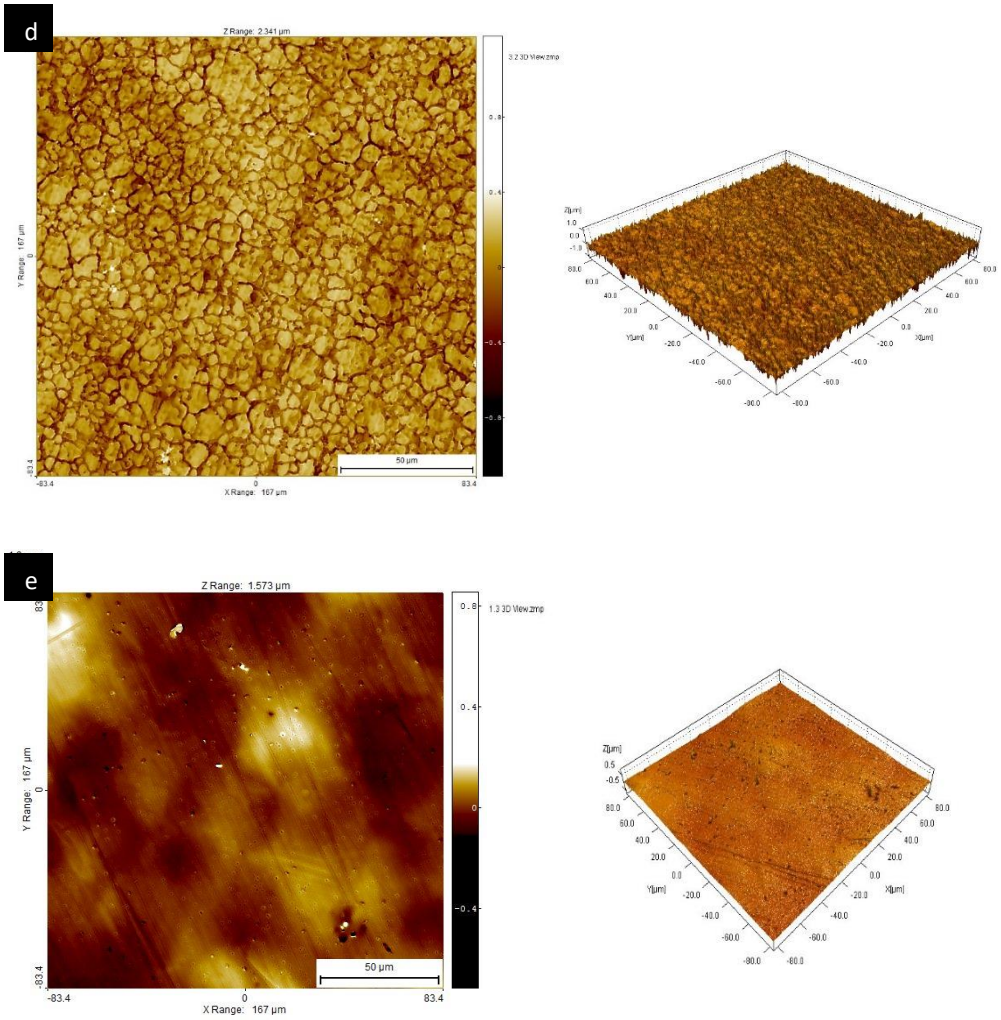


Figure 30 continued: White light profilometry pictures demonstrating the topography of the surfaces in 2D and 3D after retention assays of *E. coli* with 10 % bovine plasma on d) 316TiN, and e) TiN/15.03at.%Ag surfaces.

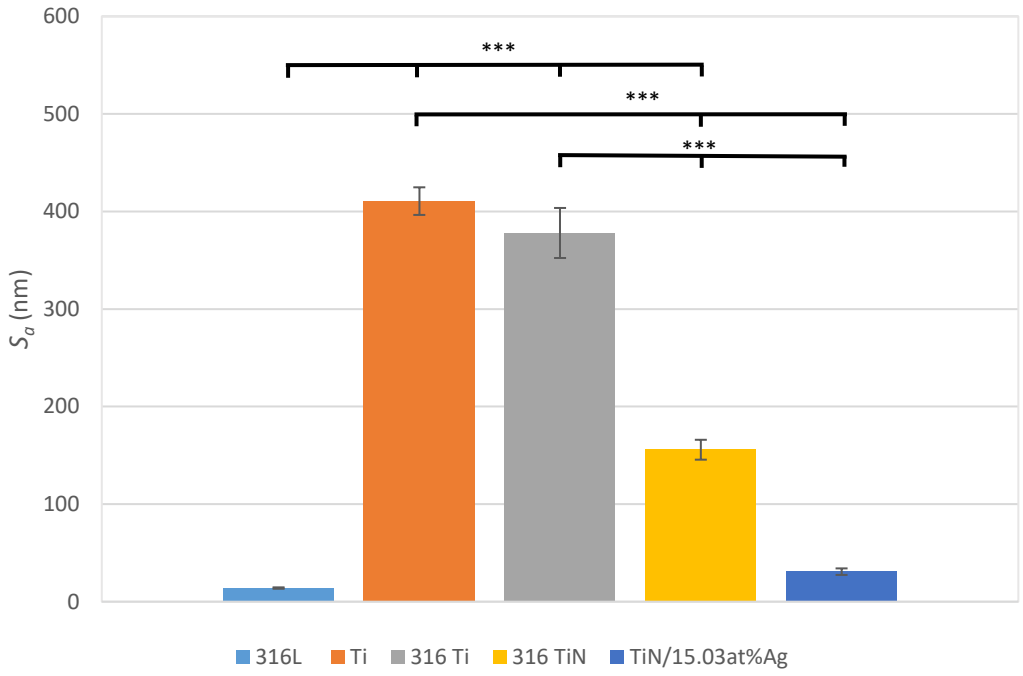


Figure 31: S_a values obtained by white light profilometry of the five substrates; 316L stainless steel, titanium, 316Ti stainless steel, 316TiN, and TiN/15.03at.%Ag. The S_a values demonstrate the differences in the roughness between the surfaces ± standard error (n = 18). * = p = < 0.05, ** = p = < 0.01, *** p = < 0.001.

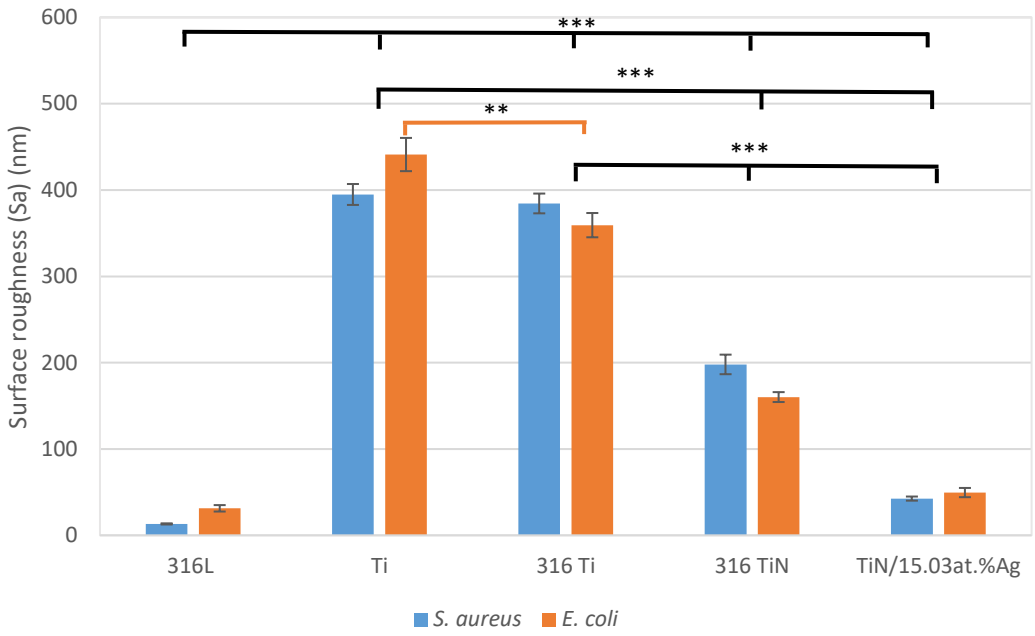


Figure 32: S_a values obtained by white light profilometry of the five substrates; 316L stainless steel, Ti, 316Ti stainless steel, 316TiN, and TiN/15.03at.%Ag after a retention assays of *S. aureus* and *E. coli* (n = 18). * = p = < 0.05, ** = p = < 0.01, *** p = < 0.001. The orange error bar demonstrates statistical difference only in *E. coli* retained surfaces.

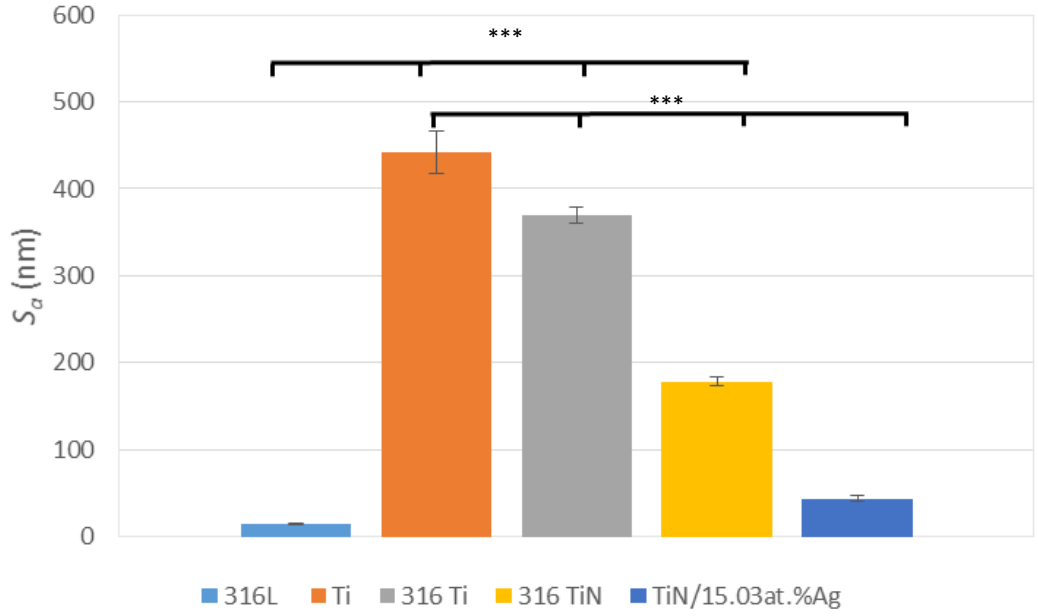


Figure 33: S_a values obtained by white light profilometry of the five substrates post retention assay of 10 % bovine plasma; 316L stainless steel, titanium, 316Ti stainless steel, 316TiN, and TiN/15.03at.%Ag (n = 18). * = p = < 0.05, ** = p = < 0.01, *** p = < 0.001.

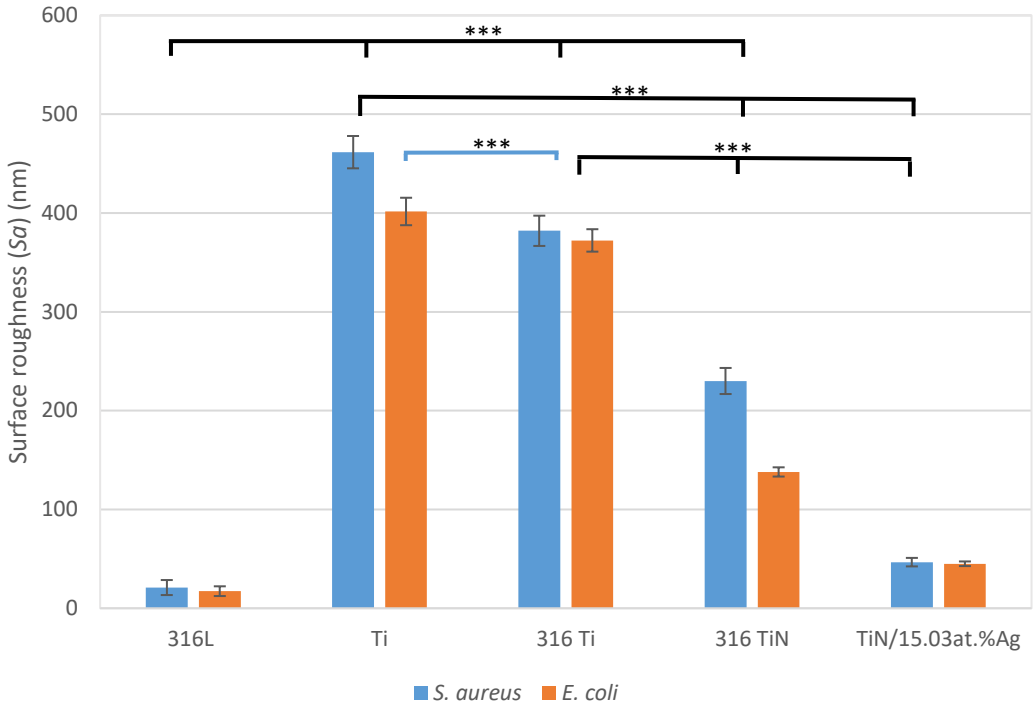


Figure 34: S_a values obtained by white light profilometry of the five substrates; 316L stainless steel, titanium, 316Ti stainless steel, 316TiN, and TiN/15.03at.%Ag after a retention assays of *S. aureus* and *E. coli* with 10 % bovine plasma (n = 18). * = p = < 0.05, ** = p = < 0.01, *** p = < 0.001. Note the blue bar demonstrating that the statistical significance is related to the *S. aureus* retained surfaces.

3.3.3 Line Profiles from White Light Profilometry.

Line profiles obtained from the white light profilometry two-dimensional images provided a cross sectional image of the surface topographies allowing for quantification of the heights and widths of the peaks. This data was displayed in box and whisker plots to demonstrate the distribution between the largest and smallest values. Average, minimum, and maximum values were also calculated and displayed.

The line profiles generated from the pristine surfaces (Figure 35) were first analysed and demonstrated that the 316L stainless steel and TiN/15.03at.%Ag contained regular valleys consistent with the linear striations observed from the two dimensional WLP images whilst the titanium and 316Ti stainless steel contained irregular valleys with taller peaks. The 316TiN surface had smaller widths between the peaks, larger peaks and fewer large valleys. Numerical data from the line profiles was analysed for the heights of the peaks (Figure 41) and widths between peaks (Figure 42).

The 316L stainless steel surfaces were found to have the smallest variation in peak height distribution which correlated with its low S_a value (13.98 nm ± 0.7 nm), and a larger variance in the widths between its peaks with a low median value. However, 316L stainless steel also had a greater distribution from the median value to the maximum value (Figure 42) suggesting that whilst the average widths in any given section were of a smaller value; the surface also had occasional large widths between peaks in intervals. The titanium and 316TiN surfaces had a similar distribution of peak heights with a greater proportion of value from the median to Q3 values (Figure 41), but titanium had larger values for the widths between peaks describing a greater propensity for large valleys in the topography than the 316TiN (average values = 324.89 nm ± 84.85 nm, and 161.06 nm ± 22.99 nm respectively). The 316Ti stainless steel surfaces had the largest distribution in peak heights between the median, quarter 3 and maximum values, and the largest average peak height values (439.78 nm ± 55.4 nm). The widths between peaks for 316Ti were still relatively larger in size in comparison to the other metals (average = 27.39 nm ± 8.34 nm) which, as visible on the line graphs, demonstrated that even the small

valleys were pronounced. The TiN/15.03at.%Ag surfaces had the most equal distribution between its peak heights and widths with (average largest = 81.29 nm ± 30.60 nm, and 86.25 nm ± 18.80 nm respectively) fewer variation between the largest and smallest values and fewer outliers in data.

Qualitative assessment of the line profiles for the metals following retention assays of either *S. aureus* or *E. coli* demonstrated only a few differences in the appearance of the peaks and valleys when compared to the pristine metal. Those present could be explained as expected variance in the metal surface due to the location sampled due to variations in the manufacturing process.

Quantitative assessment of the minimum and maximum peak heights and widths demonstrated the differences between the surfaces post the retention assays (Table 6 and 7). Similar trends were seen between the bacterial species regarding whether or not the bacterial cells presence increased or decreased the sizes the peak heights and peak widths from the pristine metal surfaces. Differences produced by the bacterial species were more prominent on the 316L stainless steel and TiN/15.03at.%Ag surfaces which saw an increase in peak heights in the presence of *E. coli* (average reduction = 121.96 nm and 39.43 nm respectively) in comparison to much smaller increases of only 10.70 nm for the 316L stainless steel and a reduction of 53.99 nm for the TiN/15.03at.Ag in the presence of *S. aureus*.

A similar trend was seen for titanium and 316Ti stainless steel which both saw a reduction in the size of their peak heights with the addition of *E. coli* (average reduction = 189.81 nm and 13.07 nm), and widths between peaks (average reduction = 154.43 nm and 19.21 nm), however, these still maintained their rank as having the largest figures for the five metals. No significant difference was seen for the 316TiN surface between its peak heights and the widths when compared with measurements from the pristine surfaces.

Analysis of the distribution of peak heights was carried out to determine how the bacterial retention affected the surface topography and it was demonstrated that the addition of *S. aureus* (Figure 43 and 44) reduced the number of upper quartile peaks (median value to quarter 3). This gave the surfaces a greater density of

smaller peaks (quarter 1 to median value) over the 166 nm line trace, with the occasional large peak, as demonstrated by the quarter 3 to maximum whiskers for the titanium and 316Ti stainless steel surfaces. The addition of *S. aureus* to the surfaces produced a similar trend in all five metals in the widths measured between peaks (Figure 44) as there was a reduction in the numbers of larger widths and an increase in the number of smaller widths between peaks. The addition of *E. coli* to the surfaces produced a similar distribution in the heights of the peaks, comparable to that demonstrated on the pristine surfaces. It was found that following the retention assays, there was a greater propensity towards large peak values (median value to quarter 3) along its line trace

Visual assessment of the line profiles obtained from the surfaces after retention assays with the 10 % bovine plasma conditioning film (Figure 38) demonstrated that the metal surfaces were visually similar to the pristine surfaces. Numerical data from the line profiles was analysed for the heights of the peaks (Figure 47) and the widths between peaks (Figure 48) and displayed in box and whisker plots to show the distribution of the line profile values. The average, minimum and maximum values were calculated (Table 8).

Analysis of the distribution of peak heights demonstrated that the surfaces followed the same trend that was seen in the analysis of the roughness parameters (Figure 47). The titanium surfaces had the greatest distribution of peak heights, with a greater propensity towards larger surface features. In contrast, the 316Ti, 316TiN and TiN/15.03at.%Ag surfaces demonstrated a tendency towards smaller surface features with the occasional larger peak measurement visualised through the amplitude of the median to maximum ranges. The 316L stainless steel surfaces were found to have the smallest variation in peak heights which correlated with the smaller roughness values and visually sparse surface features.

Assessment of the distribution of widths between the peaks demonstrated that all the surfaces had similar measurements between the minimum and maximum measurements with few changes in the distribution of quartile measurements. This correlated with the average peak measurements (Table 8) and with the assessment

of the surfaces retained with bacteria alone but not with the original measurements of the surfaces prior to any retention assays or conditioning films

Qualitative analysis of the line profiles obtained after retention assays with the two bacterial species, *S. aureus* and *E. coli* with 10 % bovine plasma (Figure 39 and Figure 40), demonstrated that those surface contaminants made few visual differences to the surfaces when compared to where the surfaces were pristine, soiled with bacteria, or soiled with conditioning film alone.

Assessment of the of the average minimum and maximum peak heights, and the average widths between the peaks (Tables 9 and 10), was performed to give quantitative assessment of any changes to the surface profiles that may have occurred as a result of the retention assays containing the bacterial species *S. aureus* or *E. coli* in the presence of the 10 % bovine plasma. On assessment of whether or not the addition of the bacterial species with conditioning film increased or decreased the values obtained, comparison of the *S. aureus* plus 10 % bovine plasma results to those obtained from the pristine surfaces, demonstrated that the surfaces followed similar trends as to when the *S. aureus* was retained upon the surface alone.

Analysis of the distributions of peak heights demonstrated that the addition of the *S. aureus* with the 10 % bovine plasma followed a similar overall distribution from the minimum to maximum values to those previously from the pristine surfaces, surfaces with bacteria, and surfaces with conditioning film. However, the surfaces did demonstrate a smaller distribution of median to upper quartile peaks. This demonstrated that the surfaces had a greater propensity towards smaller peak heights in the line graphs with the occasional irregular large peak. The distribution of the width values also followed the trend of a reduction in the numbers of larger valleys (median to Q3) to the previous chapters with the pristine surfaces, unsoiled surfaces with bacteria, and surfaces with conditioning film.

The addition of the *E. coli* plus 10 % bovine plasma to the surfaces produced a similar trend to that of the *S. aureus* with 10 % bovine plasma in demonstrating a greater propensity towards smaller peak distribution within both the heights and

width measurements when compared to the results with the *E. coli* on the surfaces alone.

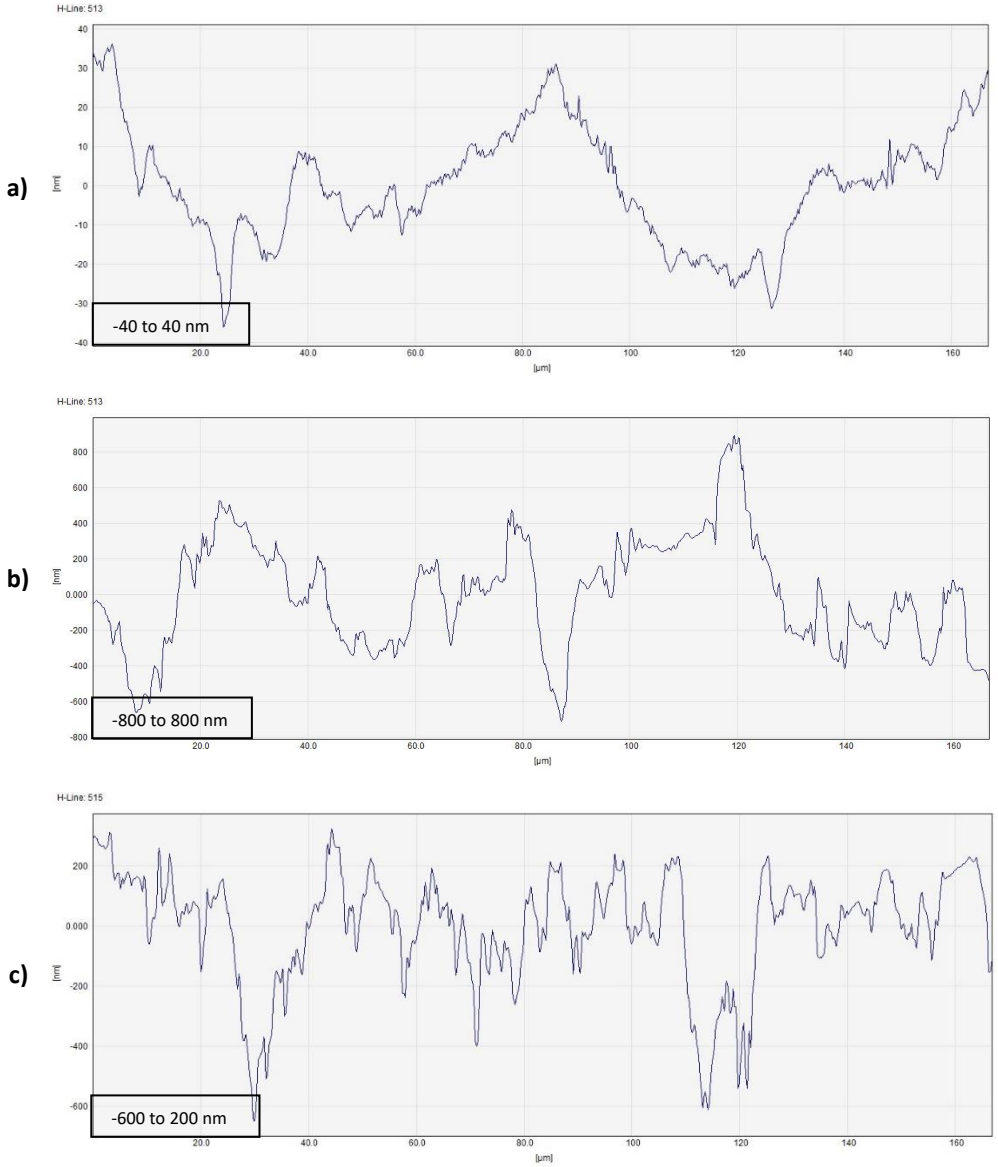
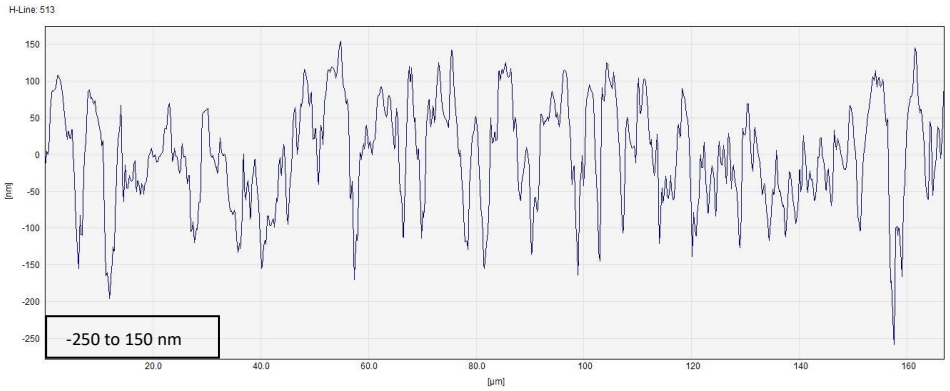


Figure 35: Line profiles obtained from white light profilometry scans of a) 316L stainless steel, b) titanium c) 316Ti stainless steel surfaces displaying a cross-sectional profile of the widths and depths of the peaks and valleys of the surfaces over 160 µm line trace. Three areas of three coupons were analysed (n = 9). Note the differences in the Y axis scale.

d)



e)

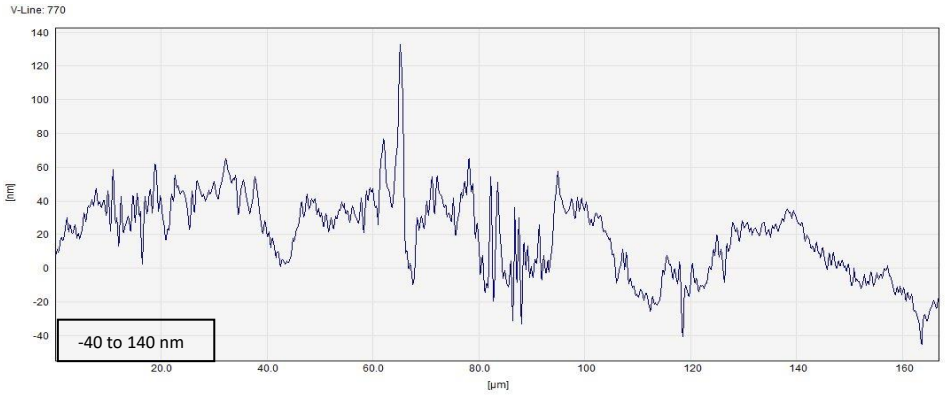


Figure 35 continued: d) 316TiN and e) TiN/15.03at.%Ag surfaces demonstrating the microtopographies of the surfaces. Three areas of three coupons were analysed (n = 9). Note the changes to scale on the Y axis.

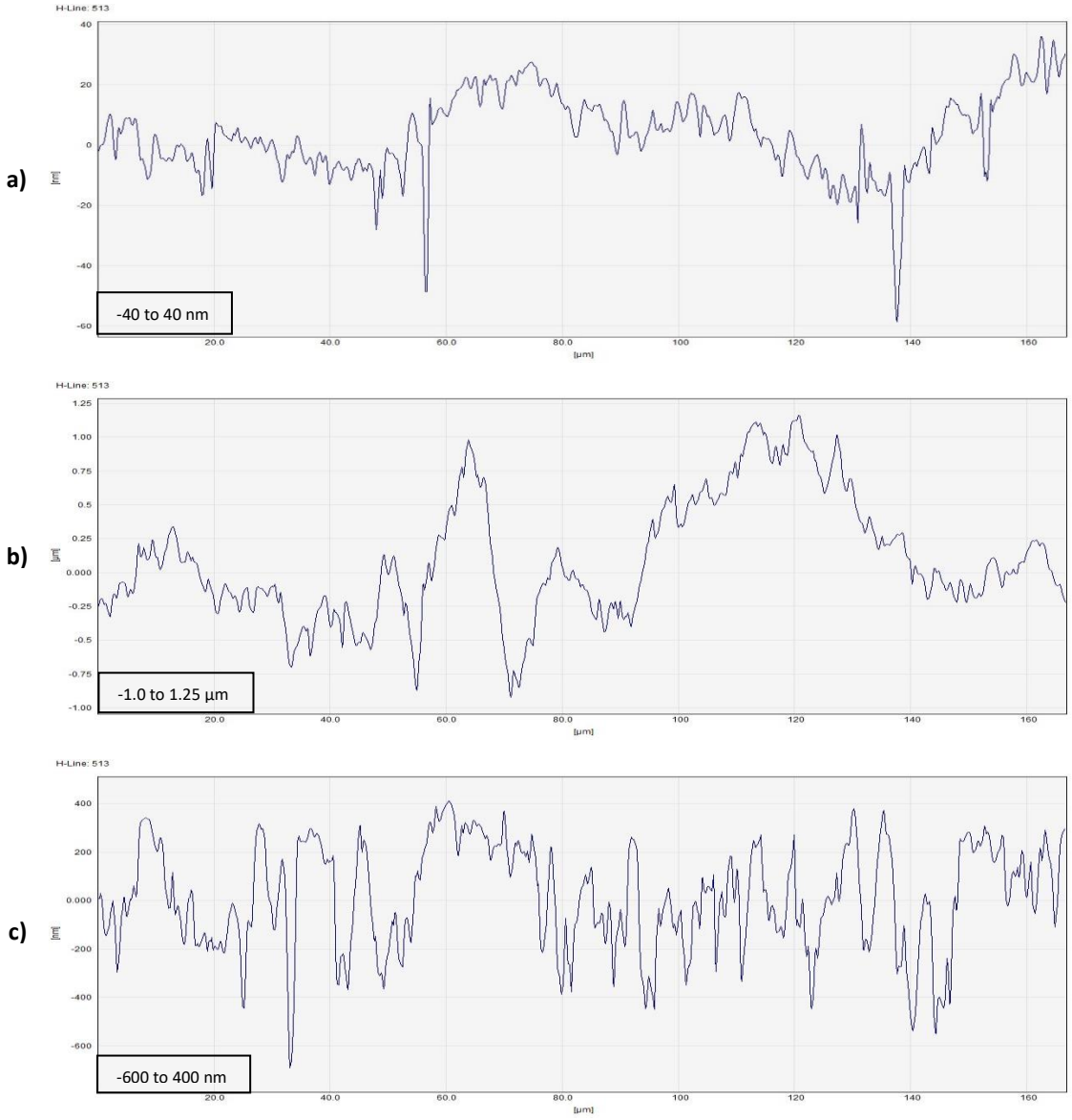
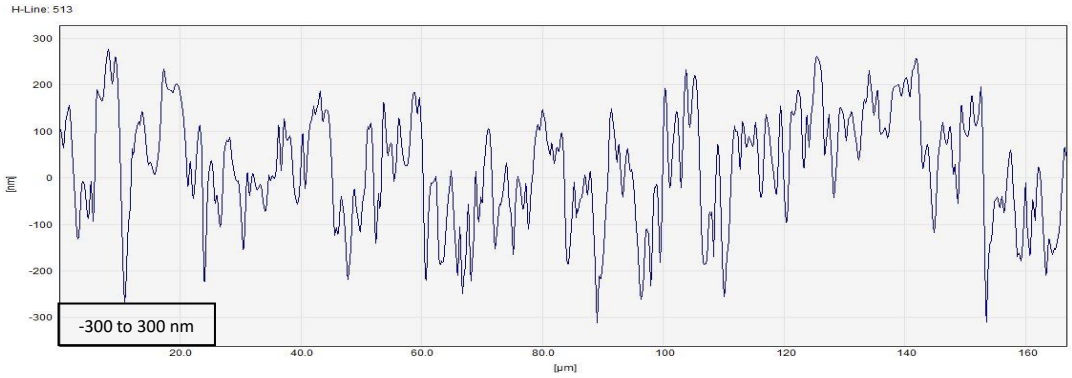


Figure 36: Line profiles obtained from white light profilometry scans of the surfaces after retention assays of *S. aureus* a) 316L stainless steel, b) titanium, c) 316Ti surfaces demonstrating the microtopographies of the surfaces. Three areas of three coupons were analysed (n = 9). Note the changes to scale on the Y axis.

d)



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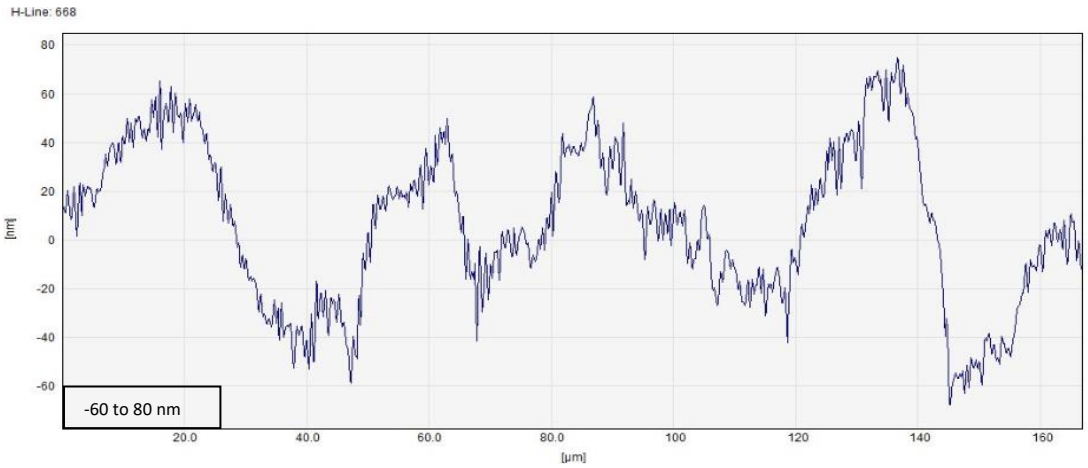


Figure 36 continued: d) 316TiN and e) TiN/15.03at.%Ag surfaces demonstrating the microtopographies of the surfaces. Three areas of three coupons were analysed (n = 9). Note the changes to scale on the Y axis.

