FACTORS AFFECTING SURVIVAL OF BACTERIA ON ABIOTIC SURFACES

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Abstract

The ability of pathogenic bacteria to be retained on the surfaces of processing equipment constitutes a potential health problem in the food industry. Stainless steel is commonly used in the food industry, but there is an increasing demand for surfaces with enhanced hygienic properties. One way to combat microbial surface fouling is the use of novel antimicrobial alloys. Although many metal ions in solution demonstrate a significant antimicrobial effect, in the relative absence of moisture, surface release rates and the efficacy of the antimicrobial agent are altered. The aim of this study is to define conditions that minimise survival of bacteria on stainless steel and stainless steel coated with titanium nitride, alloyed with silver, a putative antimicrobial surface.

Characterisation of the test substrata revealed smooth, thus hygienic surfaces with no leaching of silver observed. A method was developed to assess survival of *Escherichia coli* and *Listeria monocytogenes* on substrata under different equilibrium relative humidities (ERH): 11%, 52% and 86%ERH. Any cells remaining on the surfaces post-swabbing were detected by epifluorescence microscopy, or by metabolic dye. The survival of both microorganisms on surfaces was recorded via live/dead staining. The effect of surface re-use was also assessed. Gram positive *L. monocytogenes* survived better than *E. coli* in the highest 86%ERH and silver concentration TiN/Ag 120W, presumably due to its thicker cell wall. The increase of humidity did not affect any antimicrobial effect, but increased concentration of silver in the surface coating reduced the viability of bacteria. Re-use of the surfaces showed similar results to first time use in all experiments. The presence of meat extract increased the time of survival of *E. coli* from 4 hours to 16 hours on the stainless steel but the presence of silver decreased cell numbers when compared to other coatings. Finally, a differential staining method was developed to detect live/dead *E. coli* cells as well as meat extract to simulate contamination *in situ*. This novel method may be used in future studies to investigate the survival of microorganisms in food soil.

Surfaces show potential being able to retain antimicrobial activity post use, low wear and no release of silver ions. Future experiments may include the use of the silver containing coating surfaces *in situ*, in different food industries, to evaluate their potential for reducing outbreaks.

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Chapter 1

Introduction

1.1. Hygienic surfaces in the food industry: an overview

The ideal hygienic food processing surface would be one on which no food material or microorganism would be retained and which is easily cleaned. However, the food industry potentially presents the perfect environment for bacteria to grow with a fresh and continuing source of nutrition, moisture, a range of temperatures and plenty of surfaces to attach to (food, processing surfaces, environmental surfaces) (Siegumfeldt and Arneborg, 2011). Thus cleaning and hygiene regulations and procedures are in place, to reduce spoilage and prevent disease outbreaks (Jullien *et al*, 2003). However, even the best hygienic protocols cannot prevent microbial deposition via air dust, spray and food remains invisible to the naked eye (Marriott, 1994). These deposits or accumulations of microorganisms are defined as 'contamination'. The presence of food soil residue may enhance their survival. Viable microorganisms may subsequently come into contact with other materials, therefore providing a 'biotransfer potential' (Hood and Zottola, 1995; Bower *et al*, 1996; Wirtanen *et al*, 1996).

Thus, the food processing plant is recognised to be a major source of microorganisms of which some may potentially be pathogenic (Welshimer, 1981; Lundén *et al*, 2003; Cagney *et al*, 2004; Bielaszewska *et al*, 2011). Outbreaks of foodborne illness may result from consumption of contaminated food and food spoilage can occur during production, via cross-contamination from 'open' surfaces such as working surfaces, and 'closed' surfaces such as pipes or conduits (Verran *et al*, 2008).

Open surfaces present solid-air or solid-liquid-air interfaces which are less conducive to bacterial survival and growth than closed surfaces. This is due to lower water availability, and also because they are subject to daily cleaning and disinfection (Gram *et al*, 2007). Nevertheless, open surfaces such as conveyors, sinks, work surfaces

and feed boxes are likely sources of contamination (Marriott, 1994; Sveum *et al*, 1992; Taylor and Holah, 1996). Closed surfaces are difficult to access for cleaning and present a solid-liquid interface, which provides excellent conditions for the development of biofilms (Verran *et al*, 2008; Martí *et al*, 2011).

In a 'closed' system, there is conditioning of the surface and cells by adsorbing molecules from fluids, enhancing the binding of the cell to the surface. This often results in the development of three dimensional biofilms which are composed of patches (cells, exopolymers, and food residues) and/or isolated cells (Shi and Zhu, 2009). In an 'open' system, depending on the food material, the surface conditioning may vary (Verran *et al*, 2008). Open surfaces are either always or intermittently wet. If moisture levels are low, cells on the surface are less likely to be growing. However, these attached cells may survive very well, and are able to grow if water becomes available, for example if cells are transferred to a food material or if food soil is retained on the surface (Yabunchi *et al*, 1992).

Microbial adhesion to a surface is linked to different parameters including the physiological and structural features of the cells, the composition of the liquid and properties of the environment such as temperature and humidity. Moreover, the physical and chemical features of the substratum such as hydrophobicity, porosity, roughness, geometry and composition will also play a part (Briandet *et al*, 1999; Cunliffe *et al*, 1999; Hood and Zottola, 1997; Flint *et al*, 2000; Jullien *et al*, 2002). Stainless steel is one of the most commonly used types of material in food industries. It is hard, inert and stable at different temperatures, but is not innately anti-microbial. It remains resistant to corrosion, and is easy to manufacture (Verran *et al*, 2000). Stainless steel has been shown in previous studies to be highly hygienic (Holah & Thorpe, 1990). However,

over time, metal surfaces may wear, with pits and scratches being created, and these features may retain cells and soil, making the surface less easy to clean (Verran *et al*, 2010). Stainless steel is produced in a wide range of grades and finishes, thus giving different properties to the stainless steel and potentially affecting bacterial adhesion through these different chemistries, topographies and physico-chemical properties (Bellon-Fontaine *et al*, 1990). The major variation between stainless steel grades is in the quantities of iron, chromium and nickel used in the composition.

In the food industry, most surfaces used are made of stainless steel. Most surface modifications will be done by physical methods. Stainless steel, with a surface finish such as 2B or 2BB, has an excellent gloss and low surface roughness which is a perfect material for kitchen hoods (www.cmigroupe.com). A fine polish finish is obtained by mill methods and its smooth surface makes it very hygienic and easy to clean (www.outokumpu.com).

Stainless steel 304 is widely used in the food industry and is mainly composed of iron with 0.04% carbon, 18.1% chromium and 8.1% nickel (www.outokumpu.com). Its composition offers a good corrosion resistance to food products and detergents. In the dairy industries, stainless steel 316 composed of 0.04% carbon, 17.2% chromium, 10.1% nickel, 2.1% molybdenum, in addition to iron, is used in more corrosive environments (also with exposure to chemical, solvent or salt water) for its improved anticorrosive properties via the addition of molybdenum, thus improving the hygienic status of the surface (www.outokumpu.com). The real difference between stainless steel 304 and 316 is the presence of molybdenum with a lower chromium content in the latter and higher nickel content. Chromium is one of the most important components of stainless steel. It forms stable metal carbides at the grain boundaries, thus increasing

corrosion resistance (Fang *et al*, 2007). Nickel is used to enhance the crystal structure; in this case, the percentage of nickel used reduces the tendency of the metal to crack due to stress caused by corrosion (Fang *et al*, 2007). Carbon elements make the stainless steel stronger. Different grades of stainless steel can be obtained giving more or less rough finishes such as pickling finish (2B) and bright annealed (2R), depending on the final steel making process (Boulangé-Petermann, 1996). Additional surface treatments can be applied to reduce roughness, such as mechanical polishing or electropolishing. The topography of stainless steel surfaces may vary depending on intended use, for example, super brushed stainless steel comprises parallel linear features, giving the surface a matt finish (which does not show fingerprints). Thus, a range of stainless steels may be used in the food industry. Interaction with microorganisms and ease of cleaning are key factors (Faille *et al*, 2000; Flint *et al*, 2000; Sinde and Carballo, 2000; Frank and Chmielewski, 2001).

Other commonly used 'hygienic' materials are glass and ceramics. They have similar hygienic properties to stainless steel, being smooth and hard, but they are used more on wall and floor covering rather than direct contact with food. The wear of ceramic and glass is described as 'microcracks' (Verran *et al*, 2008; Li *et al*, 2010). Materials such as plastic components, resins and rubbers provide flexibility at joints and curves. However, these represent a much higher risk to contamination due to their softness and sometimes porous characteristics (Minagi *et al*, 1985; Stepanovic *et al*, 2004; Verran *et al*, 2008).

1.2. Approaches to maintaining hygienic status

1.2.1. Cleaning and disinfection

The contamination of food-processing equipment and its control has always presented a challenge. There are national regulations regarding the hygienic design of food machinery (Holah & Timperley, 1999). Principles such as good hygiene practices (GHPs), hazard analysis critical control point (HACCP) and cleaning-in-place (CIP) protocols were implemented between the 50s and 90s in food processing industries to help the maintenance of hygiene (APHA, 1972; Walker *et al*, 2003; Fryer *et al*, 2011).

The HACCP process encompasses seven principles which are essential to the functioning and maintenance of hygiene in a given environment (www.food.gov.uk). The first principle requires performing a hazard analysis to locate and potential microbial contamination, growth and survival. Then, the critical control points are categorised in order to create critical limits or standards for each one of them. Prerequisites are established to examine the standards or critical limits after which a correction action can be taken if needed to change the standards. Finally, a record of all the standards and potential corrections is made and implemented as a new procedure in different areas to validate that the HACCP system works as expected. (USDA, FSIS, 1997).

International collaboration is exemplified by the European Hygienic Engineering and Design Group (EHEDG), established to improve design criteria and to provide directions on apparatus, structures of edifices and processing (www.ehedg.org) across Europe. The EHEDG has devised equipment performance tests to confirm

compliance with design criteria. Their aim is to develop and implement general guidelines which would suit different conditions and factory design requirements in order to decrease potential contamination, hence reducing outbreaks linked to foodborne pathogens (Jakobsen, 2010; Jakobsen, 2011).

Cleaning and disinfecting processes are crucial to the maintenance of good hygiene in food processing industries. Regular cleaning prevents food material from attaching and potentially contaminating surfaces and entrapping microorganisms thus transmitting them to other food products coming in contact with the same surface (Wirtanen, 1995). Factory design plays a major role in making a sanitation programme effective (Van Donk and Gaalman, 2004). Even the best sanitation programme cannot compensate for shortcomings in equipment and plant design and human error. The cleaning process will be specific to the factory. It will be determined by the type of soiling and pathogens related, the surfaces used, products used for sanitation, the level of water hardness and the regulation of hygiene standard specific to the plant.

Cleaning stages involve: removing visible soiling, the use of detergent to detach invisible remaining soil, and rinsing to remove detergent and residue. Additional steps may be included to disinfect or sterilise the surfaces by using appropriate means to eradicate microorganisms present, and again rinsing the surface (Kulkani *et al*, 1975).

As there is a wide range of food, there is a wide range of soil types. This will differ depending on the food constituents and the processes they will undergo. Individual food components will behave differently regarding their cleanability. Therefore, a wide range of maintenance products is needed for their elimination. Food deposits present different properties (Holah and Gibson, 1999): dry particles such as milk powder (Henning *et al*, 2006), dried-on such as meat blood (Eustace *et al*, 2007),

cooked-on, sticky, fatty or slimy such as grilled meat, cheese, bacon and eggs. A common removal method is hot or cold water often combined with detergents. Any trace of food soil remaining affects subsequent cleaning (Van Eijk and Majoor, 2003), and increases the risk of microbial contamination and survival on the surface. Therefore, detergents appropriate to a given soil and surface contacts are required. The ideal detergent would be hydrophilic at the required temperature, non-damaging to surfaces, non-harmful for human use, eco-friendly, fragrance-free, easily washable, remain unchanged for long storage, efficient to remove wide range of soil and cheap (Van Eijk and Majoor, 2003).

Storage and transport of raw materials should ensure that residues of earlier batches are absent. Storage temperature, time and humidity should also be implemented such that growth of microorganisms and insects is halted or significantly slowed (Bili and Taoukis, 1998). Insects, rodents and birds are significant sources of microbial contamination and therefore raw materials should be protected against such pests (Campbell *et al*, 2004).

Most raw materials are contaminated on the outside only. The number of microorganisms present can be minimised by proper treatment before processes such as cutting, milling and grinding (Loretz *et al*, 2010). Such treatments include washing, blanching, flushing with water and use of a disinfectant or ultraviolet light treatment. Unfortunately, heat treatments which are intended to destroy unwanted microorganisms (pasteurisation and sterilisation) often adversely influence the characteristics of the product treated. Taste, flavour, colour and texture may be affected, as well as many nutrients (Buchin *et al*, 1998; Matser *et al*, 2003).

An alternative approach to aggressive cleaning and disinfecting processes is to modify the surface in order to enhance cleanability and reduce fouling.