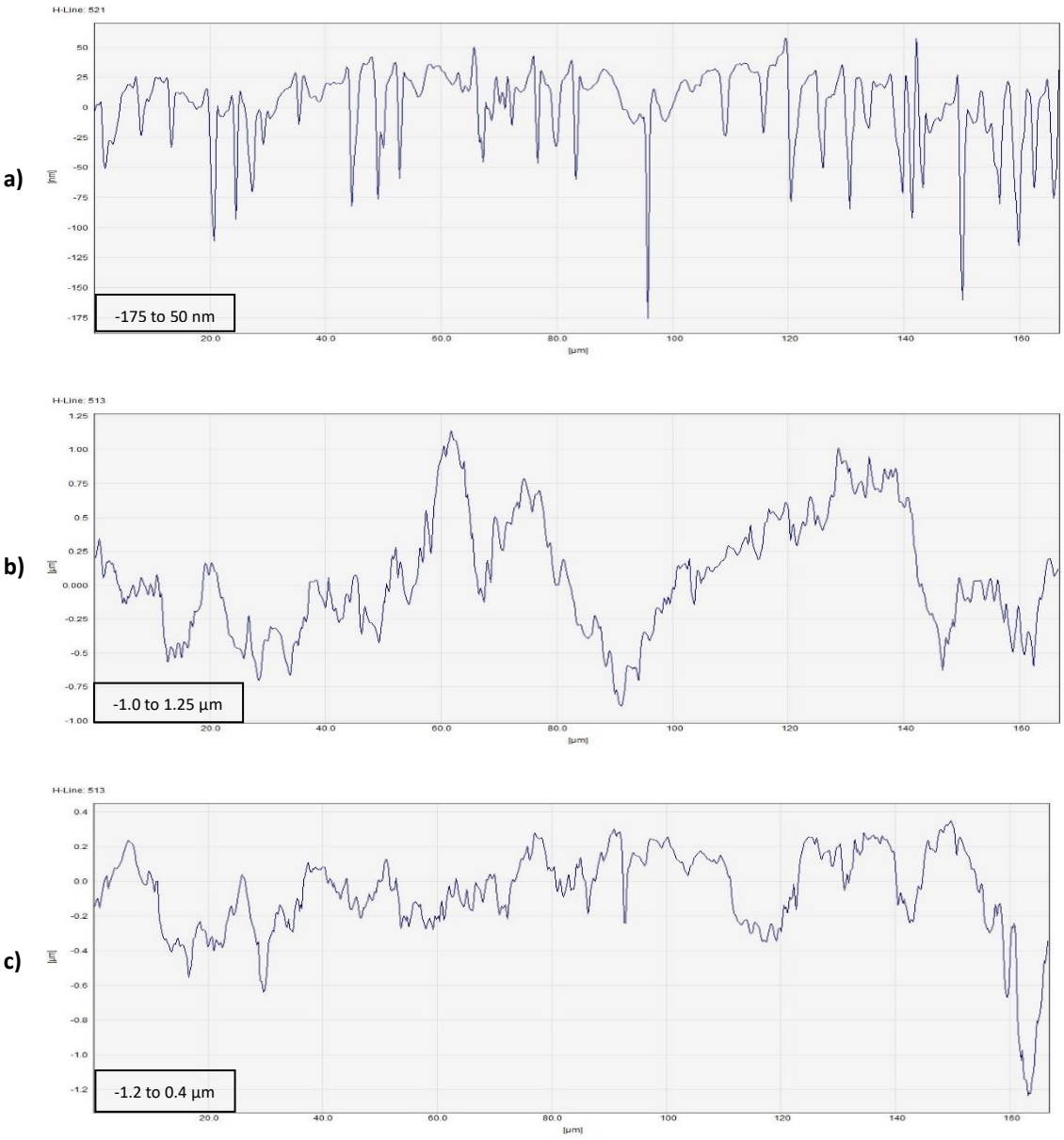
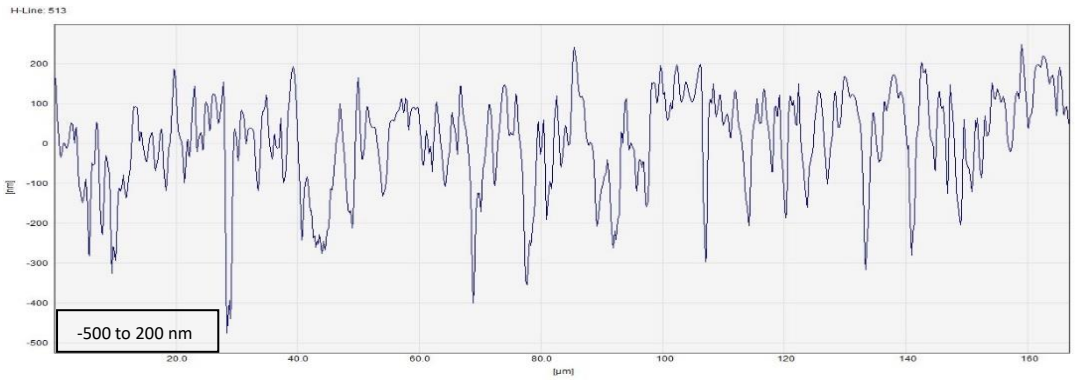


Figure 37: Line profiles obtained from white light profilometry images of the surfaces after retention assays of *E. coli* a) 316L stainless steel, b) Ti, c) 316Ti surfaces demonstrating the microtopographies of the surfaces. Three areas of three coupons were analysed (n = 9). Note the changes to scale on the Y axis.

d)



e)

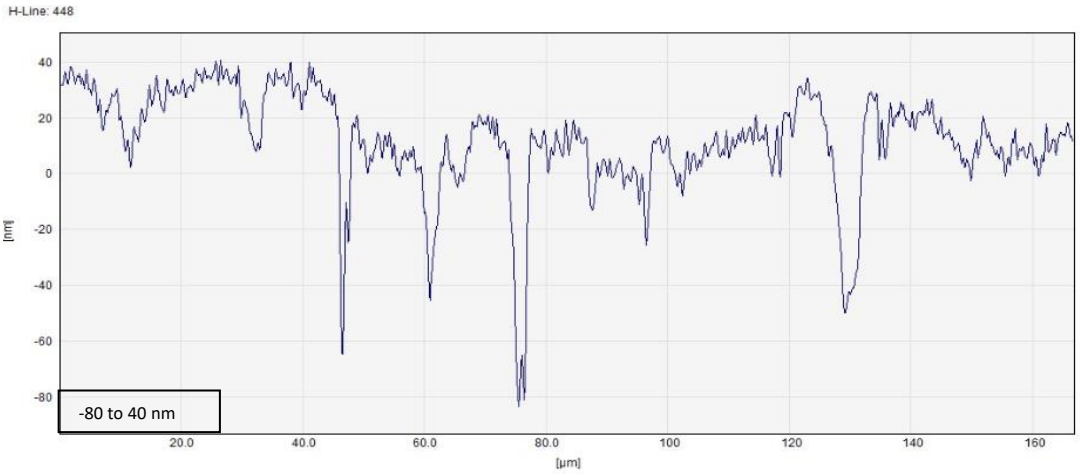


Figure 37 continued: d) 316TiN and e) TiN/15.03at.%Ag surfaces demonstrating the microtopographies of the surfaces. Three areas of three coupons were analysed (n = 9). Note the changes to scale on the Y axis.

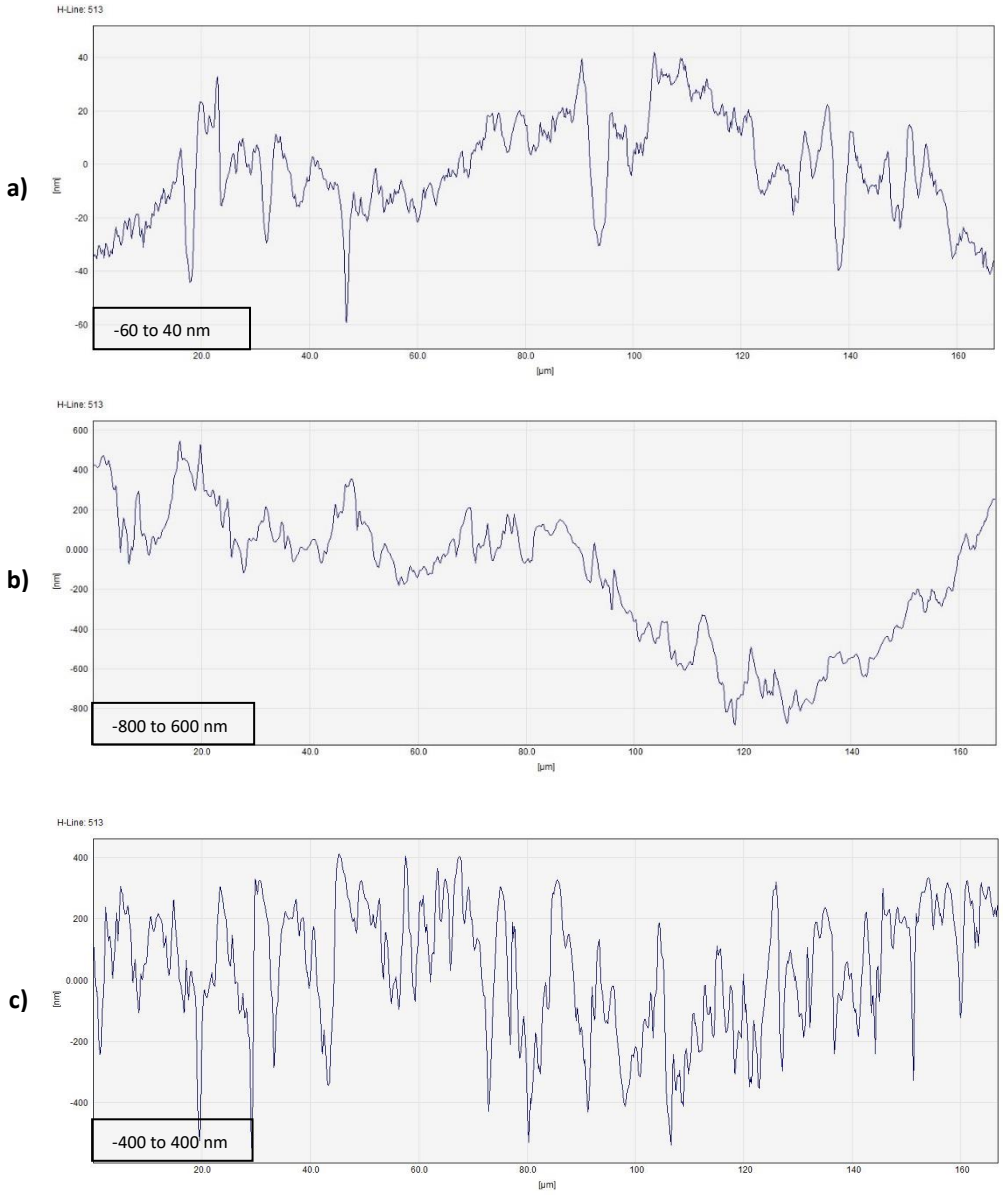
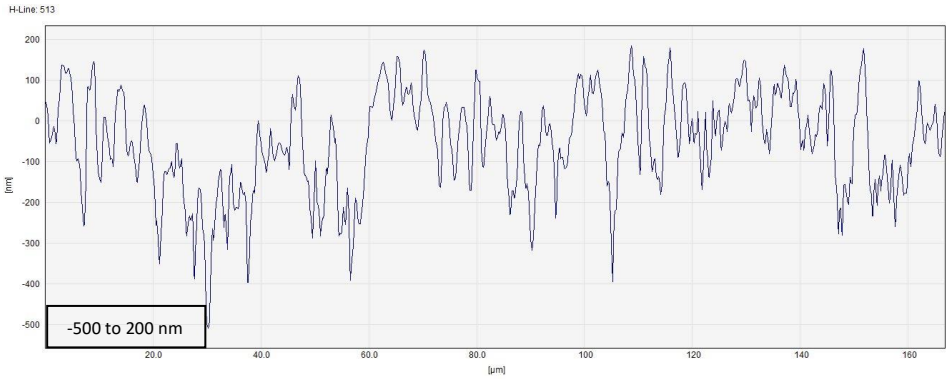


Figure 38: Line profiles obtained from white light profilometry scans of the surfaces post retention assay of 10 % bovine plasma; a) 316L stainless steel, b) titanium, c) 316Ti stainless steel surfaces displaying a cross-sectional profile of the widths and depths of the peaks and valleys of the surfaces (nm). Three areas of three coupons were analysed (n = 9). Note the changes to scale on the Y axis.

d)



e)

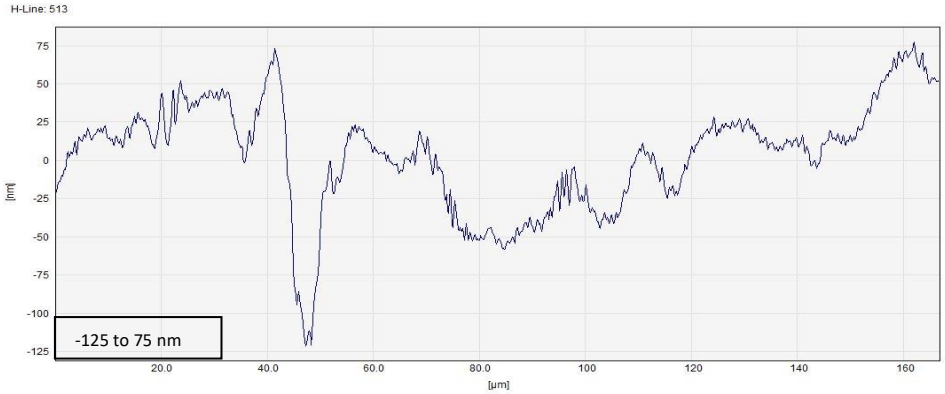


Figure 38 continued: d) 316TiN and e) TiN/15.03at.%Ag surfaces displaying a cross-sectional profile of the widths and depths of the peaks and valleys of the surfaces (nm). Three areas of three coupons were analysed (n = 9). Note the changes to scale on the Y axis.

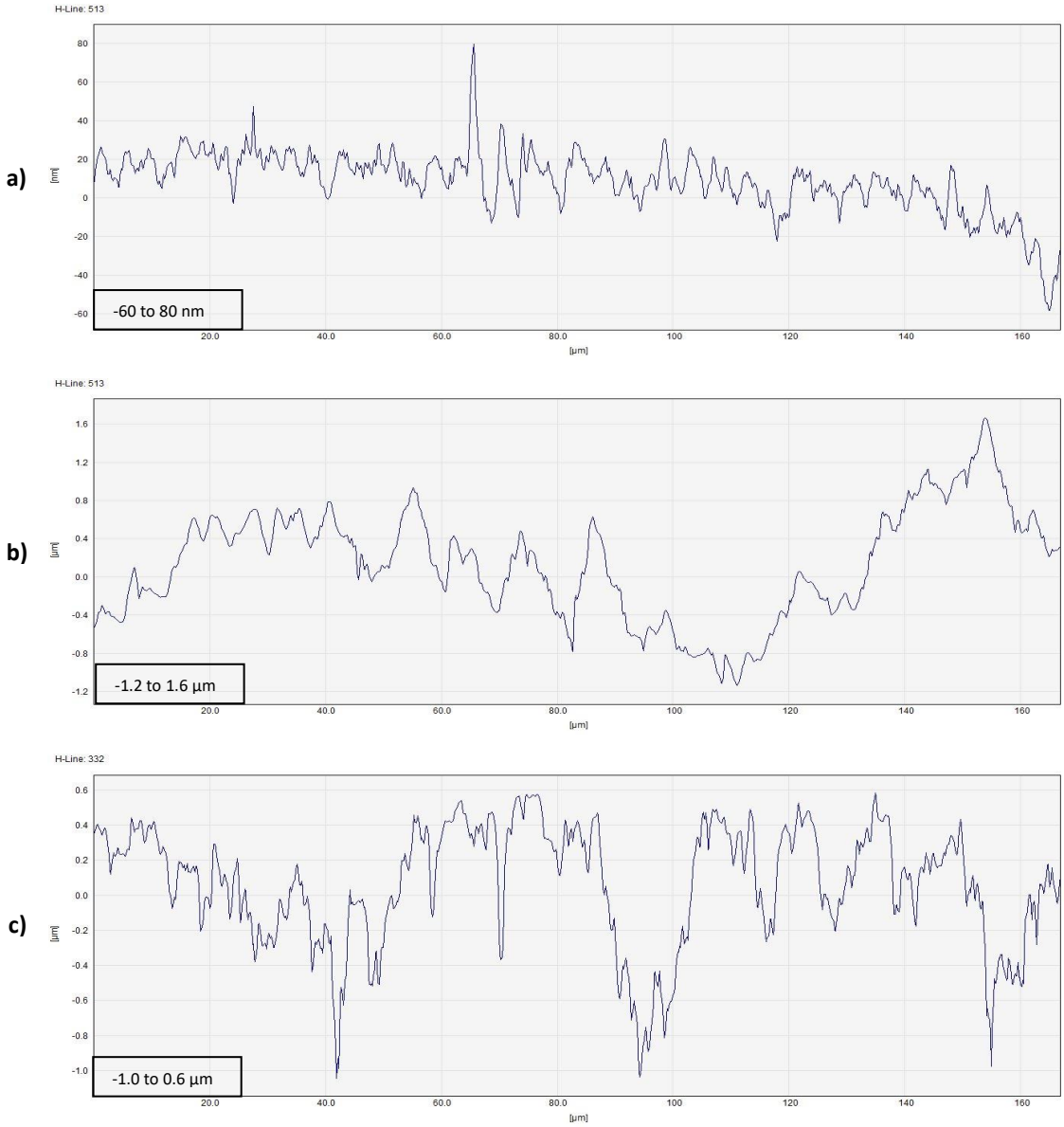
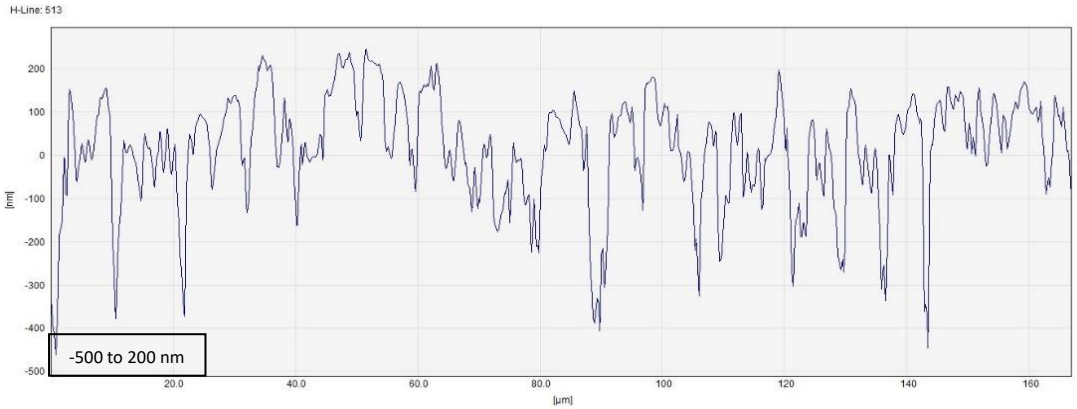


Figure 39: Line profiles obtained from white light profilometry scans of the surfaces after retention assays of *S. aureus* with 10 % bovine plasma: a) 316L stainless steel, b) titanium, c) 316Ti surfaces demonstrating the micro and nano topographies of the 160 µm surfaces. Three areas of three coupons were analysed (n = 9). Note the changes to scale on the Y axis.

d)



e)

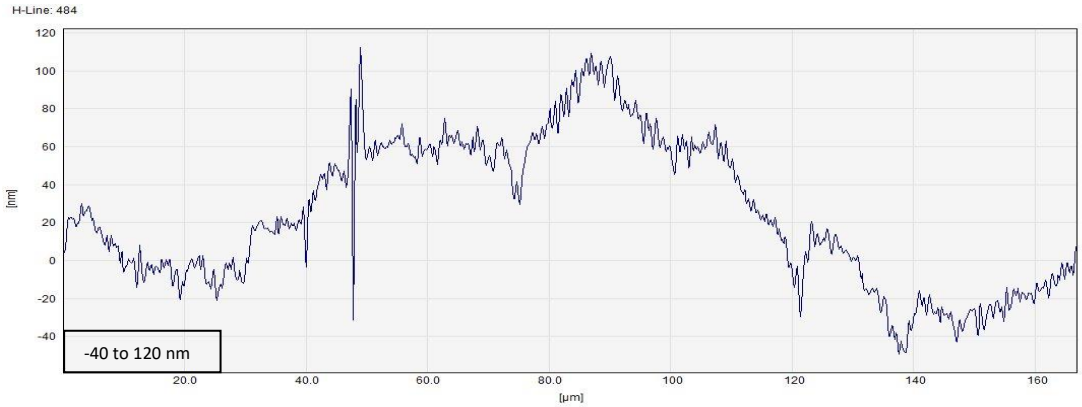


Figure 39 continued: d) 316TiN, and e) TiN/15.03at.%Ag surfaces demonstrating the micro and nano topographies of the 160 µm surfaces. Three areas of three coupons were analysed (n = 9). Note the changes to scale on the Y axis.

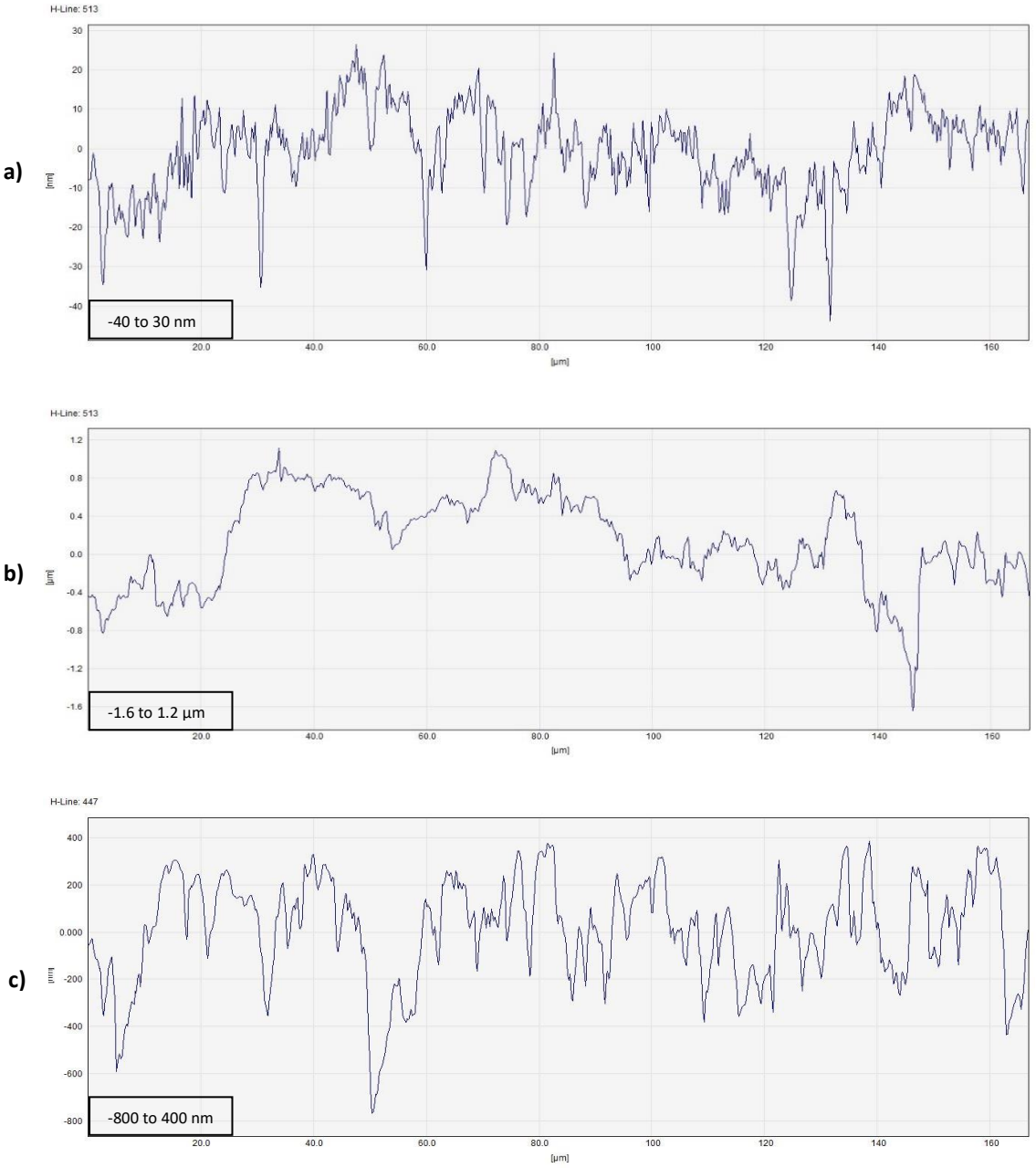
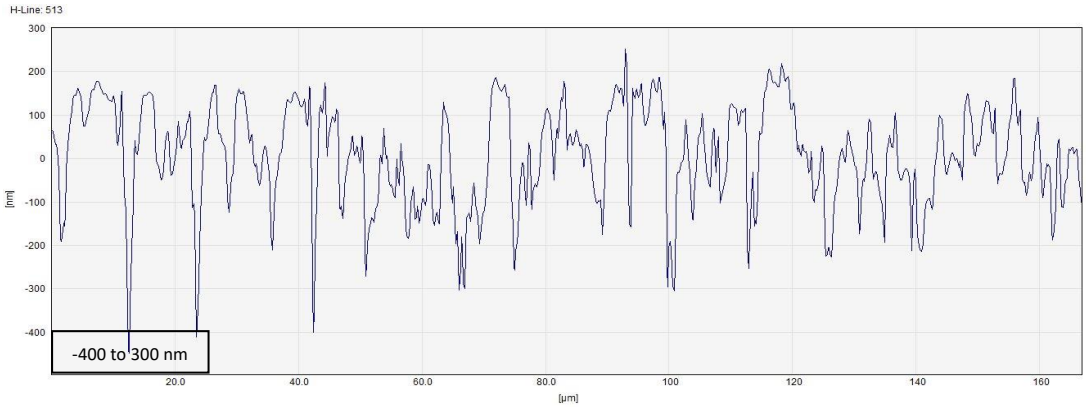


Figure 40: Line profiles obtained from white light profilometry images of the surfaces after retention assays of *E. coli* with 10 % bovine plasma: a) 316L stainless steel, b) titanium, c) 316Ti surfaces demonstrating the micro and nano topographies of the 160 µm surfaces. Three areas of three coupons were analysed (n = 9). Note the changes to scale on the Y axis.

d)



e)

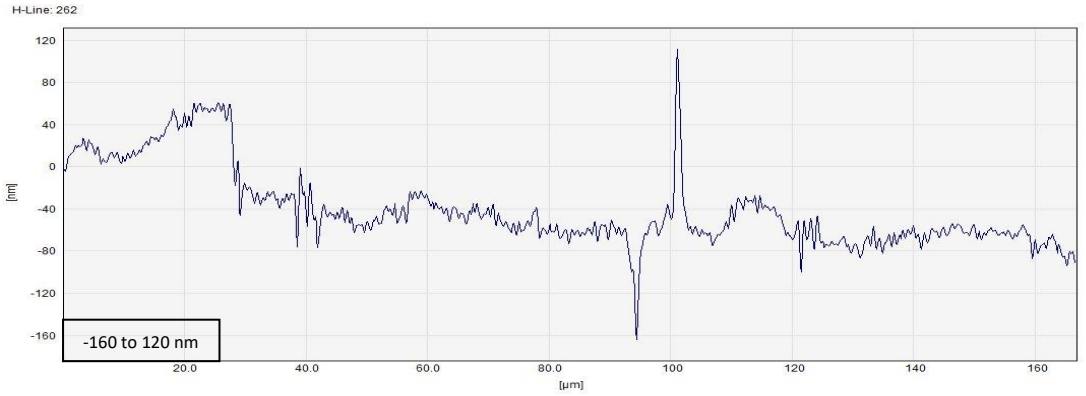


Figure 40 continued: d) 316TiN, and e) TiN/15.03at.%Ag surfaces demonstrating the micro and nano topographies of the 160 µm surfaces. Three areas of three coupons were analysed (n = 9). Note the changes to scale on the Y axis.