1.3. Surface modification

Surface modification methods of stainless steel can be divided in two broad categories: physical and chemical. Physical methods may change the surface roughness (Akamatsu *et al*, 1991; Singh *et al*, 1997), grain sizes and grain boundaries (Saul *et al*, 1970; Revathi *et al*, 2008), and faceting which is the change of morphology from a flat surface to pyramidal-like microstructure (Chen *et al*, 2003; Chandrasekharan *et al*, 2008; Wang *et al*, 2006). Physical methods often relate to use of lasers (Bauerle, 1996), plasmas (Chan *et al*, 1996; Abe *et al*, 2006), temperature (Prakash *et al*, 2005), ion beams (Yao *et al*, 2008), ball milling (Sahoo *et al*, 2008; Uzunova-Bujnova *et al*, 2008), and polishing and grinding (Prakash *et al*, 2005) to alter the surface state of a material of interest. The main intent with physical modification methods is to not alter the chemical composition of the material (Akamatsu *et al*, 1991; Singh *et al*, 1997; Sahoo *et al*, 2008; Uzunova-Bujnova *et al*, 2008), although, in some cases, physical surface modification methods can lead to changes in the chemical composition of the surface due to removal or addition of material or chemical reactions on surfaces (Chan *et al*, 1996; Prakash *et al*, 2005; Abe *et al*, 2006; Yao *et al*, 2008). For example, in the case of selective or ion-beam sputtering where high-velocity ions bombard a metal thereby displacing atoms from the target source and deposited onto the substrate in a thin film (Liu *et al*, 2008; Sanchez-Garcia *et al*, 2008). This is chemical change by a physical method.

One of the oldest methods of modifying the physical characteristics of surfaces is by polishing or grinding the surface to obtain smooth or rough finishes. A simple

process using an abrasive material to alter the surface roughness can be found dating back to ancient times where hard objects such as small pebbles and shells were attached to bark, animal skin, or paper by using gum resin, creating ancient sandpaper. When polishing, brushing and grinding, a layer of the surface is removed by a cutting or abrading action. Grinding refers to a significant material removal, often by the use of sanding paper. The brushing process involves a less significant removal of surface layer by the use of abrasive sponge to produce a smooth, polished surface. In theory, it is possible to produce an unlimited number of surface finish variations (www.outokumpu.com).

Although surface topography may be defined when new, inevitable changes will take place over time due to wear (Timperley *et al*, 1992), through abrasion, cleaning and impact damage. Thus, new surface topographical features (Verran and Boyd, 2001; Whitehead *et al*, 2006) are introduced which may increase the retention of both organic soil and microorganisms (Boyd *et al*, 2001). It is therefore of value to be able to monitor the impact of wear on the viability of microorganisms in the presence, or absence, of organic soil.

Temperature gradients and thermal treatments have often been used to change surface roughness (Banerjee *et al*, 2008) and alter the grain sizes and grain boundaries (Saul *et al*, 1970). In the past few years, thermal methods have been employed to create nanoscale features, facets, textures (Yeo *et al*, 2006; Loginova *et al*, 2007), and nanoparticles (Ahn *et al*, 2008), on a variety of metals (Otero *et al*, 1995; Wei *et al*, 1998). These methods were part of the production process of stainless steel 304 used in this study.

Chemical methods introduce a change in the chemical composition at the surface of a material. The surface may possess chemical properties that are different from the bulk material. Chemical methods include treatment with UV light which modifies the surface atomic layer to enhance adhesive bonding with other inorganic materials (Ulbricht *et al*, 1998; Vasilets *et al*, 1998; Hozumi *et al*, 2002; Bhattacharvya and Klapperich, 2007) and reactive plasmas which prime surfaces for adhesion, painting, coating or printing applications (Chan *et al*, 1996; Abe *et al*, 2006; Bodas and Khan-Malek, 2006). The chemical changes to a surface can also introduce a change in the eventual surface charge density or the surface energy, neither of which affect food product quality. This is a physical change by a chemical method.

Altering the surface chemistry by wet or dry processes is the most common methodology used for chemical modification of surfaces. The processes are so-named because of the processing methods and conditions. Modification schemes are governed by a wide range of parameters including sample type (polymers, metals, ceramics, etc.), stability to treatment conditions (for example, thermal or structural), and eventual applications.

Amongst the dry surface modification methods, use of reactive plasmas (Chan *et al*, 1996) has been gaining popularity, most likely due the wide compatibility of materials and integration with micro-fabrication processes for device development. Many different types of gas-plasmas have been cited in literature including air (Dorronsoro *et al*, 2008), oxygen (Liu *et al*, 2004), H_2O (Lee *et al*, 1991; Goldblatt *et al*, 1992; Steen *et al*, 2001a; Steen *et al*, 2001b; Steen *et al*, 2002; Long *et al*, 2006), ammonia (Schroder *et al*, 2001), and argon (Groning *et al*, 1994) for modification of surfaces. Plasma modification methods have their own sets of advantages and

disadvantages. The biggest advantage is probably that surfaces are modified uniformly and the modification is limited to a few nanometres in depth without affecting the bulk material. The main disadvantage is probably the use of vacuum equipment with system parameters that can vary, adding to cost. A detailed discussion of the various applications and advantages and disadvantages can be found, in the existing literature (Chan *et al*, 1996; Aumann *et al*, 2001; Godfrey *et al*, 2001; Vasquez-Borucki *et al*, 2001; Wilson *et al*, 2001; Zhang *et al*, 2002; Xu *et al*, 2003; Langowski and Uhrich, 2005; Zhang *et al*, 2006; Bodas and Khan-Malek, 2007; Goddard *et al*, 2007; Xue and Yang, 2007; Mireault *et al*, 2008). These methods are used to deposit metal coatings on the surfaces in order to make them more efficient to their purpose. In this study these methods were considered and the physical vapour deposition method was selected.

1.4. Microorganisms of interest

1.4.1. Microorganisms of concern in food industry

There is a wide range of microorganisms associated with food contamination: some with human disease, others with food spoilage. The first European communicable disease epidemiological report was released in 2007(Amato-Gauci and Ammon, 2007). It notes that in 2005, 200,570 cases of campylobacteriosis linked to poultry products were reported (overall incidence of 45.11 per 100,000) by 23 EU Member States, Iceland and Norway, with the highest incidence reported by the Czech Republic (296.15 per 100,000), followed by United Kingdom (87.95 per 100,000). The same year, a total of 5,215 cases of verocytotoxigenic *Escherichia coli* were reported by 25 countries. This bacterium initiated symptoms such as bloody diarrhoea, kidney damage and

haemolytic uraemic syndrome. Czech Republic (16.72 per 100,000) followed by Sweden (4.27 per 100,000) reported the highest incidence. The overall incidence in the EU was 1.17 per 100,000. Some countries did see an increase, in particular, Austria, Finland, Ireland, the Netherlands, Sweden and United Kingdom. The increase in these countries could be due to improved sensitivity of the surveillance systems, a true increase in the incidence or a combination of both. *Listeria monocytogenes* can cause spontaneous abortion, stillbirth, blood infection and meningitis. The annual incidence in Europe decreased between 1995 and 1998, but since then has shown a sustained increasing trend. The incidence in 2004 (0.28 per 100,000) was similar to that for 1995. Twenty-six countries reported 1,491 cases in 2005. Denmark (0.85 per 100,000), followed by Finland (0.69 per 100,000) reported the highest incidence rates. The overall incidence in the EU was estimated as 0.33 per 100,000 population. Other food-associated pathogens have been reported to be present but with a lower numbers: *Salmonella*, *Shigella*, *Staphylococcus* and *Yersinia* species (Amato-Gauci and Ammon, 2007).