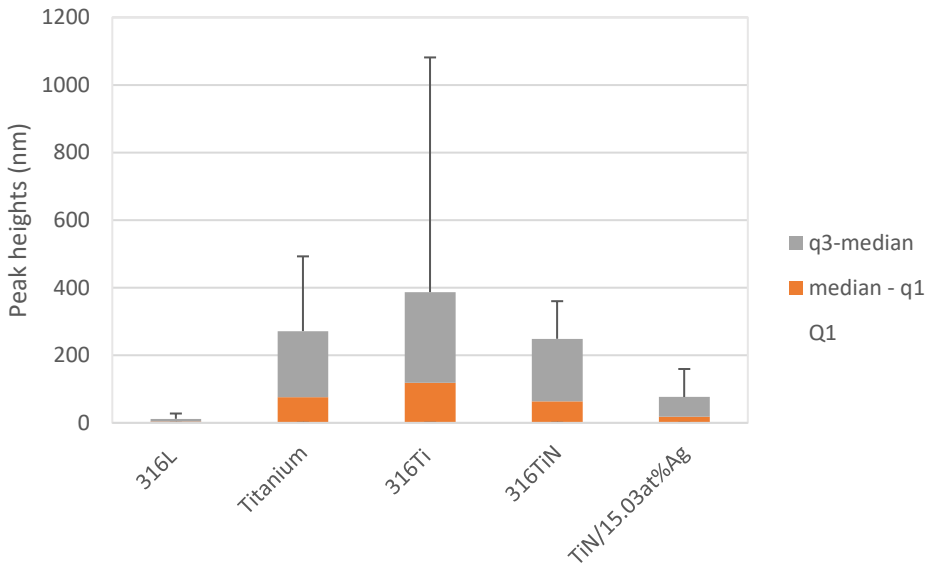


Figure 41: Distribution of peak heights obtained from white light profilometry line profiles demonstrating the distribution of peak sizes between the 5 largest and 5 smallest peaks from 3 different line profiles (n = 3).

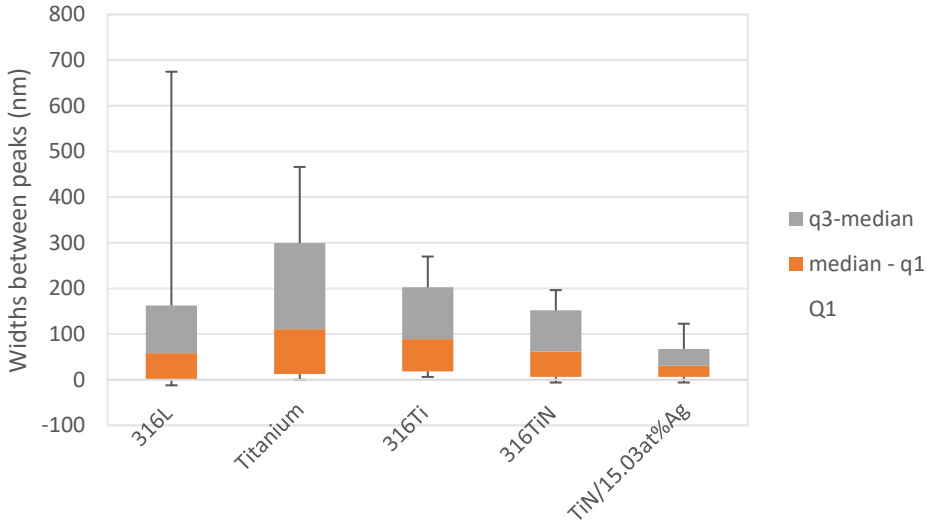


Figure 42: Distribution of peak widths obtained from white light profilometry line profiles demonstrating the distribution of peak width sizes between the 5 largest and 5 smallest widths between peaks from 3 different line profiles (n = 3).

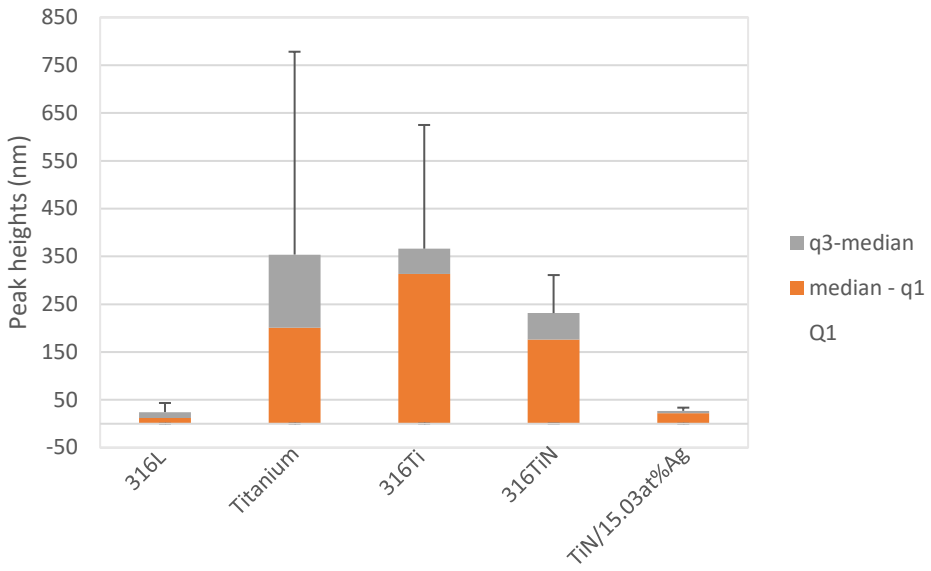


Figure 43: Distribution of peak heights obtained from white light profilometry line profiles of surfaces post *S. aureus* retention assay demonstrating the distribution of peak sizes between the 5 largest and 5 smallest peaks from 3 different line profiles (n = 3).

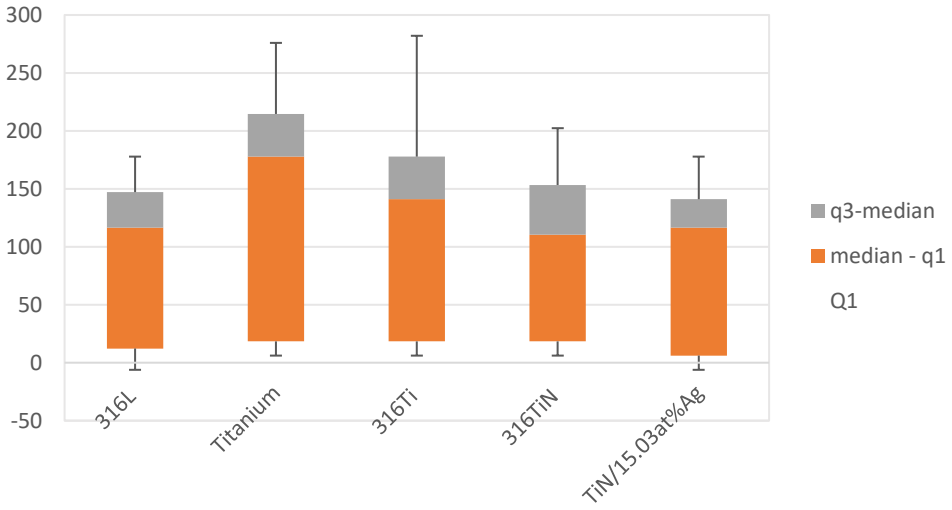


Figure 44: Distribution of peak widths obtained from white light profilometry line profiles post *S. aureus* retention assay demonstrating the distribution of peak width sizes between the 5 largest and 5 smallest widths between peaks from 3 different line profiles (n = 3).

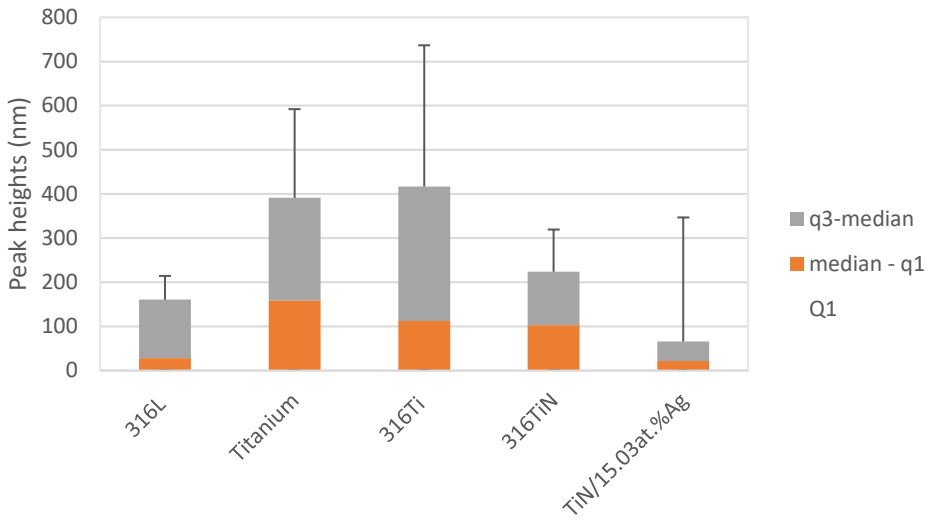


Figure 45: Distribution of peak heights obtained from white light profilometry line profiles of surfaces post *E. coli* retention assay demonstrating the distribution of peak sizes between the 5 largest and 5 smallest peaks from 3 different line profiles (n = 3).

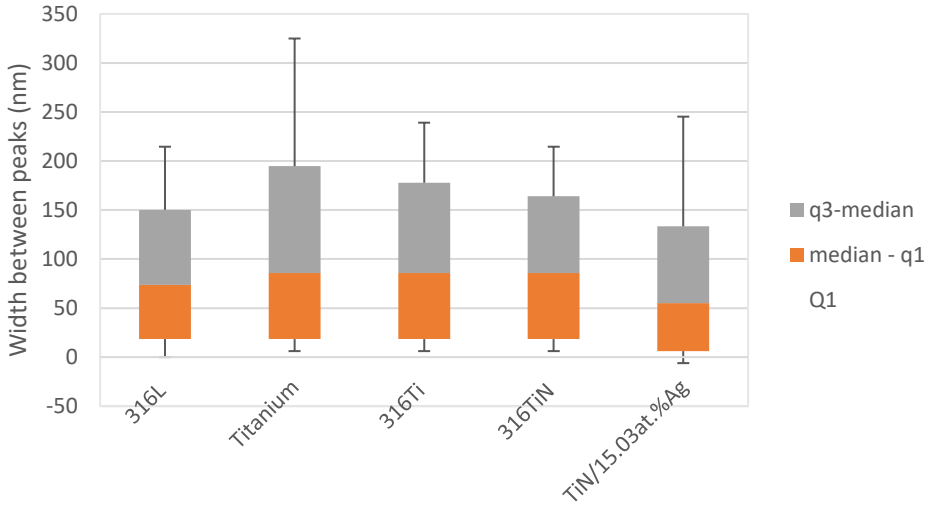


Figure 46: Distribution of the widths between peaks obtained from white light profilometry line profiles post *E. coli* retention assay demonstrating the distribution of peak width sizes between the 5 largest and 5 smallest widths between peaks from 3 different line profiles (n = 3).

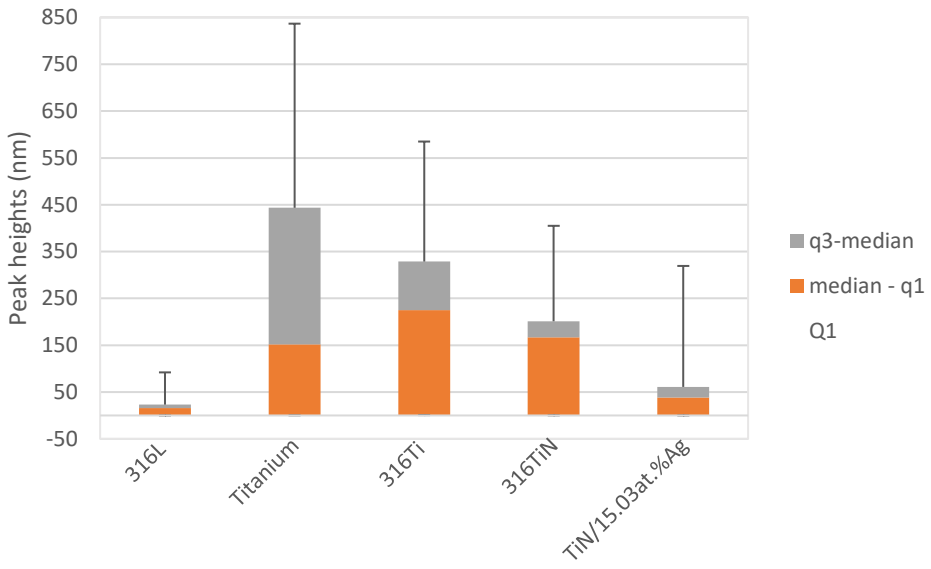


Figure 47: Distribution of surface peak heights post retention assay of 10 % bovine plasma obtained from white light profilometry demonstrating the distribution of peak sizes between the 5 largest and 5 smallest peaks from 3 different line profiles (n = 3).

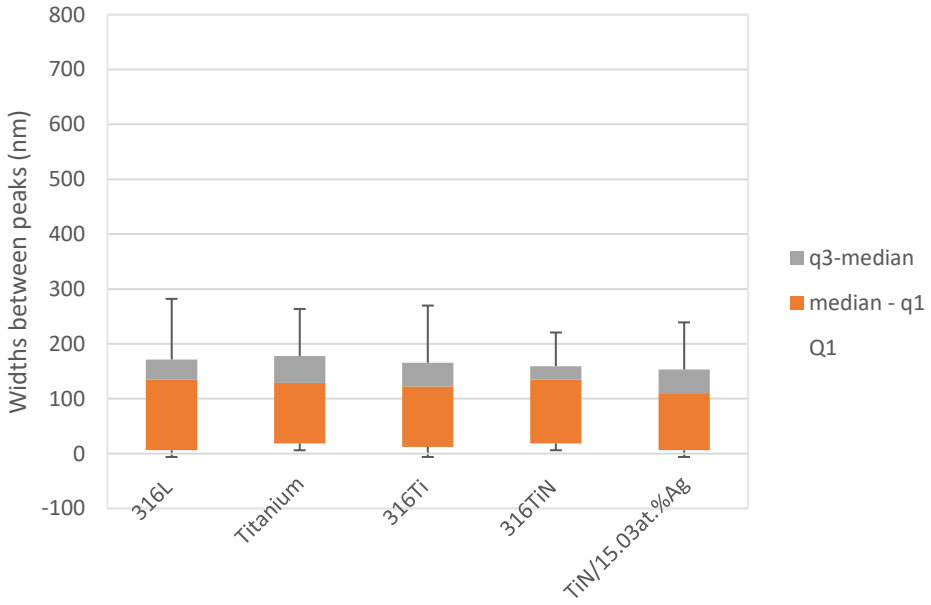


Figure 48: Distribution of surface peak widths post retention assay of 10 % bovine plasma demonstrating the distribution of peak width sizes between the 5 largest and 5 smallest widths between peaks from 3 different line profiles (n = 3).

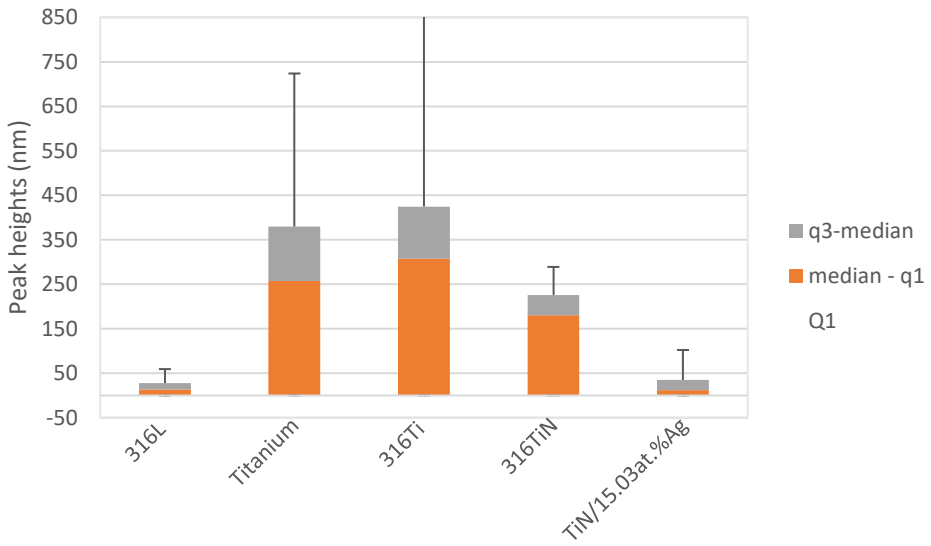


Figure 49: Distribution of peak heights obtained from white light profilometry line profiles of surfaces post *S. aureus* with 10 % bovine plasma retention assays demonstrating the distribution of peak sizes between the 5 largest and 5 smallest peaks from 3 different line profiles (n = 3).

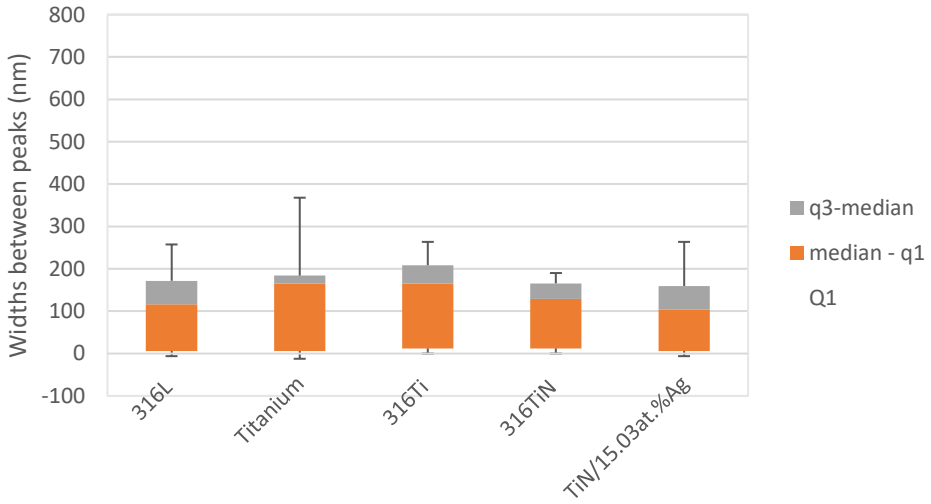


Figure 50: Distribution of peak widths obtained from white light profilometry line profiles post *S.* aureus with 10 % bovine plasma retention assays demonstrating the distribution of peak width sizes between the 5 largest and 5 smallest widths between peaks from 3 different line profiles (n = 3).

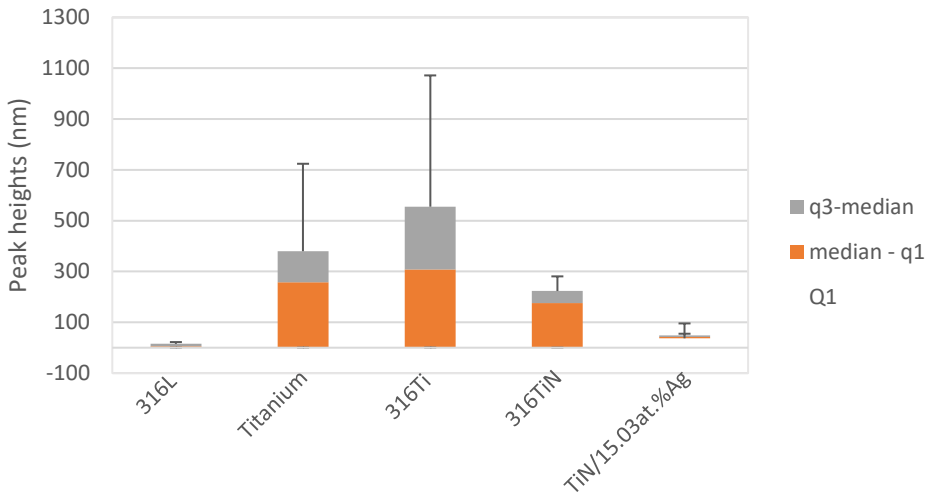


Figure 51: Distribution of peak heights obtained from white light profilometry line profiles of surfaces post *E. coli* with 10 % bovine plasma retention assays demonstrating the distribution of peak sizes between the 5 largest and 5 smallest peaks from 3 different line profiles (n = 3).

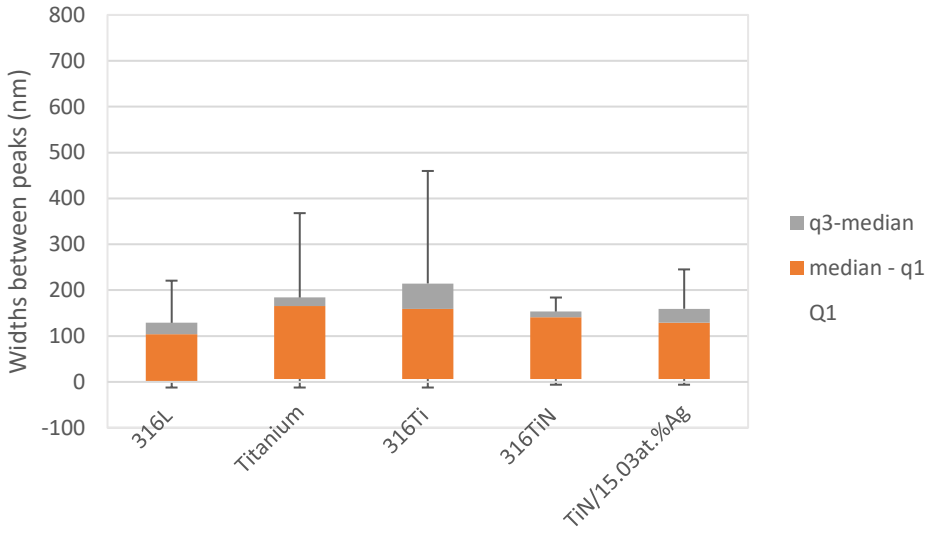


Figure 52: Distribution of the widths between peaks obtained from white light profilometry line profiles post *E. coli* with 10 % bovine plasma retention assay demonstrating the distribution of peak width sizes between the 5 largest and 5 smallest widths between peaks from 3 different line profiles (n = 3).

Table 5: Average peak heights and widths obtained from white light profilometry line profiles demonstrating the 5 smallest and 5 largest peak widths and heights from 3 lines graphs (n = 3) ± standard deviation.

(nm)		316L		Ti		316Ti		316TiN		TiN/15.03at.%Ag	
Height	Largest	13.92	± 5.78	289.70	± 100.72	439.78	± 55.4	237.97	± 80.60	81.29	± 30.60
Height	Smallest	0.01	± 0.009	0.12	± 0.10	0.24	± 0.14	0.36	± 0.29	0.08	± 0.08
Width	Largest	225.24	± 113.89	324.89	± 84.85	217.88	± 31.49	161.06	± 22.99	86.25	± 18.80
Width	Smallest	14.72	± 3.0	24.12	± 7.24	27.39	± 8.34	18.8	± 4.73	16.35	± 2.89

Table 6: Average peak heights and widths obtained from white light profilometry line profiles demonstrating the 5 smallest and 5 largest peak widths and heights from 3 lines graphs from surfaces post *S. aureus* retention assay (n = 3) ± standard deviation.

(nm)		316L		Ti		316Ti		316TiN		TiN/15.03at.%Ag	
Height	Largest	24.62	± 6.11	377.80	± 155.90	392.18	± 80.39	232.12	± 40.80	27.30	± 3.50
Height	Smallest	0.10	± 0.4	0.61	± 0.63	0.45	± 0.30	0.48	± 0.34	0.01	± 0.007
Width	Largest	163.92	± 18.00	228.10	± 29.47	194.17	± 35.60	162.30	± 21.81	153.29	± 17.63
Width	Smallest	30.70	± 9.00	29.43	± 9.30	30.40	± 9.08	27.40	± 8.03	15.53	± 3.10

Table 7: Average peak heights and widths obtained from white light profilometry line profiles demonstrating the 5 smallest and 5 largest peak widths and heights from 3 lines graphs from surfaces post *E. coli* retention assay (n = 3) ± standard deviation.

(nm)		316L		Ti		316Ti		316TiN		TiN/15.03at.%Ag	
Height	Largest	135.08	± 14.19	135.08	± 25.30	426.71	± 138.08	237.41	± 31.76	120.72	± 94.40
Height	Smallest	0.08	± 0.08	0.08	± 0.08	0.86	± 0.84	0.56	± 0.45	0.02	± 0.02
Width	Largest	170.46	± 25.30	170.46	± 25.30	198.67	± 30.68	177.82	± 20.40	150.02	± 32.00
Width	Smallest	33.11	± 5.84	33.11	± 5.83	28.21	± 11.20	29.84	± 2.76	18.80	± 4.73

Table 8: Average peak heights and widths obtained from white light profilometry line profiles demonstrating the 5 smallest and 5 largest peak widths and heights from 3 lines graphs post retention assays of 10 % bovine plasma (n = 3) ± standard deviation.

(nm)		316L		Ti		316Ti		316TiN		TiN/15.03at.%Ag	
Height	Largest	31.24	± 22.64	427.58	± 201.92	340.27	± 90.88	249.83	± 90.11	93.45	± 83.77
Height	Smallest	0.01	± 0.08	1.40	± 1.81	0.82	± 0.61	0.41	± 0.37	0.03	± 0.02
Width	Largest	194.17	± 37.78	199.49	± 41.28	198.26	± 43.68	177.41	± 25.77	165.15	± 32.25
Width	Smallest	17.17	± 2.45	29.84	± 9.75	30.25	± 6.51	24.94	± 7.91	21.67	± 4.94

Table 9: Average peak heights and widths obtained from white light profilometry line profiles from the surfaces after retention assays with *S. aureus* and 10 % bovine plasma (n = 3) ± standard deviation.

(nm)		316L		Ti		316Ti		316TiN		TiN/15.03at.%Ag	
Height	Largest	30.05	± 13.71	401.06	± 116.0	539.22	± 283.01	234.35	± 35.95	43.78	± 30.76
Height	Smallest	0.03	± 0.02	0.32	± 0.21	0.51	± 0.53	0.46	± 0.46	0.02	± 0.02
Width	Largest	185.18	± 38.92	232.60	± 55.46	224.42	± 28.21	174.96	± 20.26	181.91	± 46.95
Width	Smallest	16.76	± 4.17	31.48	± 12.44	26.16	± 10.37	25.73	± 6.41	18.0	± 3.52

Table 10: Average peak heights and widths obtained from white light profilometry line profiles from the surfaces after retention assays with *E. coli* and 10 % bovine plasma (n = 3) ± standard deviation.

(nm)		316L		Ti		316Ti		316TiN		TiN/15.03at.%Ag	
Height	Largest	14.53	± 5.28	401.10	± 115.98	567.57	± 191.43	221.45	± 35.41	32.52	± 16.27
Height	Smallest	0.015	± 0.01	0.32	± 0.21	0.52	± 0.40	0.20	± 0.15	18.46	± 25.24
Width	Largest	154.93	± 29.91	232.60	± 55.46	262.85	± 86.90	170.05	± 11.30	183.54	± 8.23
Width	Smallest	16.76	± 6.51	31.47	± 12.44	27.39	± 8.63	19.21	± 1.62	20.03	± 6.12

3.4 Discussion.

The measurement and observation of the physical parameters of surfaces has often been used to describe how likely a surface is to become soiled with bacteria and other biological conditioning agents, with many authors hypothesising a direct correlation between the roughness parameters of a surface and the retention of bacteria (Anselme et al., 2010; Puckett et al., 2010; Singh et al., 2011). However, other authors have found no relationship between the two (Hilbert et al., 2003; Whitehead et al., 2005; Milledge, 2010) and argue that the examination of one parameter alone is not sufficient when investigating such a complex issue and is rather reductive when considering the how many different bacterial species may interact with a surface during its lifetime (Zhao et al., 2008; Wickens et al., 2014).

The measurement of surface parameters is also an important factor when considering the use of metals as biomaterials as the topography of a surface plays an important role in mediating the osseointegration of bone to surfaces (Novaes Jr et al., 2010) and host tissue and immune responses (Wilson et al., 2005; Chen et al., 2010). This chapter investigated the physical parameters of the five metal surfaces, assessing what impact the addition of bacteria, conditioning film, and bacteria with conditioning film to the surfaces had upon these parameters.

The initial assessment of the pristine surfaces served as control measurements to enable comparison with assessments involving the surface conditioning treatments. Visual analysis of the surface structures of the metals by SEM demonstrated that the 316L stainless steel and the TiN/15.03at.%Ag metal surfaces visibly shared similar linear striations to one another indicating that the sputter coating process had not changed the visible underlying structures of the 316L stainless steel beneath the TiN/15.03at.%Ag coating. This assessment was confirmed by the measurement of their roughness parameters which demonstrated that there was no statistically significant difference between the two surfaces and that these surfaces were the least rough of the five tested. The titanium, 316Ti stainless steel, and 316TiN surfaces demonstrated greater surface features and structures, the most visibly different being the 316Ti stainless steel due to the differing production

processes of cold rolling which are performed to help improve the strength and hardness of the metal (Mhaede et al., 2015) but had led to the production of larger crevices within its surface. The roughest of the surfaces tested were titanium and 316Ti stainless steel (average S_a = 410.63 nm and 377.97 nm respectively) which were statistically different to all the other surfaces but not from one another and had the largest peak height and widths from all the surfaces tested. It has been hypothesized that these increases in morphological features on surfaces could provide greater locations for bacterial retention and adhesion (Flint et al., 2000; Hilbert et al., 2003; Hsu et al., 2013), as well as organic materials which would make the surface harder to clean (Gibson et al., 1999; Verran et al., 2001; Hilbert et al., 2003; Whitehead et al., 2009).

Despite the comparatively increased roughness of the titanium and 316Ti stainless steel surfaces, all the surfaces fell below the proposed parameter of 0.8 µm (R_a) for appropriate use as hygienic surfaces in the food industry (Taylor and Holah, 1996; Flint et al., 2000). However, when assessing the topography of the surfaces in view for use in the medical industry, the roughness parameters of all the surfaces were found to fall into the classification of "smooth" (S_a = 0 – 0.4 µm) for use in orthopaedic and dental orthopaedic implants as proposed by Dohan Ehrenfest *et al.,* (2010), particularly the 316L stainless steel and TiN/15.03at.%Ag surfaces which were the least rough of all surfaces tested. In a systematic review by Wennerberg and Albrektsson (2009), the authors utilised the data from 100 published papers in an attempt to propose recommendations for appropriate roughness parameters for positive bone response and reported that "smooth" and "minimally rough" (S_a = 0.5 – 1.0 µm) surfaces demonstrated less strong bone responses than "rough" (S_a = > 2.0 µm) surfaces. However, in a more recent study by Kang *et al*., (2015) into the osseointegration of titanium dioxide implant surfaces in rabbit models, the authors evaluated the effects of nanotopographies on bone response. The implants were surface treated to produce nanotubes of 30 nm, 70 nm and 100 nm, and then implanted for periods of 4 and 12 weeks prior to assessment of the osseointegration. The study concluded that that both the 30 nm and 70 nm nanotubes had positive effects upon osteogenesis and osseointegration depending

upon healing time, with the 70 nm nanotubes producing good results at 4 weeks and the 30 nm 12 weeks. This ambiguity in results is often demonstrated in this field of research, mostly due to the lack of standardised evaluation methods and lack of consensus upon the terminology used to describe surface features (Dohan Ehrenfest et al., 2010) making prediction of biological outcomes difficult. However, with consideration that these surfaces are being assessed with a view to being used as a temporary fixation device in this instance, prolonged osseointegration would not be required and so it would be feasible that these surfaces could be used, with the rougher titanium and 316Ti surfaces being of interest. If these surfaces were to be considered for utilisation in orthopaedic and dental implants, investigations into osteoblast proliferation and integration to the surfaces would be required in order to attempt to predict any biological outcome and therefore their suitability as a biomaterial.