The genus *Pseudomonas* comprises a group of Gram-negative non-spore-forming rods, which are mostly aerobic. They can be found abundantly in soils, water and many other habitats. The pseudomonads are globally active in aerobic decomposition and biodegradation, and hence play a key role in the balance of nature and in the economy of human affairs.

Some species of *Pseudomonas* are known to cause spoilage in specific types of produce. For example, an outbreak of spoilage of onion bulbs was found to be caused by *P. aeruginosa* (Cother *et al*, 1976). *Burkholderia cepacia* has been shown to cause

sour skin of onion bulb (Yabunchi *et al*, 1992) and is also able to infect patients with compromised immune system.

A common feature associated with lactic acid bacteria (LAB) responsible for food fermentation or spoilage is that the growth of rapidly growing aerobic spoilage organisms is limited by extrinsic or intrinsic factors. Food packaging providing in low oxygen content, such as a vacuum or modified atmosphere, low pH and low temperature are the most common factors selecting LAB as the main spoilage-causing bacterial group (Etchells *et al*, 1975).

Atypical flavours, such as cheesy, sour, acid and sometimes liver-like, are usually the first changes associated with LAB spoilage in raw meat (Pierson *et al*, 1970; Egan and Shay, 1982; Egan, 1983; Schillinger and Lucke, 1987). These changes have been noted, together with atypical aromas and off-odours. Similar changes also affect cooked meat products spoiled by LAB (Allen and Foster, 1960; Reuter, 1970; Egan *et al*, 1980; Borch and Nerbrink, 1989; Korkeala *et al*, 1985).

The microorganisms of particular interest in this study are *E. coli* and *L. monocytogenes*.

1.4.2. *Escherichia coli*

In 1885, Theodor Escherich described an organism he had isolated from infant stools one he named *Bacterium coli commune*. The genus *Escherichia* now comprises: Gram negative, non-sporing rods; often motile and often opportunist pathogens. Species are easy to cultivate on ordinary laboratory media and are aerobic and facultative anaerobic. All species ferment glucose with the formation of acid or of acid and gas,

both aerobically and anaerobically. *E. coli* is mesophilic and a gut commensal (Laparra and Sanz, 2010). It is found in warm-blooded mammals and in terrestrial and aquatic environments in temperate and tropical latitudes (Ingraham, 1958).

Although most *E. coli* strains are 'harmless', some are pathogenic, causing diarrhoea and urinary tract infections. Gastroenteritis is the most common disease associated with *E. coli* along with a number of diseases including septicaemia, urinary tract infections (UTIs) and neonatal meningitis. Symptoms of *E. coli* O157:H7 (first described in 1977) infection can be divided in three categories: the less severe, haemorrhagic diarrhoea; haemolytic-uraemic syndrome (HUS) caused by verocytotoxigenic *E. coli* (VTEC) which causes diverse types of bleeding, fever and may result in kidney failure, and finally thrombotic thrombocytopenic purpura (TTP) causing fever, bruises, renal failure, mental impairment due to the loss of platelets, and in severe cases death (Kaye and Obrig, 1996).

Contamination is frequent in meat and raw milk products because *E. coli* is naturally present in the intestinal micro-flora of humans and animals. It can survive and grow at temperatures between 10°C to 30°C and higher (Kasrazadeh and Genigeorgis, 1995). However, it was reported that almost instant death occurred when the temperature reached 63°C (Bell, 2002). Because physicochemical conditions in nature are rarely all optimal at the same time, growth rates of microorganisms are generally well below the maximum growth rates recorded in the laboratory. For instance, the generation time of *E. coli* in the intestinal tract is about 12 hours (two doublings per day) whereas in pure culture it grows much faster, with a minimum generation time of 20 minutes under the best of conditions (Sutherland *et al*, 1995). Its optimum growth temperature corresponds to the general body temperature of 37°C.

Cagney *et al* (2004) surveyed 1533 minced beef samples in the Republic of Ireland and found that 2.80% of the samples tested positive for *E. coli* O157:H7. A study from an outbreak in Britain showed that concentrations as low as 2 cells per 25g were enough for a disease outbreak to occur (Willshaw *et al*, 1994).

Typically, *E. coli* is an intestinal parasite of humans and animals, though some species may occur in other parts of the body, on plants and in the soil and many species are pathogenic (Wilson and Miles, 1964). The toxigenic property of the outer membrane layer (endotoxin) of these bacteria is responsible for some of the symptoms of infection. Some strains such as O157 also produces extracellular Shiga-like toxin (shiga-toxin producing *E. coli*: STEC), which inhibits protein synthesis in host cells (Moretro *et al*, 2010). Depending on the virulence genes acquired, different types of pathogenicity are conferred upon certain strains of *E. coli*. These strains are classified as enteropathogenic *E. coli* (EPEC), entero-toxigenic *E. coli* (ETEC), entero-invasive *E. coli* (EIEC), entero-haemorrhagic *E. coli* (EHEC) and entero-aggregative *E. coli* (EAEC) (Nataro and Kaper, 1998). The different virulence factors expressed by the organism, e.g. colonisation factors, ability to invade epithelial cells of the small intestine, haemolysin production and toxin production, lead to the different strains of *E. coli* being associated with a wide variety of types of disease.

E. coli is acquired by infants within a very few days of birth. The organism is acquired predominantly from the mother by the faecal–oral route, but also from the environmental surroundings. HUS is the major cause of acute renal failure in children in Britain and several other countries. Generally, about 5% of cases of haemorrhagic colitis caused by VTEC progress to HUS, in which the case fatality rate is approximately 10% (Anonymous, 1995). There are no specific treatments of the

conditions caused by VTEC and each symptom is treated as it occurs in the individual (www.food.gov.uk; Anonymous, 1995).

In some countries in which surveillance of foodborne infections has become routine and considered reliably indicative of trends, e.g. some western European countries, the USA and Canada, the numbers of cases of VTEC-related illness is steadily increasing. In the last 10 years, the incidence has more than doubled, rising between 1995 (1.4 per 100 000) and 2002 (3.2 per 100 000) and levelling off in more recent years (Amato-Gauci and Ammon, 2007). In 2011, a new strain of *Escherichia coli* called O104:H4 resulted in an outbreak from Germany through Europe causing 470 cases with 17 deaths in Germany, 15 cases and one death in Sweden, and some other cases in Denmark, the Netherlands, UK and Spain (Bielaszewska *et al*, 2011).