Understanding the effects of surface topography is also important as an increase in roughness can potentially affect the cleanability of a surface, providing greater retention points for bacteria and organic materials to accumulate. Assessment of the surfaces in the presence of the bacterial species through SEM demonstrated the bacterial responses to the structure of the surfaces, offering insights into potential issues which could arise if they were to be used as food contact areas. The addition of *S. aureus* to the surfaces demonstrated that when it was in contact to surfaces with fewer surface features the bacterial cells had a preference to clump together, and when in contact with rougher surfaces the cells would adhere inside the surface features which potentially provided a greater surface area for attachment, as well as refuge from potential sheer stress. However, the addition of *E. coli* to the surfaces did not follow the trend of the *S. aureus* cells with their locations appearing more random. This could potentially be due to the presence of their flagella providing greater anchor points for the bacterial cells to attach to the surfaces in more difficult locations. This work corroborates the findings of Friedlander *et al*., (2013) which investigated the role of *E.* coli flagella in the adhesion of cells to surfaces with defined and controlled sub-micrometer crevices which were designed to potentially reduce bacterial surface area contact and

therefore reduce adhesion. The authors discovered that during the first two hours, the numbers of adhered bacteria were reduced in comparison to smooth controls, but after that point the numbers of adhered *E. coli* increase significantly with prolonged exposure. Investigations utilising mutants lacking flagella demonstrated that the wild-type *E. coli* utilised their flagella to 'hook' onto the surface features anchoring the bacteria to the surfaces and further, producing a fibrous matrix which aided in further attachment. The authors concluded that the flagella were used for not only swimming but also for aiding surface attachment, enabling them to overcome difficult surface features.

When presented with rougher surfaces with greater surface features the *E. coli* cells appeared to have a preference towards adhesion in alignment to these surface features, likely to maximise the surface area contact with their rod-shaped cellular structure. This result was also similar to the results reported by Friedlander *et al*., (2013) in which SEM images clearly demonstrated that *E. coli* cells took preferential attachment alongside surface features rather than along the flatter tops. These results demonstrated that bacterial preference for surface location was species specific relying heavily upon which surface appendages the bacterial cells are equipped with and so it would not be possible to design a surface that would be suitable for all bacterial species, particularly in a mixed species environment such as food contact areas. However, attention should be paid to the finish of the surfaces and what structures these produce as bacteria hidden within surface features would be more difficult to disinfect and could proliferate over time, posing an infection risk to consumers. With these issues in mind, it would be suitable to assert that whilst all the surfaces did fall into the classification as "hygienic" for use in food contact areas, the 316Ti surface would not be a suitable choice due to the potential for pathogens and organic soils to attach and retain within larger crevices making the surface harder to clean and posing a contamination risk.

The issue of ease of cleaning is less of a consideration for indwelling biomaterials as they are sterile upon implantation and would not be cleaned after that point. Previous works have analysed how the properties of a surface, such as the topography, can affect the spread and clustering of bacterial cells but concluded

that this did not affect the density of the bacteria (Tetlow et al., 2017; Wickens et al., 2014).

Determination of the surface roughness in the presence of the bacterial species demonstrated that the surfaces maintained the same overall trend that was seen on the control surfaces, and that the addition of *S. aureus* only made a significant difference to the 316TiN surfaces. The addition of *E. coli* made a difference to the two least-rough surfaces, suggesting that the increased size of the *E. coli* cells in comparison to the *S. aureus* cells was responsible for this difference which correlated with the visual inspection of the surfaces. The increased size of the *E. coli* cells altered the significance in the roughness between the titanium and 316Ti surfaces, making the titanium significantly rougher than the 316Ti surface, which had not been demonstrated on the control surfaces. This may be due to the differing surface features of the metals, with the titanium demonstrating linear grooves and the 316Ti demonstrating a more pitted surfaces in which the rod-shaped cell morphology would make less of an impact upon roughness.

Assessment of the surfaces in the presence of the 10 % bovine plasma conditioning film demonstrated that the conditioning film was not apparent and made no visual changes to the structure of the surfaces or to their roughness parameters. When considering the size of the most abundant plasma protein, albumin, which is 2.74 ± 0.35 nm (Kiselev et al., 2001), it is understandable a retention assay containing a 10 % solution of plasma would not make a visibly apparent presence upon the surfaces, and also would not significantly affect topography measurements. Due to this lack of demonstrable change to the roughness parameters of the surfaces with the conditioning film present, it would be reasonable to assume that should any changes in bacterial retention be noted when conditioning film is applied, it would not be due to topography.

It has been hypothesized that the addition of a conditioning film has the potential to mask the underlying surface topographies of substrate, and so affect the way in which bacteria would interact with them (Verran & Jones, 2000). Investigations have shown that the addition of conditioning films have either increased the surface roughness (Bakker et al., 2004) or decreased (Mei et al., 2011) the

roughness of the substrates they were using, though this is largely influenced by what biological and non-biological components make up the conditioning film being used. Methodical approaches to the application of the conditioning film would also have great impact upon this parameter as, in this particular study, the proteinaceous components of the conditioning film were adsorbed to the substrates and then the excess removed. In contrast, other studies have applied thicker conditioning films and dried them to the substrata before studying the changes in the surface parameters. In a study by Moreira et al.(2017), the effects of cellular extract conditioning films upon bacterial adhesion and biofilm formation was measured. The results demonstrated that the conditioning films tested had significantly increased the roughness parameters of the surfaces. However, the cellular extracts were dried thickly to the polystyrene when those measurements were taken, and as the other parameters were measured under flow conditions of 'in static' biofilm conditions, it is arguable that those roughness measurements were not applicable to the study as they are not a true reflection of what the bacteria would encounter *in vivo*.

Visual assessment of what effect the addition of the bacterial species with the bovine plasma made to the physical parameters of the surfaces demonstrated a notable decrease in the numbers of both bacterial species adhered to the surfaces was observed. This could potentially be due to the presence of the 10 % bovine plasma which would adsorb onto the metal surfaces within seconds of its contract, which is a much faster process than the adhesion of bacteria (Neoh et al., 2012). However, where bacterial cells were found, they demonstrated the same adhesion preferences to the surfaces as when retained to the surfaces without conditioning film.

Calculation of the roughness parameters of the metal surfaces in the presence of the bacterial species with the conditioning film demonstrated that the surfaces continued to follow the same trends as the control surfaces. However, in contrast to when the surfaces were assessed with just the bacterial species present, the addition of *S. aureus* with conditioning film made the titanium and 316TiN surfaces significantly rougher than the addition of *E. coli* with conditioning film. This could be

potentially due to a reduction in the numbers of *E. coli* cells when in the presence of 10 % bovine plasma rather than an increase in the numbers of *S. aureus,* as it had previously been found that the size of the *E. coli* cell had the ability to increase the roughness parameters of the surfaces. Assessment of the numbers of bacteria retained to the surfaces would allow confirmation of this hypothesis.

At the time of writing, the authors were unable to find any other published works which sought to measure the impact of the addition of bacteria and bacteria with conditioning film to the physical properties of surfaces and their effects upon the roughness parameters of the surfaces. Due to this, it is not yet known if the quantifiable changes to the roughness parameters of surfaces through the attachment and retention of bacteria could have further impacts upon future retention of bacterial species and their interaction with surfaces which highlights the unique importance of this initial work.

Analysis of the surface's physical parameters in both a pristine state and post retention assays of bacteria, conditioning film, and conditioning film with bacteria demonstrated how each of these surface conditioning treatments affected the surface properties. Assessment of these changes to physical properties in conjunction with assessment of the numbers of bacteria retained to the surfaces will allow for assessment of any correlation between the two with an aim to discovering some of the driving factors of bacterial retention to surfaces.

The work in this chapter has highlighted the importance of the assessment of the physical properties of the surfaces for both the food industry and the medical industry, demonstrating the differences in requirements of surfaces dependent upon their use as one surface does not fit all requirements. Further assessment of the surfaces for their chemical properties as well as their antimicrobial efficacy and bacterial retention numbers will aid in these assessments.

Chapter 4

Bacterial Retention and Antimicrobial Activity of the Surfaces

4.1 Introduction

Assessment of the numbers of bacteria that can adhere to a surface (their bioburden) allows for direct comparisons of the fouling potential of different surfaces. The information gained from these assessments allows not only for informed choice of suitable surfaces which can aid in the reduction of bacterial burdens and the healthcare associated issues that arise from them, but also for the deduction of the main driving factors of the adhesion process; i.e. physicochemical, topographical, molecule / adhesin interactions, or species-specific interactions of different bacterial.

Investigations into the antimicrobial efficacy of a surface allows for assessment of the different conditions in which a surface may produce its antimicrobial effect, i.e. through direct contact with the bacteria or through leaving into the surrounding liquid areas, and to how strong the effect may be; is the surface killing the bacterial cells or inhibiting them?

The aim of this chapter was to investigate the surfaces for antimicrobial action and to assess how the bacterial species retain to the surfaces under different conditions.

4.1.1 Bacterial Adhesion to Surfaces in the Presence of Conditioning Films

As previously discussed in Chapter 2, bacterial attachment and adhesion to a surface is a multi-step process relying upon the cellular interactions with the surface features. One of the largest factors which could affect the adhesion of cells to a surface is the presence and composition of a conditioning film which can modify the physicochemical properties and topographical features of a surface, resulting in varying local adhesion efficiency (Berne et al., 2018). Conditioning film composition can vary greatly and is dependent upon the molecules present in the bulk liquid environment surrounding the surface, any of the molecules present can settle upon the surface producing a film of varying molecular consistency and composition (Geng and Henry, 2011) which can augment the growth of bacterial communities (Garrett et al., 2008).

The interaction of the bacterial cells to these surface adsorbed molecules is regulated through the presence of species specific adhesins located on the surface of the bacterial cell. Bacterial adhesins are typically either protein or non-protein based adhesins which can be displayed across the surface of the cell or attached at the terminal end of flagella or fimbriae. Adhesins are a key virulence factor enabling the bacteria to adhere, invade and damage host cells, and evade host defences and immune responses (Wilson, 2002; Solanki et al., 2018). The highly specific and selective nature of the adhesin targets molecules on the surface of the host cell, and attach in a 'lock and key' mechanism, playing a key role in their pathogenesis and effect upon the host's immune system (Solanki et al., 2018), and it is speculated that removal of the bacteria's ability to adhere and colonise specific tissue through removal of its adhesin mediated receptor recognition would render the bacteria non-virulent (Klemm and Schembri, 2000; Wilson, 2002).

Different species and strains of bacteria express different quantities and specificity of adhesins which potentially explains the variances in their virulence (Patel et al., 2017). For example, the surface adhesins displayed by strains of *S. aureus* are all grouped into a single family named microbial surface components recognising adhesive matrix molecules (MSCRAMMs). However, the role of each of these MSCRAMMs is specific to the virulence and pathogenicity of the strain; those expressing greater quantities of fibronectin and fibrinogen binding proteins (FnbpA and -B) having greater affinity to skin tissues and are commonly associated with abscesses and infective endocarditis, and those expressing adhesins for sialoprotein, collagen and fibronectin holding greater affinity to bone tissue and are commonly associated with infectious arthritis (Tristan et al., 2003). It is that specificity for particular proteins and molecules that is an important factor when considering the components of conditioning films as bacteria could potentially have preferential binding to the molecular component of the conditioning film.

Investigations into the pre-treatment of surfaces to resist the absorption of proteins and molecules, and therefore reduce bacterial burdens has demonstrated some positive results. Polyethylene glycol polymer brushes which are often considered the gold standard for antifouling surfaces, are a modification which has

previously demonstrated excellent resistance to protein adsorption and subsequent microbial adhesion (Park et al., 1998; Shaffer et al., 2015). However, the results are often species-specific demonstrating that this is not a one size fits all answer to the problem. In a study by Zeng *et al*., (2015) the addition of the polyethylene glycol coatings demonstrated that whilst the coating successfully prevented the colonisation of the surfaces by *S. aureus* and *Pseudomonas aeruginosa*, it was not successful against strains of *Staphylococcus epidermidis*. This was cited as a species-specific adaptation of the *S. epidermidis* cells which produced larger quantities of extracellular DNA that was able to infiltrate the polyethylene glycol coatings and lead to desorption and coating failure. This data demonstrates the difficulties faced when trying to find workable solutions outside of single species assessments of surfaces. This also demonstrates the importance of considering what sort of environment, and therefore what conditioning film a surface may come into contact with and assess the surfaces accordingly.

4.1.2 Retention Assays with Conditioning Films

Due to the interactions between the molecular content of the conditioning film and the bacterial adhesins which mediate adhesion, the method of application of a conditioning film to a surface should also be deliberated upon prior to microbial assessment of the surfaces.

Surfaces utilised in high contact areas of the food industry would come into contact with relevant conditioning films and bacteria with regularity and undergo multiple cleaning protocols throughout the day whilst environmental surfaces may only undergo cleaning protocols once per week (Gibson et al., 1999) leading to greater propensity of mature biofilm growth. The surfaces would never be encountered by bacterial cells in a pristine state. Contamination onto the high contact surfaces may come from either products and packaging sliding over the surface or from indirect vector transference from the environment. As such, microbial contaminants are likely to have already come into contact and been conditioned by molecules from their surroundings prior to their arrival upon the surface.

Similarly, surfaces utilised in the medical industry, particularly for external fixation, should not come into contact with microbes prior to the insertion of the metal into the body. Bacterial contamination of external fixation devices is most commonly sourced post operatively at the pin site which creates a permanent interface between the external skin and the internal soft tissues (Jennison et al., 2014). For bacteria entering the pin track site, it would come into contact with secretions and plasma proteins which would condition the bacterial cells and would already have conditioned the metal pin.

There are two main approaches which could be utilised for the application the conditioning films to test surfaces. The difference between the two are based around what point the bacteria is introduced to the molecular content of the conditioning film. Some studies (Moreira et al., 2017; Slate et al., 2019) have investigated conditioning film parameters upon bacterial retention through adding the conditioning film to the surfaces and allowing it to dry before inoculating them with the bacterial strains. Whilst this method does allow investigation into the precise interactions of the bacteria to the molecular makeup of the conditioning film through adhesin responses, it does not however replicate real world interactions between bacteria and surfaces as it assumes that bacterial cells are washed and unconditioned. It also risks masking many of the surface's physical and chemical parameters by producing overly thick hard dry films on a surface.

The second approach utilised (Saubade et al., 2018) mixes the conditioning film into the bacterial culture and then performs retention assays by submerging the surfaces into the mixture which still allows the chemical and physical features of the surfaces to interplay with bacteria. However, it should be noted that as much as the binding sites of bacteria can adsorb to molecules in conditioning films on a surface and increase bacterial adherence, molecules in liquid can also attached to the binding sites on the planktonic bacterial cell's surface and potentially reduce the ability of bacteria to attach (Berne et al., 2018). This highlights the importance of fully assessing how the surface is going used to allow for appropriate assay choice.

4.1.3 Chapter Aims

This aim of this chapter was to assess the five surfaces for potential antimicrobial properties and to measure the ability of bacteria to retain to them. The retention assays would be performed both with and without a conditioning film in an attempt to assess the effect that the conditioning film may have bacterial attachment and adhesion to surfaces.

4.2 Methods

4.2.1 Maintenance of Microbiological Cultures

The microorganisms S*taphylococcus aureus* NCTC 10788 and *Escherichia coli* NCTC 10418 continued to be used for microbiological assays in the extended study. As previous stock cultures were stored at -80 °C. Cultures were thawed as required and inoculated onto nutrient agar (Oxoid, UK) media and incubated overnight at 37 °C. Stock cultures were re-frozen following use. To maintain cell physiology, inoculated plates were replaced every 4 weeks and stored (4 °C) in a fridge.

4.2.2 Preparation of Cultures for Microbiological Assay.

Cultures were prepared as per method 2.1.9 (preparation of cultures for microbiological assay) except that colonies of *S. aureus* and *E. coli* were inoculated into 10 mL of sterile nutrient broth (Oxoid, UK).

4.2.3 Zones of Inhibition

Coupons which had been previously cleaned were aseptically adhered to the bottom of sterile Petri dishes with double sided sticky tape (Guilbert Niceday, UK) and 25 mL of moulted nutrient agar (Oxoid, UK) was poured over the top of the coupons and allowed to solidify. Cell suspensions were prepared (Method 4.2.2) (between 10^5 and 10^6 CFU/mL) and 100 µL was spread over the surface of the nutrient agar. The samples were then incubated at 37 °C overnight before the zones of inhibition were measured using digital Vernier callipers (Mitutoyo CD- 6” CP, Japan). Three coupons were tested per run and the tests performed in duplicate (n = 6).

4.2.4 Cell Viability Assay (Nitro tetrazolium violet)

The cell viability assays were performed as per Method 2.2.12 utilising nutrient agar (Oxoid, UK) instead of brain heart infusion. Three coupons were tested per run and the tests performed in duplicate (n = 6).

4.2.5 Retention Assay

Retention assays were performed as described in Method 2.2.13 (n = 6) with microorganisms prepared using Method 4.2.2. Cell-F software was used to visualise and capture the images (n = 60). Fiji Image J software was used to calculate percentage coverage of the coupons using the auto thresholding function with a visual assessment to ensure that all the appropriate areas had been correctly detected, correcting manually if required. False colouring to the black and white images was also added using Image J software.

4.2.6 Retention Assay - Adsorption of Conditioning Films

The methods for a retention assay (Method 4.2.5) were adapted and modified to include 10 % bovine plasma solution (conditioning film) rather than the bacterial cell solution. The conditioning film solution was prepared using commercially available bovine plasma (Sigma Aldrich, UK) and diluted to the appropriate concentration (10 %) (v/v) by adding 3 mL of 100 % plasma to 27 mL of sterile purified water. Once dry, the surfaces were ready for analysis.

4.2.7 Retention Assay - Adsorption of Conditioning Films with Bacteria

The method for the retention assay (4.2.5) was modified to include the addition of a 10 % bovine plasma conditioning film with the bacterial cells. The solution of bacteria and bovine plasma (10 %) was produced by mixing 27 mL of previously prepared bacteria (OD = 1.05 nm) with 3 mL of 100 % bovine plasma (Sigma Aldrich, UK). Once dried, the surfaces were ready for analysis.

To allow for visualisation of both the conditioning film and the bacteria with an epifluorescence microscope, the samples were stained using a dual staining method from Whitehead *et al,* 2009. The conditioning film was stained using 5 µl of 0.1 mg/mL solution of Rhodamine B (Molecular probes, ThermoFisher, USA) and the

bacteria was stained using 5 µl of 1 µg/mL solution of 4', 6-Diamidino-2-Phenylindole Dihydrochloride (DAPI) (ThermoFisher, USA) solution, and then visualised using an epifluorescence microscope (Nikon Eclipse E600, Tokyo, Japan) at 560- 580 nm, and 358 – 461 nm wavelengths respectively. Images were captured using Cell-F software. Fiji Image J software was used to overlay the dual colour images and calculate the percentage coverage of the coupons through the automated thresholding function, with visual inspection to ensure that the appropriate areas have been correctly detected and manually adjusted if necessary.

Percentage coverage was chosen as a more appropriate method of assessment in comparison to the individual cell counts which were performed in Chapter 2 due to difficulties encountered in distinguishing cells apart when they were clumped together. It should also be noted that in this extended study, there were a greater number of surfaces to assess under a greater number of parameters which would not have been time efficient.

4.3 Results

4.3.1 Zones of Inhibition

Zones of inhibition assays were performed upon the cleaned surfaces to assess any antimicrobial action and leaching potential the surfaces might have had upon *S. aureus* and *E. coli*. Qualitative and quantitative assessment of the surfaces demonstrated that none of the surfaces produced a zone of inhibition against either bacterial species.

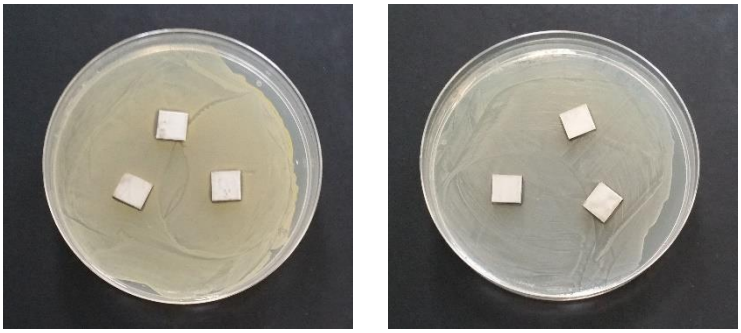


Figure 53: Example of Zones of inhibition assays used for qualitative assessment demonstrating no zones a) TiN/15.03at.%Ag against *S. aureus,* b) 316L against *E. coli.*

4.3.2 Nitro Tetrazolium Violet Assays

Nitro tetrazolium violet assays were performed using *S. aureus* and *E. coli* on the metal surfaces and growth was measured through colony counts of the respiring cells highlighted by the tetrazolium violet. The assays demonstrated that the *S. aureus* was not killed or inhibited by any other surfaces except for the TiN/15.03at.%Ag surface, and respired and increased upon the 316TiN surface. Statistical analysis of the data demonstrated that there was no significant difference between the 316L stainless steel, titanium and 316Ti stainless steel surfaces, but there was between the 316L stainless steel, 316TiN and TiN/15.03at.%Ag surfaces.

In contrast, the *E. coli* did not grow as well on any of the other surfaces and failed to grow at all on the 316Ti surface despite multiple attempts. The TiN/15.03at.%Ag surface also demonstrated an antimicrobial effect upon this bacterium. Due to the small figures obtained for all metals even when growth was apparent, there was no significant difference between any of the surfaces.

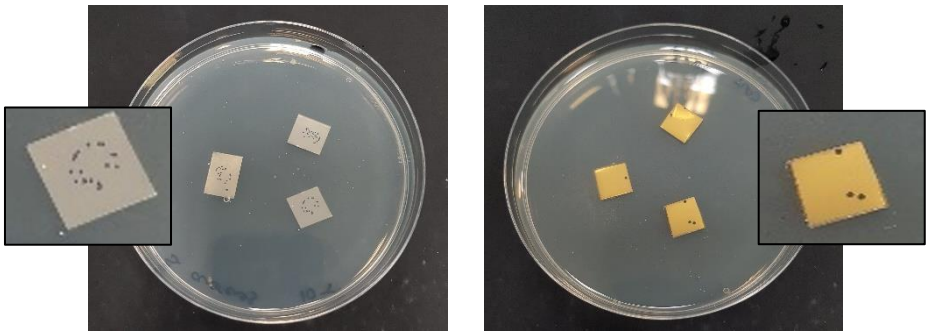


Figure 54: Example of NTV assay. a) *S. aureus* on titanium, b) *E. coli* on 316TiN, both with magnified cut-outs demonstrating the re-dox reaction illustrating the respiring colonies which were counted.

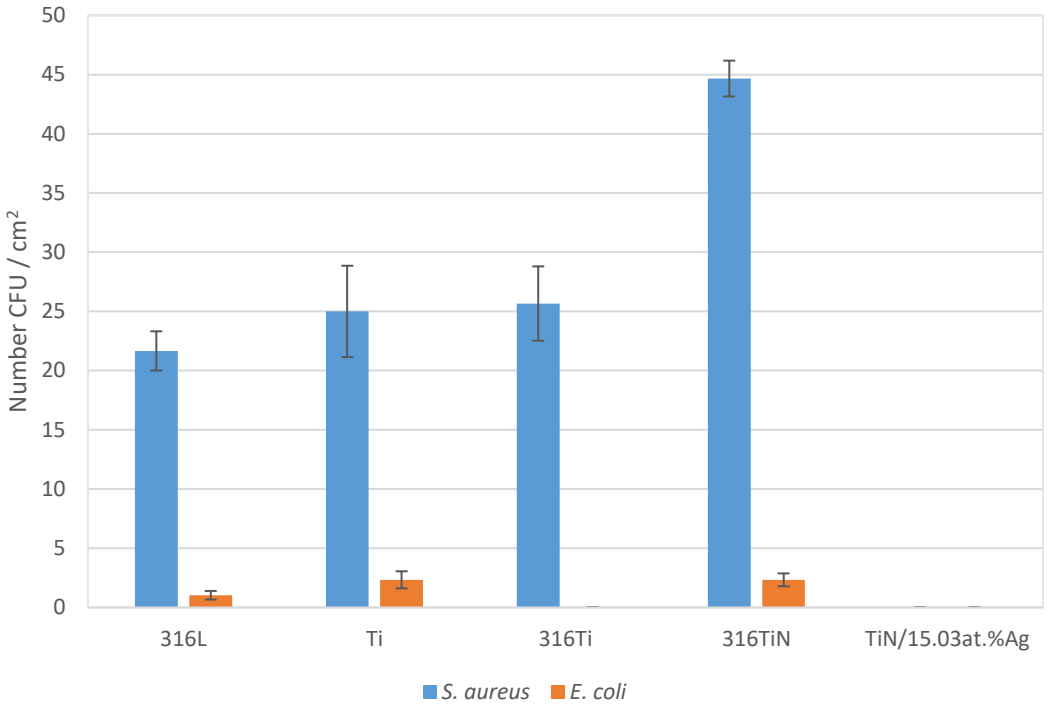


Figure 55: NTV assays demonstrating the numbers of *S. aureus* and *E. coli* colonies present when in direct contact with the metal surfaces ± standard error (n = 6).

4.3.3 Retention Assays

Retention assays of *S. aureus* and *E. coli* were performed both in the absence and presence of 10 % bovine plasma to enable the quantification of bacteria cells that had retained upon the five metal surfaces (Figure 58) and allow comparison between the conditioning treatments. The numbers of retained cells were observed through epifluorescence microscopy (Figure 56) and calculated from their percentage coverage.

The retention assays were first performed in the absence of the 10 % bovine plasma conditioning film. The numbers of *E. coli* retained upon the 316L stainless steel, titanium, 316Ti, 316TiN and TiN/15.03at.%Ag surfaces were significantly higher than the numbers of *S. aureus* retained (% increase in surface coverage = 7.35 %, 11.35 %, 2.35 %, 9.73 %, and 1.57 % respectively) demonstrating a difference in the retention preference between the species of bacteria. The numbers of retained *S. aureus* upon the different metals demonstrated that the bacterial cells had a

greater affinity towards the titanium, 316Ti and 316TiN surfaces. Statistical analysis demonstrated no significant difference in the numbers of retained *S. aureus* upon these three metals. However, analysis did demonstrate that the 316L stainless steel and TiN/15.03at.%Ag surfaces did retain significantly fewer bacteria compared to the titanium (p = < 0.00), 316Ti (p = < 0.00), and 316TiN (p = < 0.00) surfaces. The analysis of the numbers of *E. coli* retained onto the surfaces demonstrated that it also had a greater affinity towards the titanium and 316TiN surfaces, but did not maintain the same trend as the *S. aureus* upon the surfaces showing a greater affinity towards the 316L stainless steel than *S. aureus* had. Statistical analysis of the data demonstrated that there was no significant difference in the numbers of retained *E. coli* between the titanium and 316TiN surfaces (p = 0.754). The TiN/15.03at.%Ag surface produced the lowest percentage coverage against both the *S. aureus* (0.32 % ± 0.02 %) and *E. coli* (1.89 % ± 0.09 %) of all the metals.

Assessment of the numbers of *S. aureus* retained to the surfaces in the presence of 10 % bovine plasma (Figure 57 and 59) demonstrated that when co-adsorbed onto the surfaces, the *S. aureus* cells retained in the greatest numbers to the 316TiN surface and 316L stainless steel (0.83 % ± 0.17 & 0.81 ± 0.08), followed by the titanium and 316Ti surfaces (0.74 % ± 0.11 & 0.57 % ± 0.06). The TiN/15.03at.%Ag surfaces had the smallest percentage coverage of *S. aureus* cells, and was the only surface to demonstrate a significant difference to the 316L, titanium and 316TiN surfaces (p = 0.003, p = 0.014, & p = 0.002).

The addition of the *E. coli* with 10 % bovine plasma (Figure 60) to the surfaces demonstrated that there was no statistically significant difference in the retention of the *E. coli* between any of the surfaces. The titanium surfaces demonstrated the greatest percentage coverage (0.32 % ± 0.07) with the 316TiN and TiN/115.03at.%Ag surfaces retaining slightly less (0.32 % ± 0.05 0.32 % ± 0.04). The 316Ti and 316L stainless steel surfaces retained the fewest numbers of *E. coli* (0.25 % ± 0.03 & 0.21 % ± 0.08). None of the surfaces demonstrated any significant difference in the numbers of *E. coli* with 10 % bovine plasma.

Statistical analysis of the numbers of *S. aureus* retained upon the 316L stainless steel, titanium, 316Ti and 316TiN surfaces demonstrated that they were

significantly higher than the numbers of *E. coli* retained after retention assays with the 10 % bovine plasma conditioning film (p = < 0.00). This demonstrated that *S. aureus* had a greater affinity to the metal surfaces when co-adsorbed with the 10 % bovine plasma than the *E. coli*.

Assessment of what impact the addition of the 10 % bovine plasma had upon the retention of the bacterial species was made through comparisons to the results obtained through analysis of the bacterial species without conditioning film. Statistical analysis of the percentage coverage demonstrated that the titanium, 316Ti, and 316TiN surfaces had a significant reduction in both the *S. aureus* and *E. coli* with the 10 % bovine plasma (p = 0.00). However, the 316L stainless steel and TiN/15.03at.%Ag surfaces only exhibited a significant reduction in the numbers of *E. coli* retained to their surfaces (p = 0.00). This overall demonstrated that despite the greater numbers of *S. aureus* in comparison to *E. coli,* all the surfaces had a reduction in the numbers of both bacterial species in the presence of the 10 % bovine plasma in comparison to after the retention assays of just bacteria alone.

Calculation of the percentage coverage of the conditioning film was made in a similar way to the quantification of bacterial cells with the aid of a duel staining technique upon the surfaces. The quantities of conditioning film present upon the different metal surfaces significantly altered dependent upon which bacterial species it had been co-absorbed alongside. The data collected suggested that the surfaces had more conditioning film when in the presence of the *E. coli,* with the exception of the TiN/15.03at.%Ag surfaces were no statistical difference was observed. So overall, the coverage of the conditioning film upon the surfaces was dependent upon the bacterial species was present. However, the amounts of conditioning film detected appeared low, and analysis of the surfaces with conditioning film alone without the bacterial species was not successful.

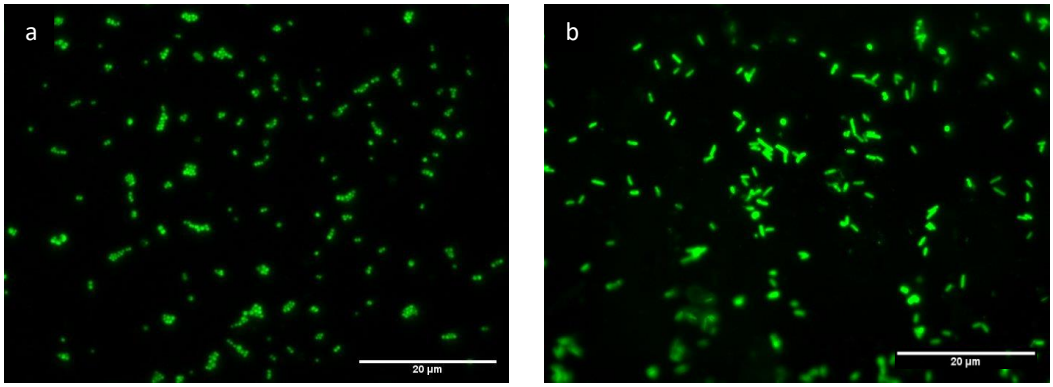


Figure 56: Example of typical epifluorescence images obtained for quantitative measurement of bacterial retention a) *S. aureus* retained upon 316L stainless steel, b) *E. coli* retained upon 316L stainless steel.

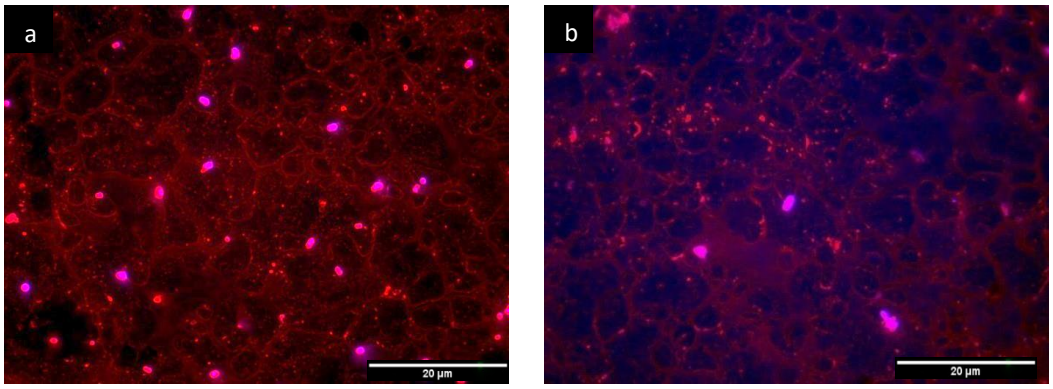


Figure 57: Example of dual colour epifluorescence images obtained to allow for quantitative measurement of bacterial and conditioning film retention. The pink colours demonstrate the presence of bacteria whilst the red colouring in the background demonstrates the presence of conditioning film adsorbed onto the surface: a) *S. aureus* with 10 % bovine plasma retained upon 316L stainless steel, b) *E. coli* with 10 % bovine plasma retained upon 316Ti stainless steel.

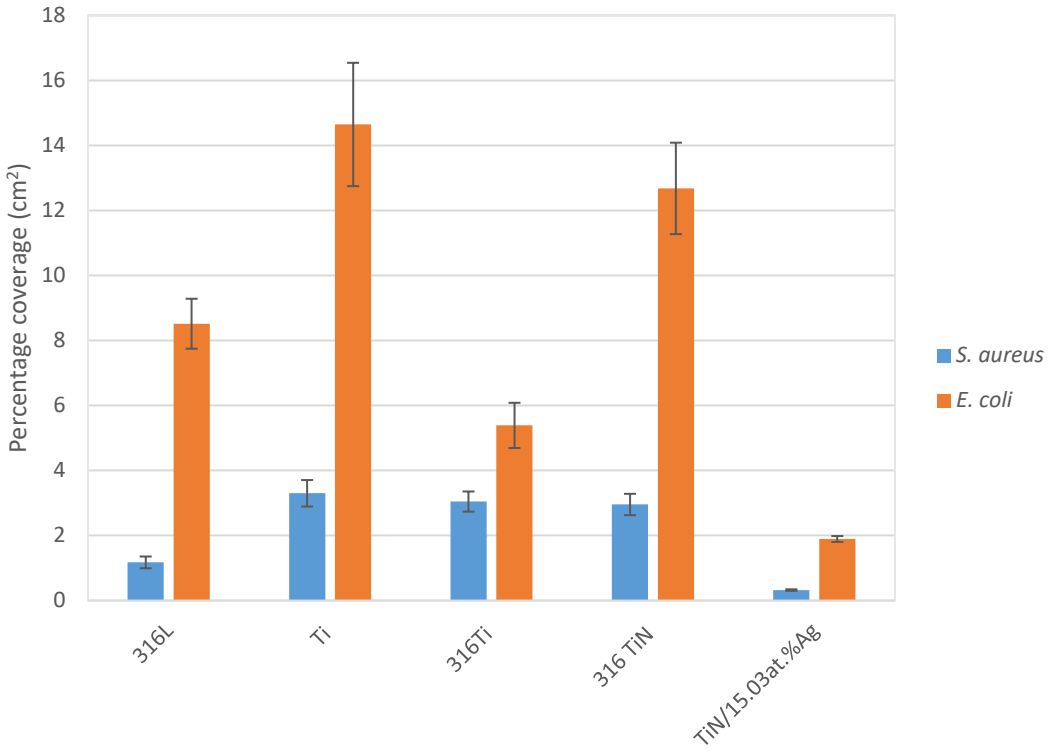


Figure 58: Percentage coverage of *S. aureus* and *E. coli* on the five metal surfaces post retention assay ± standard error (n = 60).

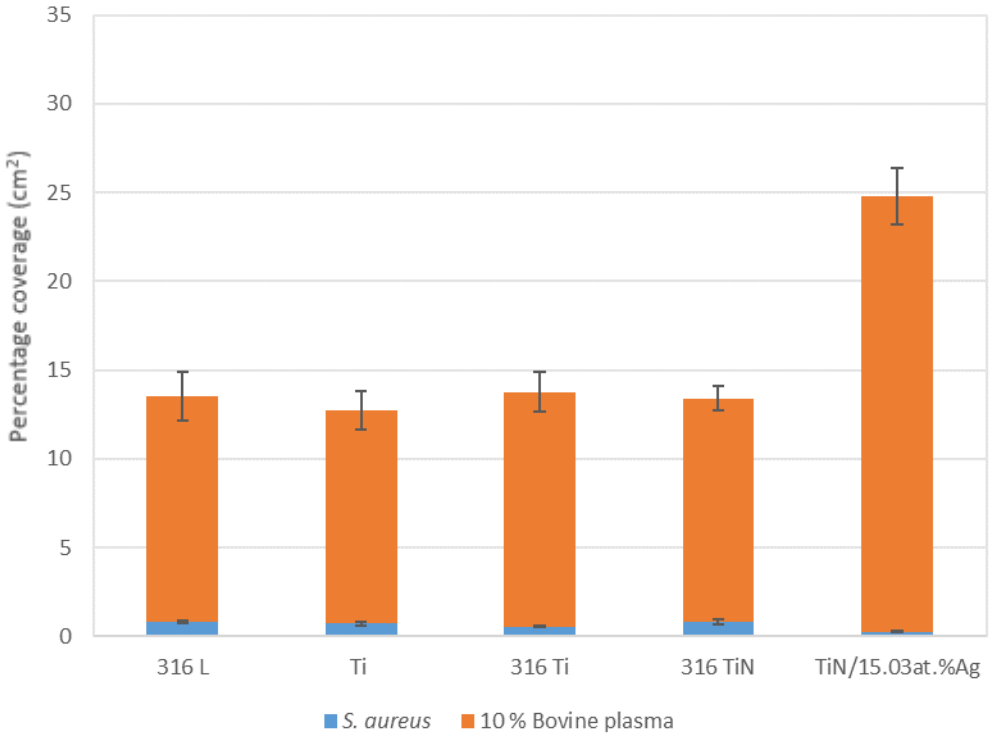


Figure 59: Percentage coverage of *S. aureus* with 10% bovine plasma on the different metal surfaces ± standard error (n = 60).

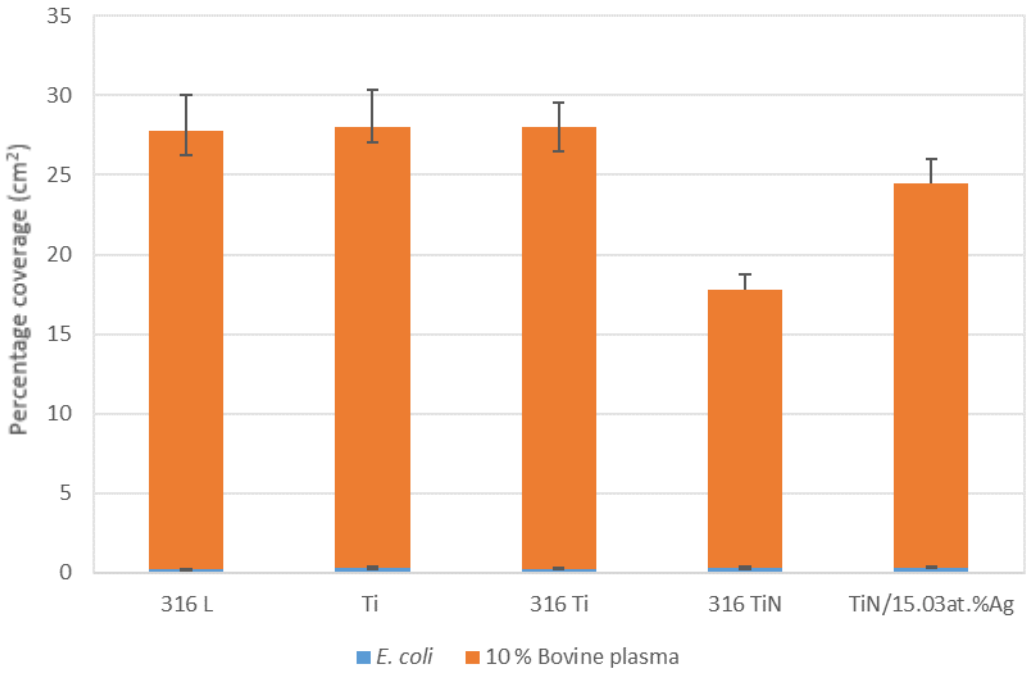


Figure 60: Percentage coverage of *E. coli* with 10% bovine plasma on the different metal surfaces ± standard error (n = 60).

4.4 Discussion

The assessment of surfaces for their potential to retain bacteria which could potentially cause infection and disease is important when assessing which surfaces are a suitable choice for their intended purpose. In this chapter the five surfaces were assessed for their antimicrobial efficacy and for their propensity to retain bacteria to their surfaces both in the absence and presence of 10 % bovine plasma conditioning film.

Assessment of the surface's antimicrobial efficacy was performed utilising zones of inhibition and nitro-tetrazolium assays which provided insights into their leaching potential and contact kill efficacy. Results of these assays demonstrated that no zones of inhibition were produced by any of the surfaces against either of the bacterial species. This result is similar to a study by Whitehead *et al*., (2011) in which surfaces of a similar composition, manufacture and silver content (TiN/16.07at.%Ag) were assessed for their antimicrobial action against *S. aureus* and *P. aeruginosa,* producing no zones of inhibition. Similarly, works by Ahearn *et al*., (1995) reported that negligible zones of inhibition were produced by ion beam

deposited silver surfaces. This may be as a result of the concentration of silver content in the surfaces which may be too small to affect the viability of the bacteria, or that ions were not successfully leaching from the surface (Whitehead, Li. H., et al., 2011). Results gained in Chapter 2 supported the former statement of causation due to the ability of the TiN/25.65at.%Ag surface to produce a zone of inhibition against the *E. coli* strain. However, its inability to affect the growth of *S. aureus* also suggests a species-specific response to the antimicrobial action, most likely due to the composition of the Gram-positive cell wall.

Examination of the NTV results demonstrated that only the TiN/15.03AT.%Ag surface demonstrated any antimicrobial action when in contact with the bacterial species. None of the other surfaces demonstrated any antimicrobial action, although the *E. coli* did not grow as well upon the surfaces as the *S. aureus* when overlaid with agar, and we were not able to get the *E. coli* to grow upon the 316Ti and TiN/15.03at.%Ag surfaces despite multiple attempts. Some research has reported the bacteriostatic qualities of titanium surfaces (Leonhardt and Dahlén, 1995) against strains of oral bacteria, however, others have refuted such claims (Elagli et al., 1992). Considering this data, the lack of growth upon some of the surfaces is not thought to be a result of antimicrobial activity but a problem in methodology and this strain for *E. coli.*

Studies into the effects of conditioning films upon the antimicrobial efficacy of nanocomposite silver content surfaces have demonstrated that the results vary dependent upon the method of administration of both the conditioning film and bacterial species. In works by Slate *et al*., (2019) the authors pre-treated Zirconium-nitride silver composite surfaces with a whole blood conditioning film and allowed it to dry in a class II cabinet prior to the application of the bacterial species. Their study demonstrated a reduction in the antimicrobial activity of the surface, suggesting that the layer of dried blood prevented moisture from reaching the silver and therefore preventing the release the silver ions from the nanocomposite material. Similarly, works by Saubade *et al*., (2018) attempted to assess the effect conditioning films upon the antimicrobial efficacy of titanium-nitride silver composite surfaces, also demonstrating that surfaces pre-treated with whole blood

and allowed to dry prior to application of bacteria had reduced antimicrobial efficacy against *S. aureus* and *S. epidermidis*. However, surfaces similarly pre-treated with bovine serum albumin did not reduce the antimicrobial efficacy against *S. epidermidis*, suggesting that the bovine serum albumin acted as an adjunct for the effect of the antimicrobial silver. These works demonstrate the importance of selecting the correct application method for assessments of conditioning films dependent upon the usage of the metal, and that selection of the appropriate conditioning film is critical due to individual species responses. Whilst the effect on the conditioning film upon the antimicrobial efficacy of the surfaces within this study was not assessed, these works demonstrate the importance of this assessment and so this should be studied in any future works.

Quantification of the numbers of bacteria present after retention assays onto the pristine surfaces demonstrated how the bacterial species interacted with the surfaces prior to the addition of conditioning film. This would provide a baseline from which we could assess what effect was exerted by the addition of the 10 % bovine plasma. The results demonstrated that the *E. coli* retained to the surfaces in greater numbers than the *S. aureus,* however, it should be noted that the size of the *E. coli* is larger than the size of the *S. aureus* cells which would not be accounted for by this metric of measurement. Assessment of the surfaces with the samples of *E. coli* retained demonstrated greater coverage of the titanium, 316L and 316TiN surfaces, with fewer bacteria retained to the 316Ti and TiN/15.03at.%Ag surfaces. The *S. aureus* retained to the surfaces also demonstrated preference towards the titanium and 316TiN surfaces, but also demonstrated a preference towards the 316Ti surface. Fewer *S. aureus* retained to the 316L and TiN/15.03at.% surfaces.

These assessments of the pristine surfaces do not differentiate between live and dead cells adhered to the surfaces, acting simply as a total count of cells retained. For this reason we cannot assess the efficacy of the antimicrobial action of the TiN/15.03at.%Ag surface from this data, it does however demonstrate that this surface had the greatest anti-adhesive / repellent properties towards both bacterial species. This data is comparative to a study by Whitehead *et al*., (2011) in which the authors assessed the percentage coverage of bacteria to TiN and TiN/Ag

(concentrations = 4.6 %, 10.8 % and 16.7 % Ag) surfaces through the same method of retention assay as utilised by this study. The authors reported that the numbers of *S. aureus* retained to the TiN surface were higher than the TiN/Ag surfaces, which decreased with the incremental increase in silver content. However, this study also noted that despite an initial reduction in the numbers of *P. aeruginosa* with the TiN/Ag surfaces, the bacteria then retained in greater numbers towards the surfaces with a higher content of silver. This demonstrates that despite the benefits which can come from surface modifications, caution must also be taken as not all bacterial species will demonstrate the same responses to the surfaces.

The numbers of the bacterial species retained to the titanium surfaces were the greatest of all the surfaces assessed in this study which was an unexpected result when compared to other works (Cheng et al., 2007; Veerachamy et al., 2014) which reported that titanium surfaces typically demonstrated properties which could lead to lower rates of infection than stainless steel surfaces when used as a biomaterial. This result highlighted the often-contradictory results demonstrated from these types of studies into bacterial retention and adhesion to surfaces for use in the food and medical industries. It is likely that the finish or grade of titanium utilised in this study differed greatly enough from others to render direct comparison of the results impossible (Bekmurzayeva et al., 2018).

The quantities of the bacterial species retained to the 316L stainless steel demonstrated that the species interacted with the surface in opposing ways, with the *S. aureus* retaining in low numbers and the *E. coli* retaining in significantly higher numbers. It is possible that the bacteria responded to the surface differently due to parameters such as physicochemistry or topography which will be examined in the final discussion (Chapter 6). Species-specific adaptations such as the flagella of the *E. coli* may also be a factor in differing adhesion results, with the *E. coli* utilising the flagella in order to anchor to the surface in a way that the *S. aureus* is unable to (Friedlander et al., 2013; Belas, 2014).

The results obtained from retention assays of the bacterial species in the presence of a conditioning film demonstrated a significant reduction in the coverage of both species, particularly the surfaces retained with *E. coli*. Analysis of the data

demonstrated that *S. aureus* retained in the greatest numbers to the 316TiN and 316L stainless steel surfaces, followed by the titanium and 316Ti surfaces, and retained in the fewest numbers to the TiN/15.03at.%Ag. The retention assays of *E. coli* in 10 % bovine plasma demonstrated that due to the low numbers of cells retained to the surfaces, no singular surface had a significantly higher or lower number of cells upon it. Statistical analysis between the bacterial species demonstrated that there was greater percentage coverage of *S. aureus* cells retained upon the 316L stainless steel, titanium, 316Ti and 316TiN surfaces than *E. coli* cells, confirming that the *S. aureus* exhibited a greater affinity towards attachment to the surfaces when co-absorbed in 10 % bovine plasma compared to the *E. coli*.

This conditioning film mediated reduction in bacterial numbers validates other similar works assessing the effects of conditioning films upon the adhesion of bacteria to surfaces. However, at the time of writing we were unable to find published works utilising bovine plasma as the conditioning film source to offer full comparison due to the possibility that the different molecular components of the conditioning films could promote different adhesin mediated binding to the surfaces. Similar works utilising blood component conditioning films and single protein sources have demonstrated mixed results. In a study by Saubade *et al*., (2018) the authors utilised bovine serum albumin and whole horse blood as conditioning agents against *S. aureus* and *S. epidermidis* utilising the same method of application of bacteria to conditioning film as used in this study. Their results demonstrated that as with this study, the addition of both the conditioning agents reduced the amounts of bacterial fouling upon the surfaces with the exception of the *S. epidermidis* when assessed in conjunction with bovine serum albumin upon stainless steel which demonstrated an increase in adhesion which was not demonstrated by the *S. aureus.* This individual species response is likely to be adhesin mediated due to the different composition and quantity of MSCRAMMs presented by the *S. epidermidis* and *S. aureus* despite both being *Staphylococcus* species bacteria. Another factor to be considered is the use of bovine serum albumin as singular protein sources from albumin do not fully represent the

intricate interplay of adsorption and replacement of specific molecules from mixed protein sources (Vroman and Adams, 1969; Vroman et al., 1980).

The effects of conditioning films against *E. coli* were assessed in a study by Moreira *et al*., (2017) were the authors assessed various cellular extract conditioning films upon the adhesion and subsequent biofilm formation of *E. coli* 96 well plates and flow cell chambers. The authors demonstrated a reduction in the numbers of *E. coli* when surfaces were conditioned with total cell extract or cytoplasm with cellular debris but increased with the addition of periplasmic extract. Under flow conditions all conditioning films exerted a reduction in biofilm growth in comparison to the control. The use of 96 well plates for static biofilm work is very popular, however it should be noted that the 96 well plate composition of polystyrene is known for forming strong aromatic bonds with molecules, often a preferential trait for some biochemistry work, however, it would affect the ability of bacteria to behave / adhere naturally, and as such it could be argued that this form of work does not provide results which could be applied to real world situations.

Berne *et al*., (2018) hypothesized that it was possible for molecules of conditioning agents to bind to bacterial adhesins prior to surface adhesion, potentially reducing the cells ability to attach to surfaces. As such, the reduction in the numbers of bacteria to the surface demonstrated in this study may be due to the co-absorption of conditioning film molecules to the adhesins located on the cell surface preventing the bacteria from utilising the adhesins for binding purposes to the surface. However, it may also be possible that the addition of the conditioning film to the surface modified the physicochemistry creating a less desirable surface for bacterial attachment and adhesion.

Quantification of the amounts of conditioning film retained upon the surfaces after retention assays demonstrated that the results obtained varied dependent upon which bacterial species was co-absorbed with the conditioning film. Surfaces with *E. coli* and conditioning film retained demonstrated greater quantities in comparison to those with the *S. aureus* retained. This could potentially be due to the differences between the cell surfaces of the two bacterial species. *Staphylococcus aureus* is well documented for its abundance of MSCRAMMs which aid in its

adhesion to proteins and acts as one of its key virulence factors. In particular, *S. aureus* has an abundance of adhesins able to bind to host macro-molecular matrix ligands such fibronectin, fibrinogen and collagen providing increased ability to adsorb the plasma proteins from the aqueous solution which could be occurring during the planktonic stage before retention to the surfaces. Alternatively, it is also possible that the technique utilised in this study is not sensitive enough to measure the fluorescence produced by singular layers of molecules adsorbed to a surface with great enough accuracy. Attempts to assess surfaces with just conditioning film alone were found to be too variable due to the capture camera on the epifluorescence microscope changing aperture in an attempt to autogain the brightness with the lack of brightly fluorescing bacteria to prevent this. As such, it is not possible to assess whether the difference in conditioning films is species related or a limitation in the study methodology.

The work in this chapter highlighted the importance of the assessment of surfaces for their antimicrobial action and for their propensity to retain bacteria, demonstrating that some surfaces do retain greater numbers than others tested and as such the choice of surface utilised should be informed by its suitability for purpose. This study also demonstrated the importance of conditioning films, demonstrating the effect they may have upon the retention of bacteria to surfaces, and importantly, how this effect is largely mediated by the bacterial species present.

Chapter 5

Characterisation of the Chemical Parameters of the Surfaces

5.1 introduction

Measurement of the chemical parameters of surfaces allows for assessments to be made regarding the potential driving factors responsible for bacterial adhesion to them. Through assessments of the physicochemical parameters of the surfaces in conjunction with those of the bacteria and retention assays, we are able to look for trends in adhesion behaviour and potentially look at ways to reduce it.

5.1.1 Physicochemical Factors Affecting Adhesion

Adhesion of molecules and bacteria to surfaces is an important stage in the process of infection and spread of pathogenic microorganisms and is a mediating factor for the formation and growth of biofilms (Tuson and Weibel, 2013; Flemming et al., 2016). In an aqueous environment, cells and molecules first approach a surface though natural forces such as Brownian motion, diffusion or gravity, and once within a closer proximity physicochemical parameters would come into play (Whitehead and Verran, 2009). Physicochemistry is a major influencing factor governing the initial attachment and adhesion of bacteria to surfaces, predominantly the van der Waals interactions and Lewis acid-base interactions which govern the hydrophobic component of a surface, and the electrostatic forces (surface free energy) (Bellon-Fontaine et al., 1996).

Hydrophobic / hydrophilic surface interactions are polar interactions governed by largely by Lewis acid-base interactions (van Oss et al., 1988; Van Oss and Giese, 1995). These encompass two parameters; the electron accepting potential and the electron donating potential. It is these parameters which describe the polar charge of the surface tension component (Van Oss, 1995). Low energy Lifshift van der Waals forces are also a component part of hydrophobic / hydrophilic interactions. However, as materials in aqueous environments tend to contain lower energy components and surface tension values similar to that of water, the Lifshift van der Waals forces are not able to act upon them. Lifshift van der Waals forces play a greater role in the bonding of molecules to surfaces once the Lewis acid-base forces have acted upon the molecules in question and expelled the interstitial water (Van

Oss, 1995). For that reason, Lifshift van der Waals forces are often considered short range.

Whilst the measurement of a surface's physicochemistry is important, measurements of the physicochemistries of the bacterial cells are also of integral importance when assessing the propensity at which a surface may be fouled and when attempting to reduce or prevent microbial fouling (Bellon-Fontaine et al., 1996; Zeraik and Nitschke, 2012). The surface of a bacterial cell is thought to comprise of islands of different physicochemistries due to the differing chemical species of the molecular components that make up the cell surface (Whitehead and Verran, 2009). It is the interaction of the physicochemistry of both the cell and the surface which are thought to determine adhesion behaviour.

However, studies into the effect of surface physicochemistry and bacterial retention are varied, with some studies reporting a link between hydrophobic substrata and greater propensities of bacterial adhesion (Marin et al., 1997; Zita and Hermansson, 1997; Sinde and Carballo, 2000) whilst other authors have not found this link (Chae et al., 2006). These variations in results are likely to be due to individual species preference towards the physicochemical parameters.

Modification of surface properties is often used in an attempt to produce hydrophilic and antifouling surfaces but, since every fouling environment is unique to the purpose of the surface, it is unlikely that one surface could be created to meet all the individual requirements and work successfully against multiple bacterial species (Whitehead and Verran, 2009). The surface properties of these antifouling surfaces also risk being altered by the presence of conditioning films which may render the desired effects of the surface temporary (Rana and Matsuura, 2010).

A conditioning film is produced through the adsorption of molecules to a surface when submerged in an aqueous environment, the composition of which would be unique to the environment it encounters. The adsorbed molecules upon the surface are able to modify the surface parameters, potentially promoting or inhibiting further fouling events and the adhesion of microbes to the surface (Briandet et al.,

2001). For this reason it is essential to assess surface parameters in the presence of an appropriate conditioning film, as measurements made upon pristine surfaces would not give an accurate portrayal of a surface in an applied state (Briandet et al., 2001; Bernbom et al., 2006). Works into surface modifications that may repel the adsorption of conditioning film layers to polyethylene glycol surfaces have had some success (Park et al., 1998; Shaffer et al., 2015), however, the surfaces are yet to be proven affective against all bacterial species (Zeng et al., 2015) and are far from a one size fits all answer to surface fouling.

5.1.2 Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFTS)

The use of FTIR techniques for biological and diagnostic processes is increasing due to speed and accuracy of the results (Paraskevaidi et al., 2018). The ability of the technique to identify and differentiate between different forms of pathogenic bacteria has led to the technique being utilised in clinical settings for the assessment of human pathogens (Zarnowiec et al., 2015) as well as the detection of potentially cancerous tissues during surgery (Argov et al., 2002; Romeo et al., 2003).

In FTIR, a wide spectrum beam of infrared light is passed through (or reflected from) a sample. Electrons in the sample can absorb some of this beam at specific wavelengths (energies / wave numbers / etc). The remaining light is collected and processed by a computer algorithm to produce an absorption spectrum (Schmitt and Flemming, 1998).

These qualities enable this technique to be used to assess microbial retention and organic fouling upon the surfaces and is sensitive enough to detect molecules adsorbed to surfaces, as well as their structure and conformation (Movasaghi et al., 2008; Glassford et al., 2013; Yang et al., 2015). When a protein molecule is made to orientate more of its hydrophobic sites towards a surface due to adsorption to a hydrophobic interface it demonstrates more α-helices in its secondary structure, whereas proteins adsorbed to a hydrophilic surface demonstrate fewer. It is thought that proteins which demonstrate α-helicity interact strongly with

hydrophobic surfaces, as so it would appear as though stronger α-helicity correlates with increased surface hydrophobicity (Dathe et al., 1996; Sanders et al., 1996). However it has been noted that proteins which demonstrate strong hydrophobicity prior to adsorption to hydrophobic surfaces can be prompted to convert their α-helicity to β-sheet conformation due to hyper-hydrophobicity (van Oss, 1997). This ability to identify the structure and conformational changes to the secondary configuration of protein structures once adsorbed onto surfaces through FTIR could be utilised to assess the effects of protein adsorption upon the physical parameters of surfaces.

5.1.3 Chapter Aims

The aim of this chapter was to characterise the chemical properties of the surfaces and through assessment of the surfaces in their pristine state, compare how the addition of the bacterial species, conditioning film, and conditioning film with bacterial species changes these parameters.

5.2 Methods

5.2.1 Energy-dispersive X-Ray Spectroscopy (EDX)

Analysis of the elemental content of the surfaces was performed as per Method 2.2.5. Three areas of three coupons (n = 9) were analysed and the data compiled demonstrating the average ± standard deviation.

5.2.2 Physicochemistry

Contact angle measurements for the surfaces (pristine, with bacteria, with conditioning film, or with conditioning film and bacteria) were determined using the sessile drop technique described in Method 2.2.10 The surface conditioning of the samples was prepared prior to analysis (Methods 4.2.5, 4.2.6 & 4.2.7) on 30 x 50 mm metal coupons (n = 6).

5.2.3 Microbial Adhesion to Hydrocarbons Assay (MATH) in the Presence of Conditioning Film

The method for the MATH assay (Method 2.2.10) was modified to incorporate the addition of 10 % bovine plasma to the microbial suspensions through the addition

of 3 mL of 100 % bovine plasma to 27 mL of standardised bacterial suspension. The cell suspension was washed three times in PUM buffer and re-suspended at an OD of 1.00 at 400 nm in PBS. Tests were performed twice in triplicate to ensure accuracy (n = 6).

5.2.4 Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFTS)

Diffuse reflectance Fourier transform infrared spectroscopy (DRIFTS) was performed on the sample surfaces (pristine, with bacteria, with conditioning film, or with conditioning film and bacteria) using a Thermo-Nicolet Nexus FTIR spectrophotometer (Thermo Scientific, USA) fitted with a Spectra-Tech DRIFTS cell (Spectra-Tech Inc, USA). The standard DTGS detector was used. The instrument was thoroughly purged with water and CO_2 free air (30 L/min) using a Parker Balston purge gas generator (Parker, USA). The control, recording and data analysis were performed using OMNIC ™ Spectra Software Suite (Thermo Scientific, USA). The spectra were obtained from 164 scans and the resolution was 2 cm^{-1}. The obtained spectra were baseline corrected using Kramers-Kronig baseline correction and the wavelengths limited to between 1900 and 1350 cm^{-1} for easier analysis of the Amide II bands and the peak heights measured. Two areas of three samples were analysed (n = 6).

5.3 Results

5.3.1 Chemical Composition (Energy Dispersive X-ray Spectroscopy Analysis)

EDX analysis was performed on the five metals in order to determine the percentage elemental composition of each. The data obtained (Table 11) indicated that the 316Ti stainless steel surface was similar to the 316L stainless steel surface with higher levels of chromium and nickel present than the other surfaces. Note the relatively low content of titanium (0.40 at.% ± 0.16) present in comparison to the titanium surface, 316TiN, and TiN/15.03at.%Ag surfaces (84.55 at.% ± 0.11, 50.70 at.% ± 0.02, and 46.53 at.% ± 0.01 respectively). The results from the sputter coated

silver containing surface demonstrated a silver concentration of 15.03 at.% with a low standard error suggesting that the coatings were homogenously sputtered across the surfaces. As the penetration depth of the EDX beam was between 1 µm – 2 µm, it would be reasonable to assume that the TiNAg sputter coatings were of an adequate depth due to the low content of iron, chromium and manganese present from the base substrate metal (5.44 at.% ± 0.01 %, 1.61 % ± 0.04 %, and 0.21 % ± 0.02 % respectively).