The very low infective dose of some VTEC (<100 cells of the organism), particularly VTEC O157:H7, underlines the importance of ensuring that the highest possible standards are maintained in agricultural practice and that food processors consistently operate well-designed and effective hygienic food production processes based on HACCP assessments of each food process (www.food.gov.uk). In addition to attention to the detail of cleaning and hygiene procedures, the treatment and formulation of food products are important for controlling any residual *E. coli* and preventing their potential to cause harm to consumers.

In the food industry, the absence of *E. coli* is commonly included in buying specifications relating to raw materials and finished food products as an indicator of the hygienic status of the food (Kiermeier *et al*, 2006) and faecal indicator in water (Paruch and Mæhlum, 2012). It is also included in some industry guidelines and legislation but, currently, there is limited food-related legislation that refers to pathogenic *E. coli*,

although, these were included in the generic statements made in some food- and water-related legislation concerning microbiological safety requirements (Anonymous, 1980; Anonymous, 1992). In 2000, the final text of the new European Water Directive stated, among other requirements, that 'Member States shall take the measures necessary to ensure that water intended for human consumption is wholesome and clean' and that this means the water must be free from any microorganism and parasites and from any substances which, in numbers or concentrations, constitute a potential danger to human health (ec.europa.eu).

Thus, the food producer or manufacturer needs to demonstrate well-structured and reliably operated procedures targeted to control *E. coli* O157 and other VTEC.

1.4.3. *Listeria monocytogenes*

Listeria monocytogenes, first described in 1924, is, like *E. coli* O157, an emerging pathogen, but it is associated with some different foods such as dairy products, raw and undercooked meats and seafood. *L. monocytogenes* is a pathogenic, Gram-positive, nonendospore-forming bacillus. It is the main human pathogen of the *Listeria* genus (Jones, 1990). The symptoms linked to the infection from this pathogen involve several inflammatory infections (listeriosis) in humans as well as animals. Bacteria of the genus *Listeria* are widely distributed in nature and have been isolated from soil, vegetation, sewage, water, animal feed, fresh and frozen poultry, slaughter-house waste and healthy human and animal carriers (Welshimer, 1981). It is a facultative intracellular parasite that binds to the surfaces of a macrophage or liver cell, triggering its own endocytosis which is a process by which cells absorb molecules such as proteins. Once inside a human cell, *Listeria* synthesises a pore-forming protein,

called listeriolysin O, which forms a hole in the membranes, releasing the bacterium from the internalisation vacuole and enabling its spread intracellularly and intercellularly using actin-base motility process. *Listeria* then grows and reproduces in the cytosol, sheltered from the humoral immune system (Cossart and Roy, 2010).

Virulence of *Listeria* is directly related to its ability to live within cells, a property conferred by listeriolysin O and the membrane protein that triggers endocytosis; it produces no toxins or enzymes that make it virulent. *Listeria* is inactive as a pathogen until it is at 37ºC in a human body. As a result, the bacterium reproduces rapidly only when it is in a host, thus exerts pathogenic effects (Cossart and Roy, 2010).

The factors predisposing infection are not fully understood but include host immunity, level of inoculum and virulence including haemolytic activity of the specific *L. monocytogenes* strain. The UK Public Health Laboratory Service defines a case of listeriosis as a 'patient with a compatible illness' from whom *L. monocytogenes* was isolated from a normally sterile site (usually blood or cerebrospinal fluid, CSF) (Anonymous, 1997). *L. monocytogenes* is transmitted via three main routes; contact with animals, cross-infection of new-born babies in hospital and foodborne infection. The latter two sources account for the majority of cases of listeriosis in humans. It is a facultative anaerobic bacterium (Buchanan *et al*, 1989) which may survive on food-processing equipment (Lundén *et al*, 2003). Studies have also shown contamination and survival of the bacterium on hands and gloves used to handle food (Destro *et al*, 1996; Autio *et al*, 1999). *L. monocytogenes* may adhere to surfaces it comes in contact with and grow in low temperatures (Mafu, *et al*, 1990; Walker *et al*, 1990). The optimum growth temperature of *L. monocytogenes* is in between 30-37°C but it is also able to grow at temperatures varying from -1.5°C to 44°C (; Khan *et al*, 1972; Grau and Vanderlinde, 1990; Lovett, 1989; Hudson *et al*, 1994) such as in chilling rooms (Jeong

and Frank, 1994). It therefore belongs to the psychrotroph group but may also grow and survive at similar temperatures to the mesophile group such as *E. coli* (Lecuit, 2007). Norwood and Gilmour's study (2001) showed that two different strains of *L. monocytogenes* resisted temperatures as low as 4°C with substantial adherence to surfaces at this temperature. However, it cannot survive heating at 60°C for a period of 30 minutes (USDA, FSIS, 1990).

The symptoms of listeriosis do not usually resemble those of the more familiar types of food poisoning but there have been several episodes in the last decade where the presence of extremely high levels of *L. monocytogenes* has resulted in the rapid onset of symptoms of vomiting and diarrhoea with few apparent cases of classical listeriosis (Salamina *et al*, 1996; Dalton *et al*, 1997; Aureli *et al*, 2000). *L. monocytogenes* can cause a variety of infections but listeriosis most commonly takes the form of an infection of the uterus, the bloodstream or the central nervous system which in pregnant women can result in spontaneous abortion, stillbirth or birth of a severely ill baby owing to infection of the foetus (DiMaio, 2000). Listeriosis may also be acquired by new-born babies owing to postnatal infection from the mother or other infected babies. The mother is rarely severely affected by listeriosis as the disease appears to focus on the foetus (Rocourt, 1996). Individuals principally at risk from listeriosis have been reviewed by Rocourt (1996) and in order of descending risk are organ transplant patients, patients with AIDS, HIV-infected individuals, pregnant women, patients with cancer and the elderly, although, healthy non-pregnant adults may also suffer listeriosis. In such groups, listeriosis usually presents as meningitis and septicaemia (Radice *et al*, 2006; Tsai *et al*, 2006; Kruszyna *et al*, 2008).

Listeria species are widely distributed in the environment and the occurrence of *L. monocytogenes* in raw and processed foods has been extensively studied. Embarek (1994) summarised many reports relating to raw and processed seafoods in which prevalences up to 75% in lightly preserved (cold-smoked, hotsmoked, marinated) fish products are noted. Beuchat (1996) summarised the results of studies of the prevalence of *L. monocytogenes* in raw vegetables including bean sprouts, cabbage, cucumber, potatoes, pre-packed salads, radish, salad vegetables, tomatoes; reported prevalences ranged from 1.1 to 85.7%.

When reviewing studies of the prevalence of *L. monocytogenes* in meat and poultry products, Jay (1996) combined the reported findings from a number of countries to give an overall prevalence in the different meat products. In addition, 2–6% of healthy individuals are reported to be asymptomatic faecal carriers of *L. monocytogenes* (Rocourt, 1996).