Table 11: Mean chemical composition (atomic %) of the test coupons ± standard error (n = 3).

	316L	Ti	316Ti	316TiN	TiN/15.03at. %Ag
C	7.36 (± 0.11)	6.98 (± 0.02)	11.98 (± 0.43)	-	-
N	-	8.47 (± 0.13)	-	48.45 (± 0.76)	31.18 (± 0.05)
Si	1.55 (± 0.03)	-	1.04 (± 0.03)	-	-
Mo	1.48 (± 0.03)	-	1.30 (± 0.06)	-	-
Cr	17.25 (± 0.04)	-	15.20 (± 0.03)	-	1.61 (± 0.04)
Mn	1.76 (± 0.04)	-	-	-	0.21 (± 0.02)
Fe	62.27 (± 0.12)	-	61.07 (± 0.10)	-	5.44 (± 0.01)
Ni	8.33 (± 0.08)	-	9.0 (± 0.27)	-	-
Ti		84.55 (± 0.11)	0.40 (± 0.16)	50.70 (± 0.02)	46.53 (± 0.02)
W	-	-	-	0.85 (± 0.76)	-
Ag	-	-	-	-	15.03 (± 0.03)

5.3.2 Physicochemistry of the Surfaces

Physicochemical analysis of the surfaces was performed to determine the surface energies and hydrophobic/hydrophilic characteristics of the five metal surfaces. Following mathematical calculation using the values from the contact angle measurements, the hydrophobicity (ΔG_{iwi}) of the surfaces was determined and demonstrated that 316L stainless steel, titanium and 316TiN surfaces were hydrophobic in nature whilst the 316Ti and the TiN/15.03at.%Ag surfaces were both mildly hydrophilic (Figure 61). The surface free energy (γ_s) was calculated and demonstrated that all the surfaces had a high surface free energy (39.7 mJ/m^2 – 57.6 mJ/m^2), TiN/15.03at.%Ag having the highest (57.62 mJ/m^2). The Lifshitz Van der Waals forces (γ_s^{LW}), and the acid-base (γ_s^{AB}) interactions, both measures of interfacial tension, were determined and described all the surfaces as having

similarly high ɣsLW values (33.6 mJ/m^2 – 42.9 mJ/m^2) and low ɣsAB values (2.3 mJ/m^2 – 14.7 mJ/m^2), with the exception of the two hydrophilic surfaces, 316Ti and TiN/15.05at.%Ag which had slightly higher ɣsAB values (13.7 mJ/m^2 – 14.7 mJ/m^2 respectively). The electron accepting (ɣs+) and electron donating (ɣs-) potential of the surfaces was also calculated and demonstrated that all the surfaces had a higher inclination to be electron donors (6.7 mJ/m^2 – 29.6 mJ/m^2) rather than electron acceptors (0.5 mJ/m^2 – 2.5 mJ/m^2), especially for the hydrophilic surfaces 316Ti and TiN/15.03at.%Ag.

Analysis of the surfaces after retention assays of *S. aureus* and *E. coli* (Figures 62 and 63) assessed how the addition of the retained bacteria affected the surface physicochemistry of the five metals. Following mathematical calculation using the values obtained from contact angle measurements, the hydrophobicity (ΔG_{iwi}) of the surfaces was determined. This demonstrated that the addition of *S. aureus* and *E. coli* significantly altered the nature of the 316Ti (p = 0.04 and *p* = < 0.001 respectively) and TiN/15.03at.%Ag (*p* = 0.04 and *p* = 0.01 respectively) surfaces. This change in the ΔG_{iwi} measurements made the surfaces hydrophobic in nature, having previously been hydrophilic prior to bacterial retention assays. There was no significant difference in how hydrophobic either bacteria made the TiN/15.03at.%Ag surface (*p* = 0.175), but there was for the 316Ti stainless steel surface (*p* = 0.039) with the *E. coli* making the surface the most hydrophobic (-77.10 mJ/m^2). The addition of the bacterial species also produced a significant difference to the hydrophobicity of the 316L stainless steel surface. Whilst it maintained its hydrophobic nature, the addition of *E. coli* made the surface significantly less hydrophobic than the pristine surface (*p* = < 0.01), whilst the addition of *S. aureus* made the 316L stainless steel surface more hydrophobic, but this result was not statistically significant (*p* = 0.09). The differences produced between the bacterial species on the surfaces were statistically significant (*p* = 0.04). Changes to the hydrophobicity of the titanium and 316TiN surfaces were not significantly different from the previous measurements of the pristine surfaces, or between the different bacterial species retained upon the surface.

The addition of *S. aureus* and *E. coli* did not produce any statistically significant differences in the surface free energy (γ_s) or Lifshiftz Van der Waals forces (γsLW) for the 316L stainless steel or 316TiN surfaces, but the 316Ti stainless steel surface did demonstrate a significant difference in the Lifshiftz Van der Waals forces post bacterial retention assays ($p = 0.027$), but not in surface free energy. The addition of *S. aureus* to the titanium and TiN/15.03at.%Ag surfaces did produce significantly reduced levels of surface free energy ($p = 0.031$ and $p = < 0.01$), and Lifshift Van der Waals forces ($p = 0.041$ and $p = 0.027$). However, the addition of *E. coli* only produced a reduction in the surface free energy ($p = 0.007$ and $p = <0.001$) of the titanium and TiN/15.03at.%Ag surfaces.

Assessment of the Lewis acid – base (γ_sAB) interactions of the surfaces demonstrated a significant difference in the energies of the titanium, 316Ti and TiN/15.03at.%Ag surfaces when retained with *S. aureus* ($p = 0.015$, $p = 0.012$, and $p = 0.004$ respectively) and *E. coli* ($p = 0.019$, $p = 0.006$, and $p = 0.02$ respectively) but no significant difference was demonstrated between the bacterial species. No differences were demonstrated in the 316L stainless steel and 316TiN surfaces post bacterial retention assays.

Analysis of the electron accepting and donating potential of the surfaces demonstrated that there was no significant difference produced by the addition of either bacterial species to the electron accepting potential of any of the surfaces. Analysis of the electron donating potential of the surfaces did demonstrate some changes through the addition of the bacterial species, but all surfaces continued to be electron donors. The addition of *S. aureus* and *E. coli* reduced the electron donating potential in the 316Ti and TiN/15.03at.Ag surfaces significantly (*S. aureus* $p = 0.036$ and $p = 0.04$, *E. coli* $p = 0.010$, and $p = 0.001$ respectively), yet no difference was demonstrated by which bacterial species was assessed. *Escherichia coli* increased the electron donating potential of the 316L stainless steel surface ($p = 0.01$) and highlighted the difference that the retention of the bacterial species made to the surface properties ($p = 0.001$). *S. aureus* significantly reduced the electron donating potential of the 316TiN surfaces ($p = 0.035$). No changes were demonstrated from the titanium after bacterial retention assays.

Assessment of what effect the adsorption of conditioning film (Figure 64) made to the physicochemistry of the surfaces as performed, demonstrating that the addition of the 10 % bovine plasma made all the surfaces more hydrophobic, significantly more so for the 316L stainless steel (p = 0.023), 316Ti stainless steel (p = 0.004), 316TiN (p = 0.027), and TiN/15.03at.%Ag (p = 0.002). Despite no significant increase in the hydrophobicity of the titanium surface, it remained the most hydrophobic. Few changes were demonstrated by the addition of the conditioning film to the surface free energy (γ_s) except for in the TiN/15.03at.%Ag surfaces which significantly increased (p = 0.020). Similarly there were few significant changes to Lifshiftz-Van der Waals (γsLW) forces except for with the 316Ti (p = 0.032) and TiN/15.03at.%Ag (p = 0.009) surfaces which both saw an increase in their long-range attractive force potential. No significant changes were present in the acid-base interactions (γsAB) for any of the surfaces.

Measurements of the electron accepting potential of the surfaces demonstrated a significant increase in the 316L stainless steel and TiN/15.03at.%Ag surfaces (p = 0.018 and p = 0.0122 respectively), although all the surfaces continued to have a low electron accepting potential (between 0.35 mJ/m^2 and 2.82 mJ/m^2). Assessment of the electron donating potential of the surfaces demonstrated that all of the surfaces demonstrated a significant reduction in their donating potential (average reduction = 17.6 mJ/m^2), except for the 316TiN (reduction = 10.9 mJ/m^2), with the addition of the 10 % bovine plasma. The reduction in electron donating potential demonstrated that the addition of the conditioning film created no significant difference between the electron accepting or donating potential of the metals.

Physicochemical analysis of the surfaces was also performed after retention assays of the bacterial species and the 10 % bovine plasma conditioning film (Figure 65 and 66). Following mathematical calculation using the values obtained from contact angle measurements, the hydrophobicity (ΔG_{iwi}) of the surfaces was determined. This demonstrated that with the addition of both the *S. aureus* or *E. coli* with 10 % bovine plasma, all the surfaces continued to be hydrophobic (range for *S. aureus* = -35.0 mJ/m^2 to -62.9 mJ/m^2, range for *E. coli* = -34.3 mJ/m^2 to -71.7 mJ/m^2). There

was no significant difference in hydrophobicity demonstrated between the surfaces which contained the *S. aureus* with the 10 % bovine plasma. However, the addition of the *E. coli* with 10 % bovine plasma did create significant differences between the hydrophobicity of the different surfaces, with the 316Ti (-71.7 mJ/m^2) and titanium (-63.3 mJ/m^2) being the most hydrophobic, followed by the 316TiN (-53.3 mJ/m^2) and TiN/15.03at.%Ag (-47.6 mJ/m^2), and the 316L stainless steel (-34.3 mJ/m^2) being the least hydrophobic. Each surface was statistically different from one another, except for the 316L and TiN/15.03at.%Ag surfaces, and the 316TiN and TiN/15.03at.%Ag surfaces which demonstrated no significant differences (p = 0.181 and p = 0.830 respectively).

Few differences were noted in either the surface free energy (γ_s) (range for *S. aureus* = 39.1 mJ/m^2 to 55.6 mJ/m^2, range for *E. coli* = 38.6 mJ/m^2 to 452 mJ/m^2) or the Lifshiftz Van der Waals forces (γ_sLW) (range for *S.* aureus = 35.4 mJ/m^2 to 40.1 mJ/m^2, range for *E. coli* = 36.1 mJ/m^2 to 39.9 mJ/m^2) between the surfaces that contained both the bacterial species and the conditioning film. The addition of the *S. aureus* with 10 % bovine plasma to the titanium surfaces produced a significantly smaller surface free energy to the 316Ti surface (39.1 mJ/m^2) (p = 0.021) and significantly smaller Lifshitz Van der Waals forces (35.4 mJ/m^2) to the 316Ti, 316TiN and TiN/15.03at.%Ag surfaces (p = 0.009, p = 0.024, & p = 0.004 respectively). Similarly, the addition of the *E. coli* with the 10 % bovine plasma only produced a significant difference in the surface free energies between the 316L stainless steel (45.2 mJ/m^2) and the titanium (40.2 mJ/m^2) and 316Ti surfaces (40.2 mJ/m^2) (p = 0.004 p = 0.028 respectively). Significant differences in the Lifshitz Van der Waals were only demonstrated between the 316L stainless steel (39.9 mJ/m^2) and titanium surfaces (36.1 mJ/m^2) (p = 0.010) when retained with *E. coli*.

The addition of the *S. aureus* with 10 % bovine plasma to the different surfaces did not produce any further significant differences between the surfaces for either the Lewis acid-base interactions (γ_sAB) (range = 2.8 mJ/m^2 to 4.1 mJ/m^2), the electron accepting (γ_s+) (range = 1.3 mJ/m^2 to 2.2 mJ/m^2) or electron donating (γ_s-) (range = 1.9 mJ/m^2 to 10.5 mJ/m^2) potential of the surfaces. However, the addition of the *E. coli* with the 10 % bovine plasma did affect the electron donating potential of 316L

stainless steel surface (11.3 mJ/m^2) , making it significantly stronger than the titanium (0.9 mJ/m^2), 316Ti (0.3 mJ/m^2) and 316TiN (1.5 mJ/m^2) surfaces (p = 0.016, p = 0.011, & p = 0.024 respectively).

Changes to the hydrophobicity of the surfaces depending upon which bacterial species was retained upon the surfaces with the 10 % bovine plasma were most prevalent in the 316L stainless steel surface which demonstrated a greater hydrophobicity in the presence of the *S. aureus* with 10 % bovine plasma (increase hydrophobicity = 21.3 mJ/m^2) (p = 0.004). Conversely, the 316Ti surfaces demonstrated greater hydrophobicity after retention assays of *E. coli* with 10 % bovine plasma (increase hydrophobicity = 18.3 mJ/m^2) (p = 0.014). No significant difference was demonstrated from the differences of the bacterial species for the titanium, 316TiN or TiN/15.03at.%Ag surfaces.

Analysis of what effect the addition of the bacteria in conjunction with the 10 % bovine plasma conditioning film made upon the surface parameters was made through comparisons with previous results obtained when the bacterial species and the 10 % bovine plasma conditioning film were assessed individually. The addition of the *S. aureus* with 10 % bovine plasma to the surfaces produced a similar trend as seen when the surfaces were conditioned with the 10 % bovine plasma alone. This similarity in trends was also demonstrated with the electron donating potential of the surfaces in which all the surfaces, except the 316TiN surfaces which had reduced its electron donating potential, seen previously when the conditioning film was tested alone, which is in contrast to the results seen when the bacterial species were tested alone. This demonstrates that the physicochemical parameters of the conditioning film are more predominant in than the physicochemical parameters of the *S. aureus* cells.

The addition of *E. coli* in the presence of the 10 % bovine plasma to the surfaces also produced greater similarities to the physicochemical parameters of the conditioning film on the surfaces alone in comparison to those of the *E. coli* on the surfaces. However, the electron donating potential of the surfaces did not reduce as greatly as when the 10 % bovine plasma was retained to the surfaces on its own.

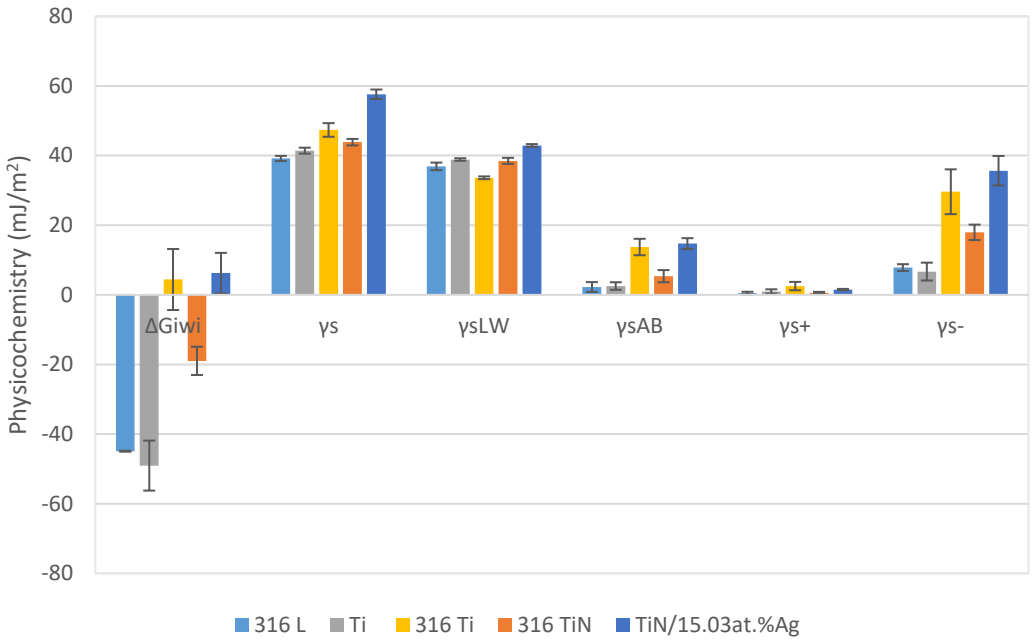


Figure 61: Physicochemical properties of the five metal surfaces ± standard error (n = 3).

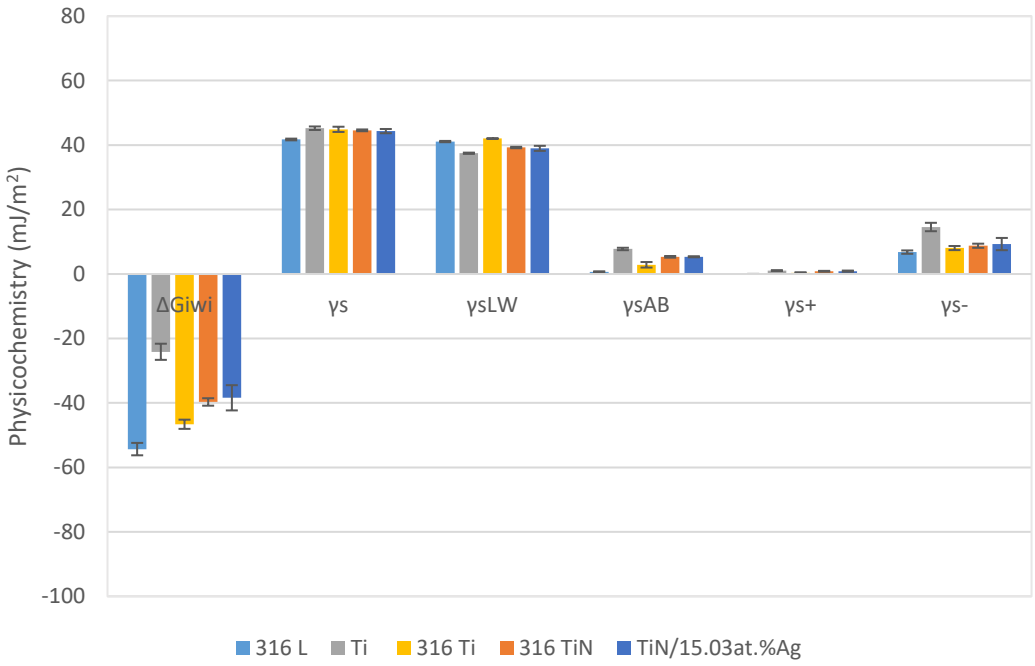


Figure 62: Physicochemistry of the metal surfaces once conditioned with *S. aureus* ± standard error (n = 3).

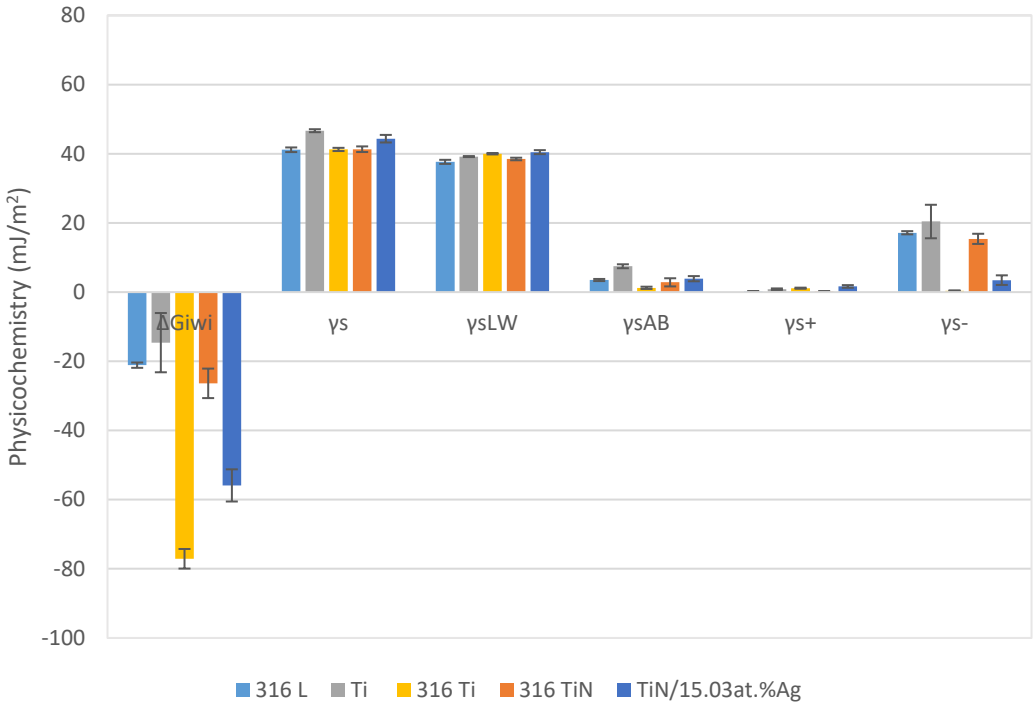


Figure 63: Physicochemistry of the metal surfaces once conditioned with *E. coli* ± standard error (n = 3).

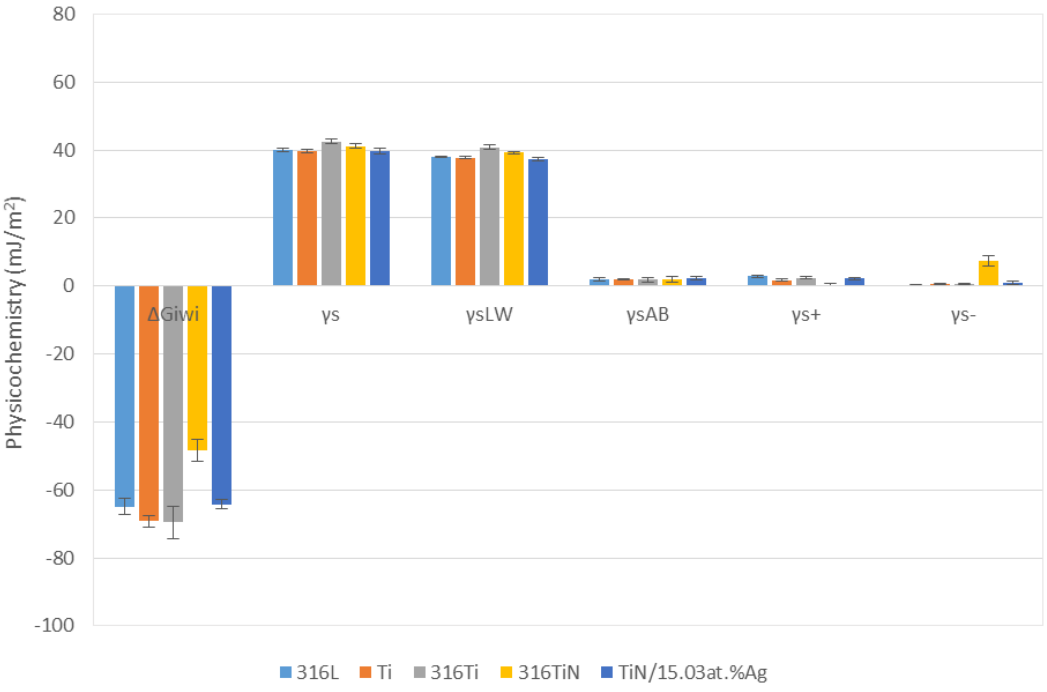


Figure 64: Physicochemical properties of the five metal surfaces post retention assay of 10 % bovine plasma ± standard error (n = 3).

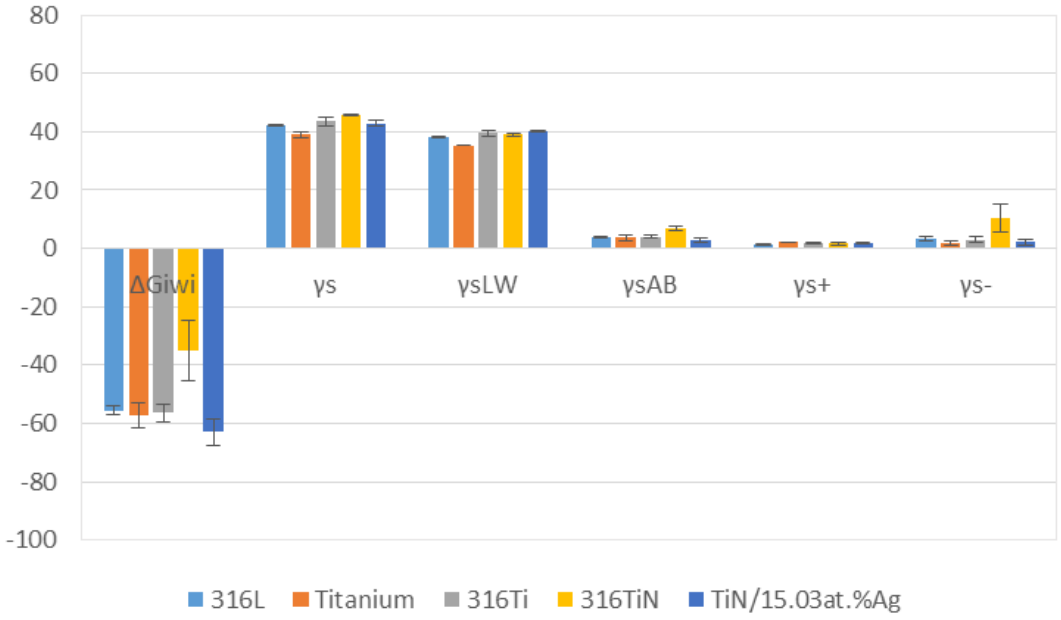


Figure 65: Physicochemistry of the metal surfaces once conditioned with *S. aureus* and 10 % bovine plasma ± standard error (n = 3).

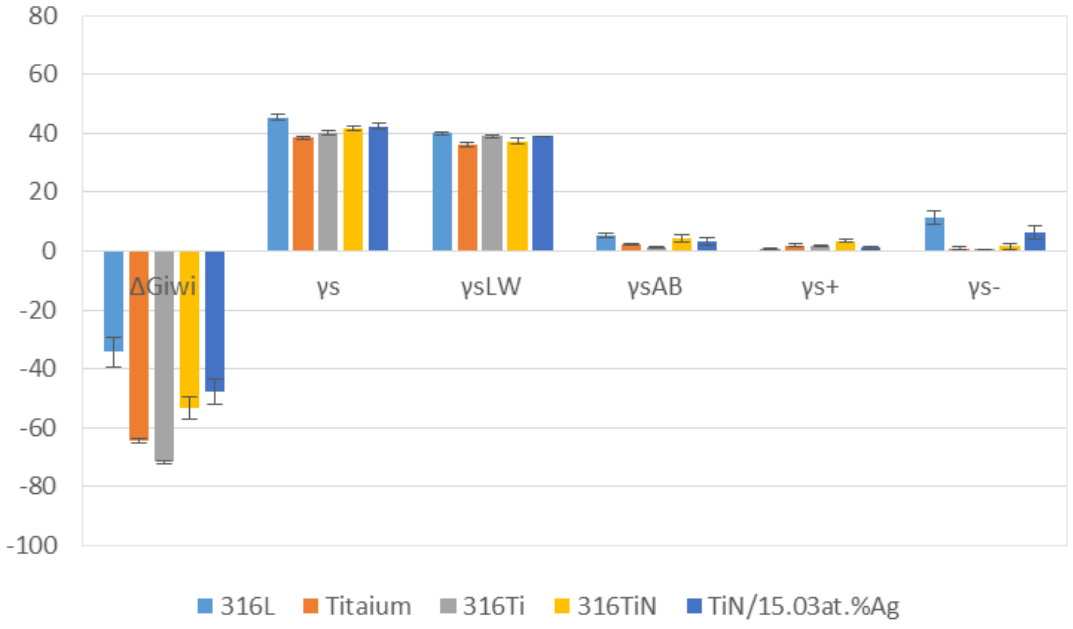


Figure 66: Physicochemistry of the metal surfaces once conditioned with *E. coli* and 10 % bovine plasma ± standard error (n = 3).

5.3.5 Microbial Adhesion to Hydrocarbons Assay (MATH Assay) with Conditioning Film.

Microbial adhesion to hydrocarbons (MATH) assays were performed upon both the *S. aureus* and *E. coli* with the 10 % bovine plasma to quantify their physicochemistries (Figure 67). Analysis of the results demonstrated that the *S. aureus* with 10 % bovine plasma had the strongest affinity towards chloroform (99.5 %), and a low affinity towards hexadecane (7.1 %) and ethyl acetate (5.5 %), with no affinity towards decane (0 %). The *E. coli* with 10 % bovine plasma also demonstrated a high affinity towards the chloroform (87.9 %) and ethyl acetate (44.3 %), but no affinity towards either the decane or the hexadecane solvents. This demonstrated that both the bacterial species, after being mixed with 10 % bovine plasma, were electron donors and strongly hydrophilic.

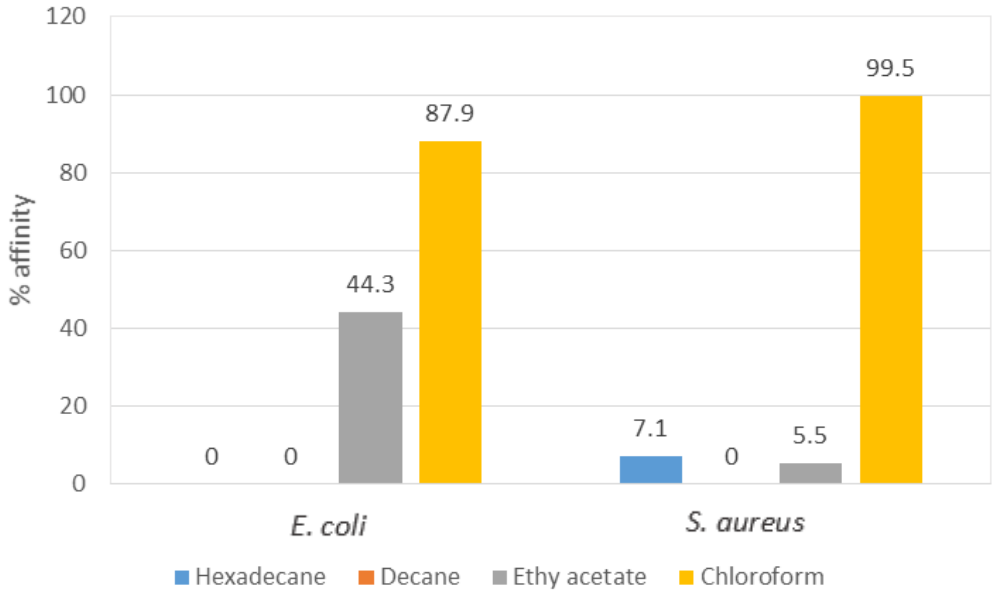


Figure 67: Math assay demonstrating the percentage affinity of the microorganisms towards the different solvents after they have been mixed with 10 % bovine plasma.

5.3.6 Fourier Transform Infrared Spectroscopy (DRIFTS)

Infrared spectral analysis of the surfaces was performed to allow for collection of data on the absorbance and the spectral location of peaks within the Amide I locations. The Amide I band was chosen as it is a specific region within the infrared spectrum which detects proteins, and surface fouling is predominantly protein based. Data was limited to allow for closer inspection of the desired wavenumber locations (1900 – 1350 cm^{-1}) and was baseline corrected to ensure that the data was easily visible to help with interpretation. Collection of this data prior to the application of conditioning films or retention assays of bacteria was important so that any shifts in the wavenumbers or intensity of the absorbance could be detected and calculated.

It was not possible to perform FTIR on the TiN/15.05at.%Ag surfaces due to chemical differences in the sputter-coated surfaces that were created due to their small-scale production. As the absorbance detected was so small, it was not possible to be certain if differences detected between the surfaces were due to the surface treatments or the composition of the surfaces themselves, and so were eliminated from this study.