Listeriosis is a rare but serious illness with an incidence of 2–3 cases per million of the total population of England and Wales (McLauchlin, 1993). In some cases, infected patients were recorded to be healthy prior to the infection (Roed *et al*, 2012). In many cases, the origin of foodborne outbreaks of *L. monocytogenes* has been shown to be related to asymptomatic carriers working in the kitchen (Hedberg *et al*, 1994). Out of 2449 cases of human listeriosis recorded in England and Wales between 1983 and 2000, a total of 739 (30.2%) were associated with pregnancy, although significant variations can be seen from year to year (11–48%).

The occurrence of listeriosis in man is sporadic although a few outbreaks have been reported notable in USA Texas in raw milk and Hispanic soft cheese in 2005 (CDC, 2009), Czech Republic with soft cheese in 2006 (EFSA, 2007) and in Canada

(Quebec) in raw milk and soft cheese in 2008 (Anonymous, 2008). The increase in consumption of ready-to-eat products and the difficulties in controlling temperature for the global trade distribution of these products could help account for the observed increase of listeriosis over the past few years (Goulet *et al*, 2008; Cairns and Payne, 2009; Kvistholm *et al*, 2010). Therefore there has been a progressive increase in the incidence of listeriosis since its discovery and a much higher rate within the last few years. The most common benign symptoms involving *L. monocytogenes* infection present as fever, headaches, muscle aches and sometimes nausea or diarrhoea (Todd and Notermans, 2010).

In the UK the levels of listeriosis have been relatively static except for a large rise in numbers of cases recorded for the period between 1987 and 1989 because of a contaminated food, pâté, from a single Belgian manufacturer (McLauchlin *et al*, 1991). The subsequent fall is believed to have been due to public health advice relating to the consumption of higher-risk food commodities including pâté and the concerted industry action at that time to control the organism (Gilbert, 1996).

The incidence of listeriosis in other countries is similar to that in England and Wales and similar decreases in infections to those noted in the UK have been reported in the USA following improvements in regulatory/industry control and the issuing of advice to susceptible groups (Tappero *et al*, 1995). In spite of the relatively low incidence of disease, i.e. 2–4 cases per million in some western countries, listeriosis is a serious illness and this is reflected by the apparent high mortality rate in many cases, with fatalities averaging approximately 30% (Newton *et al*, 1992).

It is clear that eliminating *L. monocytogenes* from most foods is both impractical and impossible but it is possible to reduce and control this hazard in foods, thereby

minimising the risk presented to public health. In order to ensure the safety of food products in respect of potential foodborne bacterial pathogens, growing, harvesting, handling, storage, processing and associated food supply systems must be managed by food producers and processors in such a way as to reliably control the growth of *L. monocytogenes* which must be prevented from multiplying to potentially harmful levels (>100cells/g) (Gilbert *et al*, 2000). To achieve this, it is necessary to understand the conditions and factors that affect growth and survival.

To control the growth of *L. monocytogenes* in chilled foods, it is crucial to operate and apply well-controlled chill temperature holding and storage systems both within the production process for component or part-processed foods and for finished product storage and distribution. This and other physico-chemical factors can control the survival and growth of *L. monocytogenes* during the manufacturing process and the shelf-life of the finished food products (Bell and Kyriakides, 1998). Temperature and humidity level were two factors used in this study to assess the survival of *E. coli* and *L. monocytogenes*.

In the food industry, different factors such as physical, chemical and biological may affect the substratum surface properties (e.g. food contact) and subsequently the survival of microorganisms on those surfaces.

1.5. Factors affecting maintenance of surface properties

1.5.1. Physical properties

Prevention of contamination of surfaces with pathogenic and spoilage bacteria by a daily cleaning and disinfecting routine is essential. Cleaning and disinfecting products must be appropriate to the type of soil and bacteria encountered (Gram *et al*, 2007). In general, 'open' surfaces will not be in constant contact with liquids. Thus during cleaning, the majority of bacteria will be removed, although retained cells and micro-colonies may remain in areas of poor accessibility. In suitable conditions, these micro-colonies may develop and accumulate (Verran *et al*, 2008). Strategies for maintaining and developing good hygienic surfaces therefore include hardness and easy cleanability. Topography should be designed to minimise retention of cells on the surface, the smoother the surface, the smaller surface contact between the substrata and the cell (page 4 and 5 of this chapter).

The surface roughness is described by a range of different parameters. The surface is defined by arithmetical mean roughness (R_a) and ten-point mean roughness (R_z) which describe the vertical deviation of the roughness profile from the mean line. The hydrophobicity of the surface may be affected by its topography. Super brushed (or called fine polish in this study) stainless steel 304 presented a topography defined by R_a = 0.3–0.5 mm and R_z = 2.5–3.8 mm, and hydrophobicity of 85.5 ± 4.5° (Whitehead and Verran, 2007). The features size/profile of the surface may also impact on cell retention. Surface imperfections of 1–2 mm wide and 0.25 mm deep were also observed on stainless steel 304 super brushed (Whitehead and Verran, 2007).

Hydrophobicity affects microbial cell survival and retention on a surface. A horizontal hydrophobic surface will affect the drying period by allowing droplets formation which take longer to dry, promoting cell deposition at the solid-liquid-air interface. However, this type of surface when placed at an angle also allows ease of cleaning because moisture rolls off the hydrophobic surface (Whitehead and Verran, 2007). The hydrophobicity of stainless steel surfaces may be modified by plasma-surface modification (Chun *et al*, 2002). Mixed gases of nitrogen and oxygen by atmospheric-pressure plasma jet method, successfully modified the stainless steel substrata into hydrophilic surfaces (Kim *et al*, 2003) which would reduce the adherence of hydrophobic cells, thus enhance cleaning and hygiene of the surfaces.

If the aim is to retain a hard metallic surface, with polymers being too soft and ceramics cracking (as previously noted), then one needs to modify the surface of metals to improve cleaning, increase antimicrobial properties and reduce retention (i.e. maintaining a smooth surface with low cell retention).

1.5.2. Chemical properties

An approach to reduce the cleaning efforts is to alter interfacial interactions between contamination and the machine surface (Förster and Bohnet, 1999; Liu *et al*, 2006): the interactions can be affected positively through the use of surface modification.

Some metals possess antimicrobial properties. However, the intended application of the surface should be considered, with issues of toxicity (particularly in the food industry), ion release, and sensitivity of likely target microorganisms taken into

account (Kelly *et al*, 2010). It may therefore be necessary to utilise different metal alloys depending on the most common type of bacteria likely to be encountered, and the intended environment in which the surface will be placed (Verran *et al*, 2008).

Copper is an essential micronutrient required in very small amounts for survival of most aerobic organisms. At higher concentrations it can become toxic and inhibit microbial growth; hence, it is essential for cells to maintain appropriate intracellular copper concentrations. The mechanisms of copper-mediated inhibition of cell growth may include substitution of essential ions and blocking of protein functional groups, inactivation of enzymes, production of hydroperoxide free radicals by membrane bound copper, and disruption of membrane integrity (Ohsumi *et al*, 1988; Rodriguez-Montelongo *et al*, 1993; Nies, 1999; Macomber and Imlay, 2009; Grass *et al*, 2011).

Copper has been utilised by human civilisation for more than 10,000 years. Although the exact antimicrobial mechanisms are not fully understood, its benefits have long been recognised (www.copperinfo.co.uk). A study describes how in ancient Greece, Hippocrates recommended the topical application of copper to treat leg ulcers, and, in the pre-antibiotic era of the nineteenth and twentieth century, copper preparations were widely used in the treatment of skin conditions, syphilis and tuberculosis (Dollwet and Sorenson, 1985).

Copper is most often used in small quantities when combined with other metals if used in food contact surfaces (www.fda.gov). However, this inhibition may be altered when copper is associated with other metals such as TiN (titanium nitride) because the two metals react when alloyed together, thus changing their properties (Kelly *et al*, 2010). *L. monocytogenes* was unable to survive over a period of 60 min on a copper surface when incubated at room temperature (Wilks *et al*, 2006). Copper alloys

are effective in rapidly killing bacteria on contact by ion release and this inactivation is enhanced by low moisture conditions, minimal media, and high copper corrosion rates (Elguindi *et al*, 2011). Copper alloys also inhibited the adhesion of bacteria during biofilm development (Kielemoes and Verstraete, 2001). Copper alloys reduced the viability of *E. coli* O157:H7, *L. monocytogenes* and methicillin-resistant *Staphylococcus aureus* (Michels *et al*, 2005), and these authors suggested that copper alloys be used in surfaces exposed to human touch or food contact. Noyce *et al* (2006) showed that copper cast alloys significantly reduced the population of *E. coli* O157:H7 and concluded that these alloys have the potential to aid in food safety. In the 1990s, researchers in Japan were the first to fabricate a copper-stainless steel antimicrobial composite by adding copper to stainless steel, followed by a complicated heat treatment (Silver *et al*, 2006). The Kawasaki Company has produced two types of silver-stainless steel using a similar method (Sreekumari *et al*, 2003).

The antibacterial effects of metallic silver have been known for centuries. Wasif and Laga (2009) described the history of silver as an antimicrobial agent dating as far as the ancient Egyptians period, in Roman time to prevent spoilage in wine, and during World War I to combat infection in wounds.

The silver ion exhibits broad-spectrum antimicrobial activity toward many different types of bacteria and is believed to be the active component in silver-based products (Sharma *et al*, 2009; Martínez-Castañón *et al*, 2008; Rai *et al*, 2009; Dong *et al*, 2008; Wang *et al*, 2006; Dowling *et al*, 2003). Studies on the effect of silver showed that when the surface came in contact with a living cell, ions were released which denatured the enzymes of the cell by binding to reactive groups (Shrestha *et al*, 2009). The ions released inactivate enzymes responsible for DNA replication by binding to

sulfhydryl groups in thiol groups (Modak and Fox, 1973; Fox and Modak, 1974; Flemming *et al*, 1990; Russell and Hugo, 1994; Lin *et al*, 1996; Liau *et al*, 1997; Feng *et al*, 2000; Lansdown, 2006; Silvestry-Rodriguez *et al*, 2007). It was also noticed that morphological changes in the outer layers of the cell were due to the presence of small electron dense granules, an end product from the reaction of silver ions and sulphur in bacteria (Nover *et al*, 1983; Feng *et al*, 2000).

Thin film coating is a method used to deposit metals on surfaces to alter their properties e.g. to enhance antimicrobial properties (Foster *et al*, 2010). Coating composition is enabled by co-deposition of target metal ions by pulsed magnetron sputtering where ions or atoms hit the "sputtering target" (e.g. silver or copper). Inert gas plasma is used to bombard the atoms ejected from the target and move them in all directions, thus allowing an even coating of all surfaces, directly in line or underneath overhangs (Kelly *et al*, 2009). Sputtered atoms are ejected and travel on a linear trajectory to impact the "substrate" (which is the surface to coat) thus forming a layer of coating (Kelly *et al*, 2009). Among other metals tested, it was shown that an increase in silver or copper concentration decreased significantly the colony forming units of *Staphylococcus aureus* and *Pseudomonas aeruginosa* on the surfaces tested (Kelly *et al*, 2010). As silver is widely recognised for its antimicrobial properties (Dowling *et al*, 2003; Wang *et al*, 2006; Dong *et al*, 2008; Martínez-Castañón *et al*, 2008; Rai *et al*, 2009; Sharma *et al*, 2009; Shrestha *et al*, 2009), in the present study, it was used in different concentrations as a coating alloyed with titanium nitride: the latter component providing coating strength and hardness.

Silver ions are known for their antimicrobial properties and are widely used as bactericides in catheters, burn wounds and dental work. In the presence of food

material, this effect might be prevented if the cells' surface does not come in direct contact with the putative antimicrobial surfaces due to the presence of interfering substances (Verran *et al*, 2008).

Previous work has shown that silver nanoparticles assembled on a surface of glass, ceramic and TiN can provide strong antimicrobial properties (Lv *et al*, 2008; Kelly *et al*, 2009; Lv *et al*, 2009). Its presence affects the survival of bacteria via the release of nanoparticle silver ions which inhibit bacterial growth in the presence of humidity (Juan *et al*, 2010). Therefore, silver nanoparticles appear to be an excellent choice for coating the surface of stainless steel to restrain bacterial contamination and the formation of biofilm perhaps over and above that of copper with reduced toxicity. Silver ions were shown to have more antimicrobial effects when left to react for longer than 24 hours (Chen *et al*, 2010), which is important when looking at the survival of microorganisms on alloy coatings, containing silver metals, over days or even weeks, particularly in the presence or absence of moisture. Overall, silver is less toxic to food than copper.

The physical and chemical properties of the surfaces affect the survival of the bacteria. *In situ*, in the food industry, the presence of organic matter may also affect the survival of bacteria on surfaces.

1.5.3. Biological properties

In food processing systems, the amount of food residue left on the surfaces represents a source of nutrients for microorganisms (Hood and Zottola, 1997). Two methods are necessary to enable excellent food hygiene: cleaning to remove soil and disinfecting to reduce the viability of potential pathogens present. Bacteria may come in

contact with food or working surfaces, thus posing sanitation problems as well as financial costs (Holah and Kearney, 1992; Carpentier and Cerf, 1993).

The development of adsorbed layers, termed conditioning films on a surface are considered to be the first stage in biofilm formation. On open food contact surfaces once microbial attachment has occurred a ‘true’ biofilm, that is “a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, and that are embedded in a matrix of extracellular polymeric substances that they have produced” (Zoltai *et al*, 1981; Donlan and Costerton, 2002) is unlikely to develop. However, the transfer of cells and soil to the surface, a process known as biofouling, may cause a gradual build up of material on the surface unless it is removed using a cleaning process (Verran, 2002).The presence of soil during the incubation of the bacteria in a dry environment was shown to have a ‘protective effect’ which was also enhanced in the presence of salt (Vogel *et al*, 2010). This demonstrated the importance of the source of organic material in the survival of bacteria.