The wavenumbers and absorbance of the four pristine metal surfaces were collected which demonstrated that 316L stainless steel and 316Ti stainless steel both had the same wavenumber peak location (1687 cm^{-1}) but that the absorbance attained at this location was greater for 316L stainless steel. The wavenumber peak locations for titanium and 316TiN were 1652 cm^{-1} and 1622 cm^{-1} respectively. Analysis of the surfaces in the presence of the bacterial species demonstrated that the addition of *S. aureus* and *E. coli* to the four metal surfaces (Table 12) did produce a spectral shift in the amide I peak and absorbance ratio, with a red-shift, or decrease, in the frequency for both 316L stainless steel and the 316Ti surfaces (reduced by ca. 20 cm^{-1}). This reduction in frequency demonstrated a greater distribution of β-turn in the conformation of the proteins, partly be due to the C=O stretching of the lipid component of the phospholipid bi-layer. There were few changes in the spectral location demonstrated by the addition of the bacterial species to the titanium surface which continued to demonstrate peak locations

around 1652 cm^{-1} – 1653 cm^{-1}, with no demonstrable change in the conformation of protein. In contrast, the peak locations demonstrated by the addition of the bacteria to the 316TiN surfaces increased the frequencies (blue-shift) of the peaks producing a spectral shift of 32 cm^{-1} in the presence of *S. aureus*, and 46 cm^{-1} in the presence of *E. coli* indicating a stronger shift towards the amide I band locations.

Measurement of the absorbance for each metal (Figure 69) demonstrated that both the *S. aureus* and *E. coli* produced significantly higher levels of absorbance from that of the metal prior to bacterial retention upon all the metals except upon 316Ti. The addition of *E. coli* to the surfaces produced an overall trend of higher absorbance in comparison to the addition of *S aureus*, however this was only statistically significant upon 316L stainless steel (p = 0.001) and 316TiN (p = 0.002) surfaces.

Analysis of the surfaces in the presence of a 10 % bovine plasma conditioning film demonstrated that a spectral shift within the amide I peak locations had occurred. The 316L stainless steel and 316Ti stainless steel surfaces continued to mirror one another as seen in the previous chapters, with both surfaces producing a red-shift, or reduction, in spectral frequency once conditioned with 10 % bovine plasma of ca. 35 cm^{-1} from the pristine surface. The wavenumber location demonstrated that the proteins in the conditioning film held a greater number of α-helices. Both the titanium and 316TiN surfaces produced an increase, or blue-shift, in their spectral frequency with the addition of the 10 % bovine plasma, the 316TiN increasing to 1667 cm^{-1} which was an increase of 45 cm^{-1} from the pristine surface prior to surface treatments, but this was not so dissimilar to the spectra produced when conditioned with *E. coli*. The wavenumber location for both surfaces demonstrated a greater number of β -turn in the protein conformity of the conditioning film. The titanium surface produced an increase in frequency of 20 cm^{-1} from both the pristine surface and from the ones after bacterial retention assays indicating a strong spectral shift towards the amide I band locations.

Measurement of the absorbance for each surface demonstrated that the addition of the 10 % bovine plasma adsorbed onto the surfaces did increase the absorbance

detected for all the metals, except for the 316L stainless steel which saw a reduction which is thought to be due to the conditioning film desorbing molecules that had been previously adhered to the surface due to the adhesives used in the protective films which were removed from the surface of the metal. The 316TiN surface (absorbance = 0.00523 nm) demonstrated the highest level of absorbance, as it had when conditioned with bacteria, followed by the 316Ti (absorbance = 0.00128 nm) and titanium (absorbance = 0.001913 nm) surfaces which exhibited a similar level of absorbance as when conditioned with the bacteria. Thus, it would seem that the addition of organic material resulted in changes in the amide I peaks. Statistical analysis of the data demonstrated that the increases seen in the titanium and 316TiN surfaces were significant (p = 0.01 and 0.00 respectively), whilst the increase seen in the 316Ti surface was not significant (p = 0.133). Due to inconsistencies in the data obtained from the analysis of the pristine 316L stainless steel surface, it was not thought that statistical analysis was appropriate in this instance.

Analysis of the spectra produced by the surfaces in the presence of both the bacterial species and the 10 % bovine plasma conditioning film (Table 12) demonstrated that a spectral shift had been produced within the amide I location. As demonstrated previously when the surfaces were assessed in the presence of the bacterial species and the conditioning film separately, the 316L stainless steel and 316Ti surfaces continued to follow the same spectral shifts as one another. The addition of both the *S. aureus* and *E. coli* with the 10 % bovine plasma, demonstrated a shift to a lower wavenumber, or red-shift, of –25 cm^{-1} (from 1687 cm^{-1} to 1662 cm^{-1}) for the *S.* aureus with 10 % bovine plasma, and -17 cm^{-1} (from 1687 cm^{-1} to 1670 cm^{-1}) for the *E. coli* with 10 % bovine plasma. In contrast, the titanium and 316TiN surfaces demonstrated a shift in spectral location to a higher wavenumber, or blue-shift, following the addition of the bacterial species with the conditioning film present. The titanium surfaces produced an increase of 17 cm^{-1} and 20 cm^{-1} respectively in the presence of *S. aureus* (from 1652 cm^{-1} to 1669 cm^{-1}) and *E. coli* (from 1652 cm^{-1} to 1672 cm^{-1}) with the 10 % bovine plasma, and the 316TiN increased by 45 cm^{-1} (from 1622 cm^{-1} to 1667 cm^{-1}) with both the bacterial

species and conditioning film. Analysis of what affect the addition of the two bacterial species had upon the peak locations demonstrated that the *S. aureus* with 10 % bovine plasma had produced a lower peak location than when the same metal type was retained with *E. coli* with 10 % bovine plasma. However, this trend in the effect of the bacterial species was not demonstrated by the surfaces with the bacterial species without conditioning film.

Measurement of the absorbance for each of the surfaces once the bacterial species with conditioning film had been retained (Figure 71) demonstrated that the 316TiN surface had significantly higher levels of absorbance from the addition of both the *S. aureus* and *E. coli* with the 10 % bovine plasma (absorbance = 0.0064 and 0.0051, respectively) than any other surface (p = < 0.00). The 316L stainless steel also demonstrated significantly higher levels of absorbance from the addition of *S. aureus* with 10 % bovine plasma (absorbance = 0.003) than the titanium (absorbance = 0.0016) and 316Ti surfaces (absorbance = 0.0015). However, the recorded absorbance of 316L stainless steel with *E. coli* and conditioning film was the lowest of all four surfaces (absorbance = 0.00138), significantly lower than the 316TiN (p = 0.00).

Analysis between the bacterial species for the individual metals demonstrated that both the 316L and 316TiN surfaces had significantly higher levels of absorbance when retained with *S. aureus* with 10 % bovine plasma rather than the *E. coli* with 10 % bovine plasma (p = 0.001 & p = 0.020). In contrast, the titanium and 316Ti surfaces had slightly higher absorbance from the surfaces with *E. coli* and 10 % bovine plasma, although not significantly higher (p = 0.520 & p = 0.410).

Analysis of what effects the addition of the 10 % bovine plasma to the bacterial species had upon the levels of recorded absorbance was made through comparisons to the results of the surfaces with bacterial species alone, and plotted to demonstrate which surfaces were most affected by the competitive adsorption of the plasma components (Figure 72). The addition of the *S. aureus* with 10 % bovine plasma followed a similar trend as previously demonstrated by the surfaces with *S. aureus* alone. Both the titanium and 316Ti surfaces demonstrated no significant difference from the addition of the bovine plasma to *S. aureus* (p = 0.940

& p = 0.925), and the 316L stainless steel demonstrated a significant reduction in the absorbance recorded (p = 0.019). However, the 316TiN surfaces demonstrated a significant increase in the amount of absorbance obtained from the addition of the 10 % bovine plasma to the *S. aureus* (p = 0.017). In contrast, the addition of the *E. coli* to the 10 % bovine plasma produced significantly lower levels of absorbance upon the 316L stainless steel and the 316TiN surfaces (p = 0.000 & p = 0.007). The titanium and 316Ti surfaces, as with the addition of the *S. aureus* with the 10 % bovine plasma, had no significant changes in the levels of absorbance recorded.

Table 12: Peak locations of the four metals after retention assays of *S. aureus* and *E. coli.* The numbers in red demonstrate the spectral shift (cm^{-1}) from the pristine surfaces.

Metals	**Peak location (cm^{-1})**					
	Pristine surfaces	Surface plus *S. aureus*	Surface plus *E. coli*	Metal plus CF	Metal plus CF and *S. aureus*	Metal plus CF and *E. coli*
316L	1687	1668 (-19)	1666 (-21)	1652 (-35)	1662 (-25)	1670 (-17)
Ti	1652	1652 (0)	1653 (1)	1672 (20)	1669 (+17)	1672 (+20)
316Ti	1687	1668 (-19)	1666 (-21)	1652 (-35)	1662 (-25)	1670 (-17)
316TiN	1622	1654 (32)	1668 (46)	1667 (45)	1667 (+45)	1667 (+45)

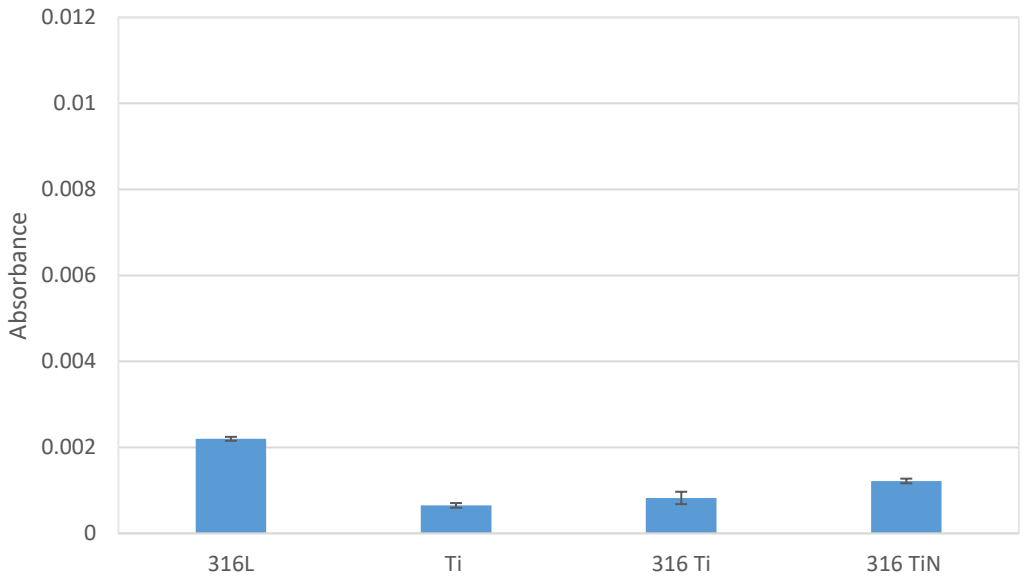


Figure 68: Absorbance from infrared spectra of the pristine metal surfaces post and prior to retention assays or application of conditioning film (n = 6).

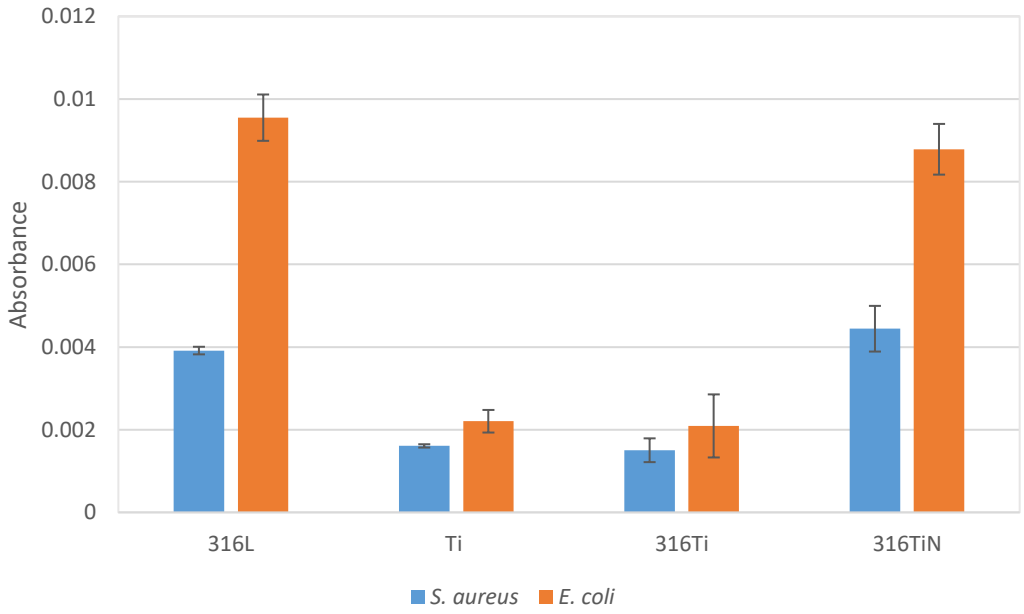


Figure 69: Absorbance from infrared spectra of the pristine metal surfaces post bacterial retention assays of *S. aureus* and *E. coli.*

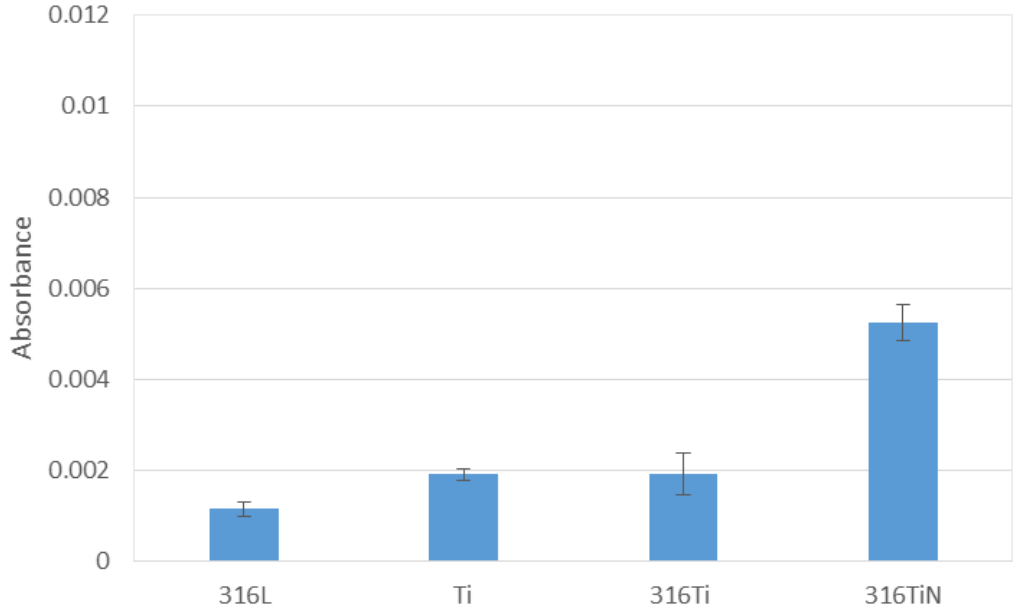


Figure 70: Absorbance from infrared spectra of the metal surfaces post retention assays of 10 % bovine plasma (n = 6).

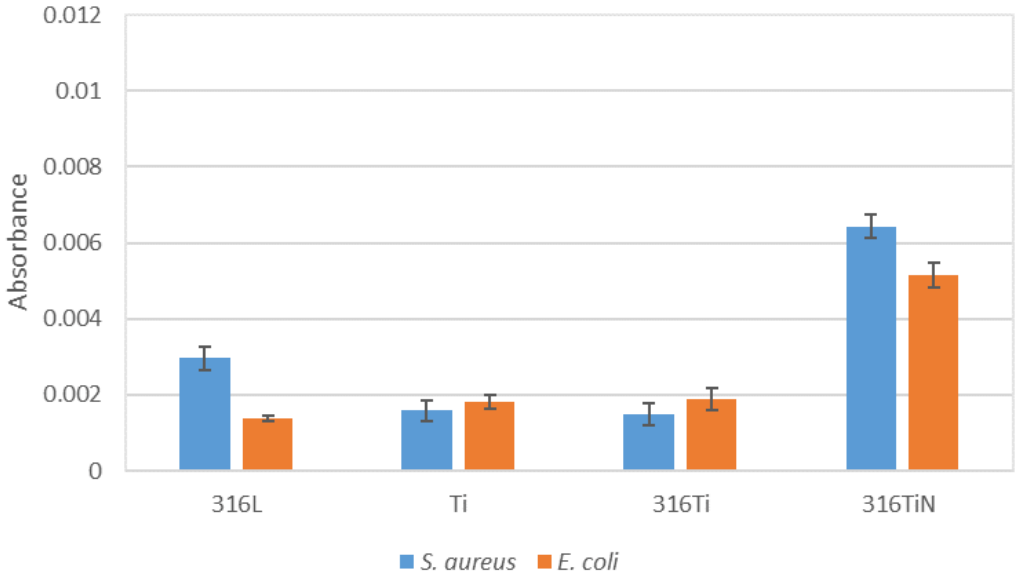


Figure 71: Absorbance from infrared spectra of the metal surfaces after retention assays of *S. aureus* and *E. coli* with 10 % bovine plasma.

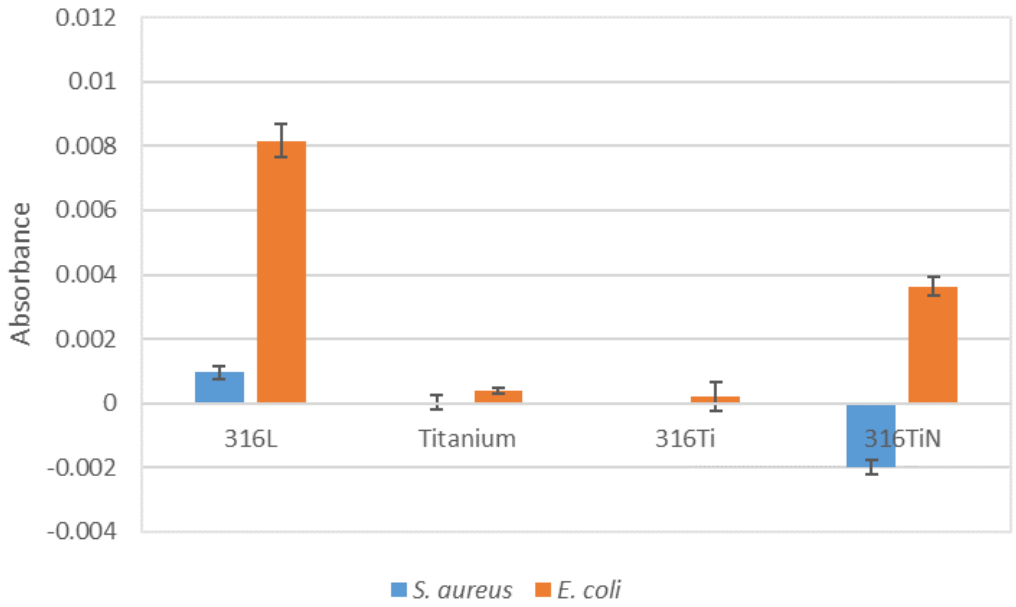


Figure 72: The differences in absorbance between bacteria alone and the bacteria plus conditioning film coated surfaces. The figures in the negative scale demonstrate that there has been an increase in the absorbance in comparison to the bacteria alone.

5.4 Discussion

The assessment of the chemical parameters of surfaces is important as it has been proposed that these parameters play a crucial role in the initial attachment and adhesion of bacteria and molecules to surfaces in an aqueous environment, and subsequently mediate biofilm proliferation (Boulange-Petermann et al., 1993; Vernhet and Bellon-Fontaine, 1995; van Oss, 1997; Whitehead and Verran, 2009). However, many of these assessments of abiotic surfaces are performed prior to the addition of a relevant conditioning film to the surface, rendering the results irrelevant in an applied setting (Whitehead, Benson, et al., 2011) as the adsorption of molecules to a surface has the potential to modify the surface parameters, potentially increasing or decreasing the abilities of bacterial species to adhere to those surface (Jones et al., 2001). It is therefore essential to measure what the effects surface fouling and conditioning films may have upon the chemical parameters of surfaces prior to surface selection. It is also possible that through investigations into the chemical parameters of a surface in relation to its potential

abilities to repel fouling, that surfaces modifications may be created to aid in the reduction in numbers of infection causing bacteria upon surfaces (Mitik-Dineva et al., 2009; Dohan Ehrenfest et al., 2010; Schaer et al., 2012; Rochford et al., 2014).

Measurements of the chemical parameters of the pristine surfaces provide the baseline control figures that future measurements would be compared to allowing for assessment of what effects the addition of the bacterial species, conditioning film, and conditioning film with bacteria made to those parameters. Initial analysis of the elemental composition of the surfaces confirmed their differing composition and that the distribution was spread homogenously across the surface, lessening the likelihood of patches of differing chemical forces being exerted across the surface. Assessment of the TiN/Ag surface demonstrated its silver content as 15.03 %, confirming a reduction in the silver content from the surfaces used in the preliminary chapter (Chapter 2).

Measurement of the physicochemical parameters of the surfaces demonstrated that the 316L stainless steel, titanium and 316TiN surfaces were all hydrophobic in nature and that the 316Ti stainless steel and TiN/15.03at.Ag surfaces were both mildly hydrophilic. Analysis of what effect the addition of the bacterial species had to the physicochemical characteristics of the surfaces were assessed and demonstrated that the 316Ti and TiN/15.03at.%Ag surfaces both became hydrophobic in the presence of both bacterial species, and the electron donating potential of their surfaces was reduced. Relating this data to the analysis of the surface charges of the bacterial species (Chapter 2 MATH assay), which demonstrated that both the bacterial species were electron donors, and that whilst the *S. aureus* was hydrophobic, the *E. coli* was hydrophilic, suggested that whilst the surface chemistry of the bacterial cells did alter the physicochemistry of the surfaces, they did not alter the surfaces in a predictable way. I.e. a hydrophilic bacterial species did not increase the hydrophilicity of a surface, and an electron-donating bacterium did not make an electron donating surface more electron donating due to an addition in surplus electrons. Works by other to measure the electrostatic charge of bacterial lawns have investigated the influence of drying times on the results and found that this is a factor to be considered (Gallardo-

Moreno et al., 2011). Although it should be noted that in this experiment, the same methodology was not utilised, it is possible that the drying time of the bacteria upon the surfaces, post retention assay, may have been a factor influencing the results obtained.

Previous works investigating the role of hydrophobic / hydrophilic surfaces upon fouling proclivity have suggested that fouling is more likely to occur upon hydrophobic surfaces than upon hydrophilic (Goulter et al., 2009; Rodrigues and Elimelech, 2009; Heilmann, 2011; Krasowska and Sigler, 2014a). However, results are often contradictory no with assertion of a common theme explaining the underlying mechanisms that would allow agreement upon a positive or negative relationship regarding hydrophobicity and bacterial attachment to surfaces. An explanation for such conflicting data could be the lack of standardisation and sensitivity in methodologies (Goulter et al., 2009).

Assessment of the surfaces via Fourier transform infrared spectroscopy was performed upon the 316L stainless steel, titanium, 316Ti and 316TiN surfaces after retention assays of *S. aureus* and *E. coli* in order to assess what changes had occurred in the peak locations and absorbance in comparison to the control group of pristine surfaces. Analysis of the peak locations demonstrated that similarly to the pristine surfaces, the 316L stainless steel and 316Ti surfaces produced the same results as one another in the presence of the bacterial species, both demonstrating a reduction in frequency. The addition of the bacterial species to the titanium surfaces did not appear to produce a spectral change in its peak locations which was in contrast to the 316TiN surfaces, which saw a large shift and increase in the frequency of the peak locations.

Whilst there was a close similarity observed in the peak locations between the samples of the Gram-positive *S. aureus* and the Gram-negative *E. coli,* most likely due to the presence of peptidoglycan and lipoproteins*,* the differences in frequency that were demonstrated would be due to the different chemical species present the bacteria such as lipopolysaccharide (*S. aureus*), teichioc acid, and lipoteichioc acid (*E. coli*). As the metal surfaces had a thin layer of the bacteria present through the retention assays, it is likely that the infrared radiation penetrated through the

bacterial cells, and then reflected back through the cells again producing a double pass in the data obtained. It is therefore likely that the entire cellular structure of the bacteria was analysed, and not just the membrane characteristics of the bacterial cells retained to the metal surfaces (Liauw et al., 2018).

The absorbance of the FTIR peaks demonstrated greater level of absorbance for the surfaces adsorbed with *E. coli* that those adsorbed with *S. aureus*. This could potentially be due to the increased size of the *E. coli* cells in comparison to the *S. aureus* (1 µm -2 µm x 0.75 µm, and up to 1 µm respectively), or due to differences in the numbers of each bacteria retained to the surface. Another factor that could affect the absorbance could be the presence of the outer membrane on the Gram-negative *E. coli* which consists of lipopolysaccharides. Whilst this difference would not be great enough to change the peak locations of the spectrum, it may affect the absorbance obtained. Future assessment of these results in relation to retention assay results may determine if the measurement of absorbance may be exploited as a method for measuring quantities of surface fouling. At the time of writing, we were unable to find any other published examples demonstrating this methodology.

The effect of the addition of the 10 % bovine plasma to the surfaces was assessed through the measurement of the physicochemistry of the surfaces which demonstrated that the addition of the conditioning film significantly increased the hydrophobicity of the surfaces. Minimal changes occurred to the other surface parameters except for with the electron donating potential of the surfaces. As previously discussed, it has been speculated that changes to a surfaces chemistry exhibited by the addition of conditioning films could influence bacterial attachment upon a surface (Briandet et al., 2001; Jones et al., 2001; Whitehead and Verran, 2015). Due to previous authors (Faille et al., 2002; Zeraik and Nitschke, 2012) asserting that bacterial adhesion to surfaces is increased by hydrophobic surfaces it is possible that the addition of the conditioning film to these surfaces has an effect of increasing the adhesion of the bacterial species. (Briandet et al., 2001; Whitehead, et al., 2011). A study by Slate *et al*., (2019) assessed the effects of blood conditioning films upon the physicochemical parameters of metal surfaces and

reported that the conditioning film did have an effect upon the surface physicochemistry, changing the previously hydrophobic surfaces to hydrophilic which was found to reduce the adhesion of bacteria to the surfaces. However, in a study into the effects of conditioning films upon bacterial attachment by Moreira et al., (2017) the authors found that the addition of cellular extracts to polystyrene increased the hydrophobicity of the surface which reduced the adhesion of *E. coli* and the proliferation of biofilms under flow conditions. Although it is not fully understood if hydrophobicity was the lone driving factor in these studies, they demonstrate the importance of measuring these parameters in the presence of conditioning films when assessing how bacteria may attach and adhere to surfaces.

Assessment of what effect the addition of the conditioning film had upon the physicochemical parameters of the bacteria was made through MATH assays after absorption of conditioning film to the cells. Prior to the addition of the conditioning film it had been demonstrated (Chapter 2) that the *E. coli* was hydrophilic and that the *S. aureus* was hydrophobic. After the adsorption of plasma proteins to the cells both bacterial species became hydrophilic. These results demonstrate the importance in considering bacterial interactions with conditioning films as the changes in the physicochemical parameters of the bacterial cells may also be a contributing factor behind the inconsistencies in some of the reported results into bacterial preference for hydrophobic surfaces.

Due to the greater numbers of plasma protein related adhesins on the surface of the *S. aureus* cells which relate to its virulence, it is likely that the effect upon the cell's hydrophobicity was due to the linking of plasma proteins to the MSCRAMMs. The increased hydrophilicity demonstrated by the *S. aureus* after adsorption with plasma proteins demonstrates the orientation of the plasma proteins once bonded to the MSCRAMMs which promote hydrophobic to hydrophobic bonding (Heilmann, 2011), leaving the hydrophilic tail of the protein protruding outwards from the surface. This change in the hydrophobic / hydrophilic nature of the *S. aureus* cells could have an effect upon the quantity and patterns of bacterial attachment, and it is thought that these parameters, along with the physicochemical parameters of the surface, should be assessed alongside the

results of retention assays in order to provide insights into the hygienic nature of surfaces (Bellon-Fontaine et al., 1996; Cunliffe et al., 1999; Habimana et al., 2014). Based upon current theories regarding bacterial preference for the same type of surface charge to their own, these results would suggest that after absorption of conditioning film to both the bacteria and surfaces, the bacteria would no longer preferentially adhere to the surfaces. This hypothesis will be assessed in the next chapter.

Analysis of the surfaces in the presence of the 10 % bovine plasma by Fourier transform infrared spectroscopy demonstrated that the conditioning film reduced the frequency of the spectra obtained for the 316L stainless steel and 316Ti surfaces when compared to the spectra taken of the pristine surfaces. Both these surfaces continued to mirror the same results as one another demonstrating a reduction of ca .35 $cm^{-1.}$ The titanium and 316TiN surfaces demonstrated an increase in the wavenumber locations (1672 cm^{-1} and 1667 cm^{-1}, respectively). The changes in the wavenumbers produced by the addition of the 10 % bovine plasma demonstrated a shift within the amide I band due to stretching of the C=O group due to the proteins in the bovine plasma. Measurements of the absorbance detected within the amide I band for each of the metal surfaces demonstrated that the addition of the 10 % bovine plasma had increased the absorbance detected for each of the metal surfaces except for in the 316L stainless steel. However, it is possible the results for its pristine surface were affected by residual adhesive retained from the protective film coating.

Assessment of the effect of the addition of bacteria in the presence of conditioning film was assessed through physicochemical analysis of the surfaces. Measurement of the hydrophobicity / hydrophilicity of the surfaces demonstrated that all the surfaces continued to be hydrophobic in the presence of both bacterial species with the 10 % bovine plasma, which was concurrent with the results previously obtained from the surfaces with bacteria or conditioning film retained alone. Changes had occurred in the measurements of how hydrophobic each metal had become, with the results appearing to follow more closely to the results obtained for the surfaces with conditioning film alone than to those of the surfaces with bacteria retained.

This is most likely because of the much smaller physical size of the proteins adsorbed to the surfaces in comparison to the bacterial cells, which would produce a more homogenous film upon the surface. In addition, these alterations in physicochemical parameters could also have been due to the adsorption of the plasma proteins onto the bacterial cell membranes. Bacterial cells membranes consists of highly complex arrangements of chemical species which would create 'islands' of different physicochemical properties in the sub-nano scale (Whitehead and Verran, 2009). If the plasma proteins had adsorbed and bound to the receptors on the cells surface, their own physicochemical parameters would be masked by those of the plasma proteins (Subramani et al., 2009; Habimana et al., 2014). The outer cellular membrane of lipopolysaccharide that envelopes the Gram-negative *E. coli* may have provided an extra layer of protection (Silhavy et al., 2010) for the cells against the absorption of the plasma proteins that the *S. aureus* cells were lacking which could explain the differences in hydrophobicity demonstrated by the different bacterial species once adsorbed with plasma proteins (Harris et al., 2002; Silhavy et al., 2010).

Analysis of the infrared peak locations peak locations demonstrated that the 316L and 316Ti surfaces continued to follow the same spectral shifts as one another as they had in the previous chapters. Both surfaces demonstrated a red shift in spectra compared to the pristine surfaces when adsorbed with both bacterial species, more so with the addition of the *S. aureus*. However, the spectral shift to a lower wavenumber had been much greater with the addition of the 10 % bovine plasma alone indicating the presence of the bacterial cells was still detected. The trend for both these surfaces exhibiting the same infrared spectra may be due to the low levels of titanium detected in the 316Ti surface, meaning that the 316Ti surfaces behaved like the 316L stainless steel surfaces.

In contrast the titanium and 316TiN surfaces demonstrated a blue shift when the bacterial species with conditioning film were applied to them. For the titanium surface, the increase observed in wavenumber from that of the pristine surface decreased slightly with the application of *S. aureus* with 10 % bovine plasma compared to those recorded for the 10 % bovine plasma alone, whilst the addition

of *E. coli* in 10 % bovine plasma demonstrated the same results to those of the conditioning film alone. The difference in the recorded wave number between the two bacterial samples could be due to the differences between Gram-positive and Gram-negative bacterial cells producing different vibrations such as the lipopolysaccharide components of the outer membrane of the *E. coli* cells which the *S. aureus* does not contain, as well as the thicker layer of peptidoglycan of the Gram-positive *S. aureus* (Davis and Mauer, 2010; Silhavy et al., 2010; Gupta and Karthikeyan, 2016).

The addition of the bacterial species to the 316TiN surfaces demonstrated the largest spectral shift of all of the surfaces, with the results for both the bacterial species concurring with those obtained where the conditioning film was analysed on its own. The high shift demonstrated by the 316TiN surfaces may be due to the top layers of the adsorbed materials being less highly bonded to the substrate, and as such, the molecules were able to vibrate more freely. When the infrared radiation comes into contact with the atoms of a sample, this causes excitation of the atoms recording the data regarding spectral location. However, this excitation also relies upon the chemical bond which could be affected by physicochemical factors of the surface. It is known that a stronger chemical bond with a smaller reduced mass does produce higher wave numbers (Reichenbächer and Popp, 2012), which was demonstrated by the 316TiN surfaces.

The results in this chapter demonstrated how the adhesion of surface foulants affected the chemical parameters of the surfaces. These changes in surface chemical parameters could have the potential to affect the attachment and retention of bacteria to the surface and subsequent biofilm formation. As the surfaces within the study were assessed with the view for use either in the food processing (meat) industry or as external fixation pins, they would come into contact with plasma proteins prior to bacterial contamination. These results demonstrate that it is imperative that surface assessments for chemical parameters are assessed in the presence of an appropriate conditioning film and surface foulants.

Chapter 6

Discussion

Biofouling is a critical problem that affects many industries and is of particular concern to both the food and associated medical professions, posing vast economical costs worldwide and ever increasing risks to public health (Whitehead and Verran, 2009). Fouling within the food industry can affect many areas of the process from operational aspects, such as blockages in heat exchangers to contamination of food contact areas which can have a direct impact upon the quality of the food product placing subsequent risks to public health through cross contamination (Verran et al., 2010; Barish and Goddard, 2013).

Risks associated from surface fouling within healthcare also affects many aspects ranging from fouling of high contact areas such as door handles and sinks which then risk cross infection to patients, to direct infection risks posed from indwelling device surfaces such as bio-implants, orthopaedic pins and catheters. Implants such as Kirschner wires, which penetrate through the skin into the fractured bone temporarily immobilising the limb with the use of an external frame, are at great risk of tracking infection into the limb from the commensal bacteria upon the skin surface. Treatment of such infections are costly and place a huge burden upon health care associations around the world; in the USA alone, it is estimated that the cost of such infections will rise to $1.6 billion annually by 2020 (Gil et al., 2017).

The choice of surface must be carefully chosen with consideration of the functionality of the surface; factors such as strength and durability, biocompatibility, ease of cleaning, resistance to corrosion, and propensity for bacterial colonisation being of importance (Wilks et al., 2006; Campoccia et al., 2013a; Schlisselberg and Yaron, 2013; Whitehead and Verran, 2015). The characterisation and development of surfaces that could potentially be used to reduce the risks of bacterial cross contamination of food contact areas and reduce infection rates for patients with indwelling metal medical devices such as orthopaedic bone pins would not only help to reduce the risks to public health, but also help to mitigate the huge economic burden associated with surface fouling.

Previous assessments of bacterial adhesion to surfaces have focused upon its correlation to a singular surface parameter, often not attempting to look at the overarching problem of surface fouling and subsequent biofilm formation as

multifactorial. Assessments have also been made upon pristine surfaces without the consideration of the effects that conditioning films may have upon the surface despite general consensus from the scientific community that the propagation of a conditioning film upon a surface is the base for bacterial adhesion and biofilm formation (Pringle and Fletcher, 1986; Jones et al., 2001; Garrett et al., 2008; Whitehead et al., 2010; Lorite et al., 2011). The aim of this study was to assess the surface parameters in conjunction with one another against pathogens of relevance to both the food and medical industries. The study attempted to elucidate the driving factors behind bacterial retention to surfaces through comparisons of assessments under different conditioning parameters; pristine surfaces, surfaces with bacteria, surfaces with conditioning film, and surfaces with conditioning film and bacteria.

6.1 The Effect of Physical Parameters of the Surfaces upon Bacterial Retention

Assessment of the effect that surface topography had upon bacterial retention demonstrated that the retention of *S. aureus* cells upon the pristine did appear to increase upon the rougher surfaces. However, the retention of *E. coli* cells did not follow any topography related trend, demonstrating a greater correlation between its retention to the surfaces and the physicochemistry measurements taken of the metals prior to any surface conditioning. This work is comparable with the works of Whitehead et al., (2015) in which there was no trend observed between the retention of *E. coli* and the surface roughness, whereas there was a trend between the retention of *S. aureus* and surface micro-topography. However, not all studies agree with this result. Wu *et al*., (2011) investigated the responses of *Staphylococcus epidermidis* cells to four titanium surfaces with different topographical finishes. They discovered that with there was no correlation between the roughness of the titanium and the quantities of bacteria adhered to them, with their roughest and smoothest surfaces (R_a = 33 µm & 0.006 µm) retaining the least bacteria (~ 10 %), and their second and third roughest surfaces (R_a = 11 µm & 0.83 µm) retaining the most (52 % & 59 % respectively). Due to the increased roughness parameters in the surfaces tested within that study in comparison to those within

this study, it could be speculated that topography does not make a difference to the retention of bacteria with surfaces that are less rough.

The effects of surface topography upon bacterial retention has been widely discussed with often-contradictory conclusions drawn from the results. Many studies have similarly reported that there are links to the increases in cell adhesion with increased topography (Boyd et al., 2002; Mitik-Dineva et al., 2009; Whitehead, Li. H., et al., 2011; Hsu et al., 2013), other works have found no relationship (Medilanski et al., 2002; Hilbert et al., 2003; Whitehead et al., 2005; Milledge, 2010). It should be noted however that there are often great variations in some of the surface parameters of the tested surfaces due to the wide variety of different test substrates used such as glass, Polytetrafluoroethylene (PTFE) or polystyrene. These differences in the surface parameters of the test surfaces would lead to discrepancies in the results when comparing to surfaces of a different chemistry, topography or physicochemistry. However, this conflicting data also supports the suggestion that the current descriptive factors that describe surface roughness are lacking or inappropriate when considering its impact upon the preferential binding of bacterial cell (Zhao et al., 2008; Wickens et al., 2014).

Cell shape and structure are also important factors to consider when assessing the influences of topography upon bacterial retention to surfaces. The individual properties of the bacterial cells, such as presence of surface adhesins or flagella are also important factors to consider as these can affect its attachment behaviour to surfaces (Faille et al., 2002; Zeraik and Nitschke, 2012). Mei et al., (2011) used time dependent force measurements with the use of an atomic force microscope (AFM) to measure the increased bonding of Staphylococcal strains to surfaces, noting an increased level of binding with time, theorising this was due to the increased attachment of adhesins to the surfaces with increased time scales. Friedlander et al., (2013) also investigated the role of *E. coli* cell appendages in increasing cellular adhesion to surfaces, reporting that whilst initial bacterial retention to rougher surfaces may be reduced due to poor cell body / surface contact, the cells overcome this with the use of flagella, which anchor the cells to the rougher surfaces and increase surface area contact. It is likely that these factors are

responsible for the species-specific response to surface roughness demonstrated in this study, and the lack or correlation between surface topography and the retention of *E. coli* to the surfaces was due to all the surfaces having roughness parameters under 500 nm.

6.2 The Effect of Conditioning Film upon the Physical Parameters of the Surface

It has been hypothesized that the addition of a conditioning film has the potential to mask the underlying surface topographies of substrates, and so affect the way in which bacteria would interact with them (Verran & Jones, 2000) through topographical change rather than assessment of the interactions with the conditioning film. Works assessing the effects of conditioning films upon the physical parameters of surfaces have produced conflicting data, often as a result of the differing methods of conditioning film application. The work in this study utilised an application method that allowed the proteinaceous components of the conditioning film to adsorb to the surfaces prior to a rinse step to remove the excess. In contrast, other studies have applied thicker conditioning films and dried them to the substrata before studying the changes in the surface parameters. In a study by (Moreira et al., 2017), the effects of cellular extract conditioning films upon bacterial adhesion and biofilm formation were measured. The results demonstrated that the conditioning films tested had significantly increased the roughness parameters of the surfaces due to the cellular extracts drying in a thick layer to the polystyrene. As such, these measurements of the roughness parameters were no longer applicable to the surface parameters but to a non-standardised and non-repeatable conditioning film aggregation from which results should not be utilised to form conclusions regarding driving factors of bacterial adhesion in the presence of conditioning films.

Results from assessments in this study of the surfaces after retention assays with conditioning film demonstrated that the conditioning film made no significant difference to the roughness of the surfaces and so any changes to the retention of bacteria with conditioning film would not be due to topographical changes.

6.3 The Antimicrobial Efficacy of the Surfaces

Assessment of the surfaces for potential antimicrobial action demonstrated that only the two surfaces which contained silver produced an antimicrobial action. The mode of action of the two surfaces did however change dependent upon the concentration of the silver within the titanium nitride matrix. Assessments of the potential leaching potential of the surfaces demonstrated that the TiN/25.65at.%Ag surfaces did demonstrate an ability for the silver ions to leach into a surrounding aqueous environment which was effective against strains of *E. coli* but not *S. aureus.* However, the TiN/15.03at.%Ag surfaces did not demonstrate an ability for its silver ions to leach into a surrounding aqueous environment in a concentration high enough to elicit an antimicrobial action against either bacterial species. Assessment of the surfaces' antimicrobial efficacy when in contact with the bacterial species demonstrated that both silver containing surfaces exhibited an antimicrobial action against both bacterial species assessed.

Whilst the TiN/25.45at.%Ag surfaces did demonstrate greater antimicrobial efficacy (leaching) against *E. coli* in comparison to the TiN/15.03at.5Ag surfaces, it was also noted that the surfaces had poorer tribology with an occasional tendency towards lamination of the surface and so would not be a suitable surface choice for either the food or medical industries where durability and strength are essential.

These results are concurrent with other similar studies (Whitehead, et al., 2011) assessing the antimicrobial efficacy of TiN/Ag surfaces where a species specific response was demonstrated by the bacteria tested. This data supports the hypothesis that the release of silver ions into an aqueous environment elicits structural changes in the bacterial cell wall, interrupting interactions of thiol groups of proteins and enzymes (Morones et al., 2005; Rai et al., 2009; Skovager et al., 2013). The structural differences between the Gram-positive and Gram-negative cell wall would account for the species-specific response towards leached silver ions.

There are however limitations in this study which would require addressing prior to recommendations being placed upon the suitability of the use of surface containing

silver in either the food or medical industries. In recent studies by Slate *et al*., (2019) and Saubade *et al*., (2018) into the effects of conditioning films upon the antimicrobial efficacy of surfaces containing silver, the authors demonstrated differing results over whether the presence of a conditioning film hinders the antimicrobial effect or acts as an adjunct for its efficacy. Whilst the method of conditioning film application between the two studies was different, with Slate *et al*., (2019) applying a conditioning film to a surface and allowing it to dry prior to antimicrobial assessment and Saubade *et al*., (2018) applying their conditioning film in a method similar to the one utilised in this study, the results demonstrate that this is an area which does need further assessment.

A further limitation in this study which would need assessment prior to recommendation of a surface would be related to whether the surface retains its antimicrobial efficacy over time with use. If the antimicrobial efficacy of a surface waned over time it would not make it suitable for use within the food industries where it would not practical or financially viable to require surfaces to be periodically replaced. Assessments utilising fouling and cleaning protocols would need to be conducted to replicate the environment that a surface may come into contact within an industrial setting. With a view to use as an external bone pin, the longevity of the antimicrobial effect would become less important due to the temporary nature of the external fixating device. Assessment of the time required in-situ for the surface to demonstrate antimicrobial action would however be beneficial.

6.4 The Retention of Microbes to the Surfaces

Analysis of the numbers of bacteria to the pristine surfaces demonstrated that the *S. aureus* retained in a greater abundance to the titanium, 316Ti and 316TiN surfaces, in comparison to the 316L stainless steel and TiN/15.03at.%Ag surfaces. Analysis of this data in conjunction with the physicochemistry and surface roughness results from the pristine surfaces demonstrated that whilst there appeared to be no correlation between the numbers of retained *S. aureus* and the physicochemistry of the surfaces, there was a correlation between the surface roughness and the percentage coverage.

However, assessment of the retention of *E. coli* to the pristine surfaces demonstrated a different pattern of surface preference, favouring adhesion to the 316L stainless steel, titanium and 316TiN surfaces. Analysis of this data in conjunction with the physicochemistry and roughness parameters of the pristine surfaces demonstrated no correlation between the retention of *E. coli* to the surfaces and the roughness parameters. However, there was a positive correlation demonstrated between the retention of bacteria and the physicochemistry of the surfaces, with the *E. coli* preferring to retain to the hydrophobic surfaces. As it had been previously ascertained through MATH assay that the *E. coli* was hydrophilic, this suggests that this particular strain prefers surfaces that have a different hydrophobicity than its own cell surface. These results are similar to those demonstrated in the initial study (Chapter 2) were the *E. coli* demonstrated preferential adhesion to the less hydrophobic surface. Whitehead *et al.*, (2014) had previously reported that *S. aureus* retained in greater numbers to rougher surfaces and produced aggregation in surface features, and that *E. coli* cells were affected not by topography but by the physicochemistry of the surface. This results from this study support those conclusions.

The lack of consensus in this topic could be due to the lack of standardisation in the test methodologies. Often the surfaces tested are not comparable due to differences in topography, chemical composition of the surfaces, method of application to assess bacterial adhesion and the bacterial species used. For example, Wu *et al*., (2011) examined the response of *Staphylococcus epidermidis* to titanium substrates of differing topographies and reported no correlation to topography. However, the substrates used varied significantly in roughness parameters (S_a = 0.006 µm, 0.85 µm, 11 µm and 33 µm) with only one of the test surfaces falling within the range of this and other comparable studies.

The species-specific response to the differing physical features of the surfaces is likely due to differing cell wall compositions and the presence of bacterial appendages. For example; the increased numbers of *S. aureus* retention to rougher surfaces is likely due to the increased surface area that a rougher surface would provide to a sphere shaped cocci cell, whilst a cell with appendages like the flagella

of the *E. coli* would be able to overcome a lack of roughness / surface area through utilisation of the flagella as an anchor to the surface (Friedlander et al., 2013; Rossez et al., 2015). For this reason, it would be unwise to make assumptions regarding the response of all bacterial species based upon the results of a few.

6.5 The Effect of Conditioning Film on the Retention of Microbes to the Surfaces

Assessment of what effect the addition of the conditioning film made to the retention of the bacteria to the surfaces was performed through the use of retention assays with the 10 % bovine plasma present. Results demonstrated a significant reduction in the percentage coverage of both bacterial species, with the greatest reductions demonstrated by *E. coli* species. Whilst it was previously discovered that the *S. aureus* strains demonstrated a correlation in adhesion in relation to the roughness parameters of the pristine surfaces, this was no longer the case when the 10 % bovine plasma conditioning film was added to the retention assay with the *S. aureus.* As it was ascertained that the addition of the conditioning film had not significantly changed the roughness parameters of the surfaces, it could be hypothesized that the reduction in the numbers of bacteria was not related to the roughness parameters, and that the conditioning film component was more important than the roughness properties of the surface (Yuan et al., 2017).

Whilst it had been previously hypothesised that the presence of organic materials upon a surface could potentially increase fouling upon surfaces, with the proteins forming a linking layer for microbial contaminants (Jones et al., 2001; Whitehead et al., 2011). This study demonstrates that the molecular content of the conditioning film is of great importance as it is this which mediates bacterial retention. Works into single protein source conditioning films, such as albumin, have demonstrated reductions in bacterial adhesion previously (An et al., 2000; Hedberg et al., 2013). Conversely, works into the effect of fibronectin upon surfaces demonstrated that it significantly enhanced and increased the adhesion of *S. aureus* to surfaces (Herrmann et al., 1988).

Whilst it has been found the addition of some conditioning films, such as those produced by milk and its constituent proteins can increase bacterial adhesion to surfaces (Speers and Gilmour, 1985), other investigations into the efficacy of mixed protein sources relevant to the food industry, such as fish extract protein or cod extract, were also examined and found that they were able to decrease bacterial attachment by a factor of 10 – 100 (Bernbom et al., 2006; Pillai et al., 2009; Whitehead et al., 2015).

Works utilising mixed protein sources represent a more realistic representation of the interactions of bacteria to surfaces with conditioning films present as the constituent components of liquids tend not to be of a single molecular content. Studies have ascertained that proteins of differing size are able to reorganise themselves to surface, with larger molecules replacing smaller ones until an equilibrium is met (Vroman and Adams, 1969; Vroman et al., 1980). For this reason, if surfaces such as bone pins were pre-treated with a conditioning film of a single protein source with the aim to reducing bacterial adhesion, the composition of the proteins on the surface would soon be replaced by the fluids in the patient.

A potential mechanism for the reduction of bacteria upon the surfaces in the presence of conditioning film in this study could be due to the interactions of species-specific adhesins to the molecular components of the conditioning film. The surface bound adhesins of Staphylococcal species bacteria play a key role in the virulence of the cells, adhering to key proteins such as collagen, fibronectin and fibrinogen to gain adhesion to host tissues. The co-absorption method of application of conditioning film during the retention assays would allow the cellular adhesins of the planktonic bacteria to bind to the conditioning film components prior to attachment and retention to surfaces. Whilst this could be a limitation of the study, the method of conditioning film application is an important factor to be considered. Other works assessing the effects of conditioning films have utilised methods which allow the conditioning film to dry onto surfaces prior to application of bacterial species (Moreira et al., 2017; Slate et al., 2019). However, when assessing the potential usage of the surfaces in this study, this method does not appear to provide a honest representation of how bacteria and conditioning film

would actually deposit on the surface. Saubade *et al*., (2018) utilised a method similar to the one in this study, allowing the bacteria and the conditioning film to co-absorb to the surfaces providing a realistic *in vitro* recreation of how bacteria would interact with a surface. As demonstrated by this study, the authors reported reductions in the coverage of bacteria and also suggested binding of the specific and nonspecific adhesion/absorption sites as a potential causative factor (Fletcher, 1976; Grześkowiak et al., 2011). Whilst it could be argued that the prior binding of molecules to these adhesion/absorption sites provides rationale for the reduction in bacterial numbers, we would suggest that this method of assessment provides a truer account of bacterial interactions to conditioned surfaces due to the unlikelihood of bacteria reaching a surface in a pristine, unconditioned state.

6.6 The Effect of Surfaces Chemical Parameters upon Bacterial Retention

Measurements of the chemical parameters of the pristine surfaces allowed for the assessment of what changes the addition of the bacteria and conditioning film made upon them, and how that might have affected bacterial retention. Many previous studies into the effects of surface physicochemistry upon bacterial retention and adhesion to surfaces have been performed, assessing the pristine surfaces against washed bacterial cells. The results of those previous studies produced a hypothesis that hydrophobic surfaces were likely to enhance surface fouling (Chae et al., 2006; Goulter et al., 2009; Rodriguez Emmenegger et al., 2009; Heilmann, 2011; Krasowska and Sigler, 2014b) and that bacteria preferentially bonded to surfaces of their own physicochemical type; i.e. hydrophobic to hydrophobic (Zita and Hermansson, 1997; Sinde and Carballo, 2000; Zeraik and Nitschke, 2012). The results of this study into that bacterial retention to pristine surfaces partly concurred with that hypothesis. Results from the preliminary study utilising only two surfaces with similar roughness parameters did suggest that the bacterial species retained to surfaces with a similar surface charge to their own but further investigation utilising a broader range of surfaces with differing topographies allowed for a more in-depth analysis. Once the bacterial species were challenged with greater diversity in both the roughness and physicochemical parameters, a species-specific response was demonstrated, with the Gram-negative

E. coli preferentially retaining to hydrophobic surfaces, whilst the *S. aureus* demonstrated no correlation in its retention behaviour towards the physicochemistry of the surfaces but to the roughness of the surfaces.

These results highlight the importance of assessing a larger set of parameters in conjunction with one another provided a potential rationale for the conflicted results of other studies; if the study parameters were too narrow the subsequent data sets would be skewed.

Measurements of the physicochemistry of the surfaces after the retention assays with bacteria demonstrated how the chemistry of the bacterial cells interacted with and affected the surface's physicochemical parameters. Initial adhesion of the bacterial species alone onto pristine surfaces demonstrated that all the surfaces became hydrophobic in the presence of the bacteria. However, there were species-specific differences in the levels of hydrophobicity expressed by the surfaces, most likely due to the differences between the Gram-positive and Gram-negative bacteria. Interestingly, the physicochemistry of the surfaces did not alter towards that of the bacteria adhered to it, i.e. the hydrophilic *E. coli* did not make the hydrophobic surfaces hydrophilic, and it did not make the hydrophilic surfaces more hydrophilic, they in fact became hydrophobic also. This data demonstrates that despite measurements of hydrophobicity being recorded for the surfaces and the bacterial species, our current understanding of these interactions does not allow for prediction of the influences of each in conjunction.

Many studies have attempted to measure the physicochemical parameters of either the surfaces or the bacteria that they were working with, yet from the literature, it seems that none have investigated the actual parameter changes produced by the interaction of the bacterial species with the metals once adhered. Whilst some authors have hypothesized that a bacterial biofilm should be regarded as a conditioning film, which would then affect the adhesion of behaviour of planktonic microorganisms (Briandet et al., 2001), none were found that discussed how the physicochemical parameters of the pioneer cells upon a surface could affect the surface parameters, and subsequent bacterial attachment and biofilm formation.

6.7 Effect of Conditioning Film on Chemical Parameters

Assessment of what effect the addition of the conditioning film had upon the chemistry of the bacterial species was assessed through MATH assays, which demonstrated that the absorption of proteins to the cell surfaces did make a significant change to the chemical and physicochemical properties of the cells. Initial assessment of the bacterial species without the conditioning film demonstrated that whilst the *E. coli* was hydrophilic, the *S. aureus* cells were hydrophobic. However, after the bacterial species were mixed with the plasma proteins, both species became hydrophilic. Closer assessment of what effect the conditioning film had upon the *E. coli* samples demonstrated that whilst the affinity to the hydrocarbons appeared masked, it had not altered the expression of hydrophilicity or electron donating potential. The most likely explanation for the differences seen in the adsorption of the plasma proteins to the bacterial species would be due to the expression of, and interaction with, different cellular appendages which are able to bind to the plasma proteins (An et al., 2000). The increased number of covalently bonded surface proteins in *S. aureus* cells such as fibronectin binding proteins A and B (FnBPA and FnBPB), which are able to overlap their affinity to target molecules, would have bonded to the plasma proteins of the conditioning film via a hydrophobic to hydrophobic interaction (Heilmann, 2011). Once bound to the outer surface of the *S. aureus* cell wall, the protruding tail of the plasma protein would then be able to interact with the hydrocarbons available in the MATH assay and illicit a change in the exhibited physicochemistry of the cells to hydrophilic. This result demonstrates the importance in the measurement of the cell physicochemistry in the presence of the conditioning film, whilst previously discussion of adhesin interaction with molecular components has gravitated around the potential of the formation of linking layers (Jones et al., 2001; Whitehead et al., 2010) to the surfaces, the measurement of the change in the physicochemistry of the bacterial cells and the consequential effect on bacterial retention has not been fully investigated.

Assessment of what effects the addition of the conditioning film made upon the chemical parameters of the surfaces demonstrated a significant increase in the

hydrophobicity of the surfaces. However, despite the proposition made from other studies suggesting that this change would increase the adhesion of bacteria to the surfaces (Faille et al., 2002; Zeraik and Nitschke, 2012), the numbers of bacteria retained to the surfaces significantly decreased in comparison to assessments with the pristine surfaces. A possible reason for the differences in results obtained in comparison to other studies are the methods utilised when applying the bacteria to the conditioned surfaces. As previously discussed, the conditioning film had elicited a change to the physicochemistry of the bacterial cells as well as the surfaces, but if the assessments made of the conditioning films involved the addition of 'unconditioned' bacteria to surfaces adsorbed with conditioning film, the bacterial adhesion may increase. However, we do not believe that this is a representative of an applied situation and suggest that the methods used in this study are more illustrative of a real-world situation. These results demonstrate the importance of appropriate assessments of physicochemistry of the surfaces and bacteria in the presence of conditioning films as parameter changes can have effects upon bacterial retention.

Analysis of surfaces once co-adsorbed with the bacterial species and conditioning film demonstrated a species-specific response. The surfaces with the *S. aureus* with conditioning film retained expressed many similarities to the physicochemical parameters of the surfaces with the conditioning film alone adsorbed to it, whilst the surfaces with the *E. coli* and conditioning film demonstrated greater variations in the surface energy parameters of the different surfaces. These results demonstrate how the bacterial cells have absorbed or bonded to the plasma proteins differently due to the presence or absence of different cellular receptors. For example, the addition of the lipopolysaccharide outer membrane of the Gram-negative *E. coli* cells may be providing the cells with protection from the plasma proteins (Silhavy et al., 2010), whilst the surface adhesins, such as FnBPA and FnBPB of the *S. aureus* cells may be binding to the proteins in the conditioning film (Clarke and Foster, 2006).

FTIR analysis enabled the assessment of how the plasma proteins or bacteria interacted with the metal surfaces. Assessment of the data for the 316L stainless

steel and the 316Ti surfaces demonstrated that the proteins interacted with the surfaces in an identical manner. This is most likely due to how similarities in the chemical composition of the surfaces, which was demonstrated through EDX analysis, and the composition of only the outermost atomic layers of the surface being analysed (Kasemo and Gold, 1999; Puleo and Nanci, 1999; Paul Roach et al., 2005). Measurements of peak locations within the amide I band demonstrated that both surfaces exhibited a red-shift, or shift to lower wavenumbers, with both the application of either bacteria or conditioning film. Variations in the peak locations demonstrated conformational changes in the proteins under the different tested conditions, with the addition of the bacteria, and the bacteria with conditioning film producing higher wavenumbers indicating an increase in β-turn components. However, assessment of the conformational changes to the plasma proteins when adsorbed to the pristine surfaces alone demonstrated that the proteins maintained their α-helix structure. The shifts produced by the addition of the conditioning film, as well as bacteria in conditioning film, produced the largest positive spectral shifts, potentially suggesting an increase denaturation or in β-turn or random structure components. Other potential explanations for this could be that the top layers of conditioning film were less influenced by the surface allowing them to vibrate more freely. It has been hypothesised that proteins would undergo a greater level of denaturation when interacting with more hydrophobic surfaces, making them lose a greater degree of the helical secondary structure due to the stronger interaction involved (Kidoaki and Matsuda, 1999; Paul Roach et al., 2005). Whilst it was previously asserted by van Oss, (1997) that these conformational changes could be related back to the hydrophobicity of the surfaces, no correlation between the FTIR data and the changes to the hydrophobicity of the surfaces could be found in this study. These interactions have however been previously recorded in single protein source interactions with human fibronectin and human albumin, and confirmed through other metrological approaches such as atomic force microscope (Kidoaki and Matsuda, 1999), but was not demonstrated in this study which utilised a mixed protein source.

Chapter 7

Conclusion

In conclusion, whilst measurements of the individual surface parameters are important, these works demonstrate the importance of surface assessment in conjunction with one another and how the presence of an appropriate conditioning film can change the parameters significantly. The addition of the conditioning film within this study mitigated the effects of any of the previous surface parameters assessed from the pristine surfaces, which demonstrates that the current process of assessing surfaces is not appropriate, as the likelihood of bacterial retention onto pristine surfaces in real life is unlikely. Assessment of the effect the retained soiling components; biological, bacterial or chemical, have on the surface parameters is important as the changes mediated through adsorption of this conditioning layer have the potential to alter the future adhesive behaviours of other biological soils. Bacterial responses to conditioning films are also an important parameter to consider, as co-absorption of proteins to the cells, which are governed by their individual surface components, can greatly affect the chemical parameter exhibited by the cells.

Based upon all the assessments from this study, we would recommend that the surfaces of greatest interest for utilisation as temporary external fixation devices would be the TiN/15.03at.%Ag due to the low numbers of bacterial retention and antimicrobial efficacy which could have a significant impact upon the rates of infection and reduce the subsequent requirement for further medical intervention and use of antibiotics. The positive that would be gained from the use of the antimicrobial coating outweigh the negatives in relation to poor osseointegration due to low topography as the surfaces are for temporary use only and so osseointegration is a less restrictive parameter.

The results of the retention assays in the presence of the conditioning film present the possibility that external fixators could be pre-conditioned with plasma from the patient prior to insertion as a way to reduce bacterial adhesion without impairing the biocompatibility of the surface through the addition of artificial coatings.

Due to regulations prohibiting use of silver-based surfaces in areas with food contact, we would recommend that the 316L or 304-2R surfaces would be best purposed for food contact surfaces use in the food industry. However, utilisation of

the TiN/15.03at.%Ag surfaces on splash backs and other non-food contacting surfaces could provide reductions in the numbers of bacterial contamination from environmental cross contamination.

7.1 Future work.

Future works should be conducted to analyse the surfaces at further time points, to assess the antiadhesive performance of the surfaces after the initial adhesion event. Analysis of how the conditioning film affects the growth and architecture of mature biofilms, and how the physicochemistry of the biofilms alter the surface parameters would help with the understanding of established infections could be treated in the presence of metal surfaces.

Different bacterial species chould be analysed to help assess the role of physicochemistry and bacterial cell chemistry. The presence of different cell appendages would produce further differences in the adhesive nature of the bacterial species. In addition, the use of mixed species bacterial cultures would provide data upon how the surface parameters are affected by retention of bacteria which exert different physical and chemical parameters to one another, and how the competition between the species could affect further adhesive behaviour of the bacteria. Assessments utilising different concentration gradients of conditioning film would also provide insights into the binding responses of bacteria and assess whether or not the concentration of conditioning film is also important.

Molecular investigations into the effects that surface parameters and conditioning films have upon the bacterial retention after none reversible adhesion to surfaces has occurred could provide insights into how to prevent or block these responses which could provide potential to create more hygienic surfaces. Multiplex PCR could be used to quantify interactions with specific adhesins on the cell surfaces in the presence of conditioning films.

Analysis of how the bacterial cells interact with conditioning films in their planktonic state, prior to attachment and retention to surfaces, should be assessed as this interaction could affect their interactions with surface properties and physicochemical parameters, and the conditioning film could mask their own cell surface chemistry. Also, assessment of the effects of conditioning films upon the antimicrobial efficacy of the surfaces should be performed to assess if the conditioning film has the potential to alter these parameters.

Cleaning protocols could be used to assess how the surface parameters change with repeated use and assess if the antimicrobial efficacy of the surfaces change over time. Live/dead assays could be used to assess if the surfaces still perform after certain time periods and a defined number of cleaning protocols.

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