The composition of a specific type of organic soil can influence the growth of a particular microorganism. As shown in the study conducted by Chung *et al* (1989), the *in situ* environment in food industry show a flora composed of more than one type of microorganism and different type of soiling. Results showed how the growth of the bacterium *P. aeruginosa* significantly overtook *L. monocytogenes* on meat because it was more adapted to this type of environment than *L. monocytogenes*. Several studies reported the persistence of some pathogens associated with food contamination such as *Listeria monocytogenes* (Farber and Peterkin, 1991), *Yersinia enterocolitica* (Kumar and Singh, 1994), *Campylobacter jejuni* (Stern and Kazmi, 1989) and *Escherichia coli* O157:H7 (Doyle and Padhye, 1989; Doyle, 1991; Dewanti and Wong, 1995).

In this study, meat extract was selected to assess its effects on growth of *E. coli* in different conditions on the selected substrata. *L. monocytogenes* was not included in this part of the project

1.6. Environmental conditions

1.6.1. Temperature

Temperature affects the potential for microbial growth, the rate of growth or death and the production of metabolites, assuming nutrients are available (Rodrigues *et al*, 2009). There are upper and lower limits to growth, at which the growth rate becomes zero, and an optimum temperature at which the growth rate is maximal. The minimum, maximum and optimum temperatures for growth are known as the cardinal temperatures for growth (Van Derlinden *et al*, 2008).

Between the minimum and optimal temperatures, growth rate increases with increasing temperature. The growth rate increase is not proportional to the temperature, but increases more rapidly as the temperature is increased. As the temperature increases above the optimum, the growth rate decreases rapidly, due to thermal inactivation of cellular macro-molecules needed for growth. At low temperatures the growth rate does not necessarily decrease indefinitely to zero and there may be a critical threshold temperature below which growth suddenly is not possible (Zwietering *et al*, 1990).

Each organism has its own preferred temperature range for growth, related to its usual growth habitat. For bacteria, the range of growth usually spans 35- 40°C, irrespective of the preferred temperature region for growth. According to the preferred

temperature region for growth, organisms are classified as psychrophiles (≤ 15ºC), psychrotrophs (25-30ºC), mesophiles (35-45ºC) or thermophiles (> 45ºC) (Ratkowsky *et al*, 1982; ICMSF, 1996).

Despite the fact that organisms have adapted to different temperature ranges for growth. Among the fastest growing organisms known are those that are selected for and cause problems in moist, proteinaceous foods. Those foods are very nutritious, temperature and air supply are the only constraints to microbial growth. Among those organisms, strains that grow fastest at low temperatures nonetheless grow more slowly at their optimum than those species best adapted to growth at higher temperature. For example, the fastest known bacterial growth rates recorded are for anaerobic bacterium *Clostridium perfringens* in ground beef, with a generation time of 7 min in the temperature range 40-45°C (Willardsen *et al*, 1978; Labbe and Huang, 1995).

The growth of Enterobacteriaceae, comprising a cocktail of five representative species (*Proteus mirabilis*, *Klebsiella pneumoniae*, *Citrobacter freundii*, *Enterobacter cloaceae* and *Hafnia alvei*) was predicted for a variety of factors including temperature (0-30°C). With regard to temperature, some species grow better, or are metabolically more active, at 25-30°C although the majority of species grow well at 37°C. However, within the Enterobacteriaceae psychrotrophic strains are reported to exist (Michener and Elliott, 1964; Kraft, 1992) and growth of some coliform bacteria at temperatures as low as -1.5 to 1.5°C in milk has been reported (Eddy and Kitchell, 1959). At these lower temperatures growth rates are slower, although in raw milk during refrigeration at 3-5°C coliforms have been reported to increase 100 or even 1000-fold in 3 days (Panes and Thomas, 1968). Reducing the temperature typically increases the generation time which results in slower growth. Generation times for some psychrotrophs have been reported between 0°C and 32°C by Tompkin (1973).

While some strains may tolerate and even grow at temperatures as high as 50°C members of the Enterobacteriaceae are not particularly heat resistant and these bacteria would be killed by pasteurisation temperatures (typically >72°C) used in many food processes. For *E. coli* is $D_{56°C} = 4\text{-}6$min and $D_{60°C} = 2$min at, whereas for *Salmonella* it is $D_{60°C} = 0.1\text{-}2.5$ min, $D_{65°C} = 0.07$ min and $D_{70°C} = 0.3$min (Mossel *et al*, 1995). Although there are occasional strains that appear to show greater tolerance of heat compared with others these are very much the exception. The majority of the Enterobacteriaceae are not exceptionally heat resistant, which therefore makes them suitable indicator organisms for contamination of cooked and other heat processed foods. A study of thermal resistance of *E. coli* 0157:H7 in beef burgers revealed greater heat resistance of this bacterium when cells had been previously exposed to frozen storage compared with those that had been stored at 15°C prior to cooking (Jackson *et al*, 1996). Under normal cooking conditions, a 4 Log_{10} cycle reduction in the *E. coli* 0157:H7 population in beef burgers was achieved when the burgers were heated to an internal temperature of 68.3°C (Juneja *et al*, 1997). These results show that pasteurising food is the best way to eliminate any viable *E. coli* cells followed by, cooking, which reduces the bacterial population. Chilling food only reduces or may stop the growth of the bacterium, however it does not eliminate the potential threat when the food is brought back to higher temperatures.

L. monocytogenes is a psychrotrophic bacterium able to grow at refrigeration temperatures, although optimal growth occurs between 30 and 37°C. The influence of temperature on the survival and growth of *L. monocytogenes* has been examined, frequently in conjunction with other environmental variables. It was reported that the mean minimum growth temperature of *L. monocytogenes* was + 1.1 ± 0.3ºC (Holt *et al*, 1994; Lou and Youself, 1999; Rocourt, 1999). Generation times in various milk

products ranged from 1.2 to 1.7 days at 4ºC, 5.0 to 7.2 h at 13ºC, and 0.65 to 0.69 h at 35°C. It was established that the minimum growth temperatures ranged from - 0.1 to - 0.4ºC in chicken broth, with generation times ranging from 13 to 24 hours at 5°C to 62 to 131 hours at 0°C (Walker *et al*, 1990). Growth temperature has also been shown to influence the virulence of *L. monocytogenes*. Growth at 4°C significantly increased the virulence of three clinical listerial isolates in mice when compared to cells grown at 37°C for intravenously, but not intragastrically, inoculated mice. Growth at 4°C significantly decreased the killing of test strains by human neutrophils (Czuprynski *et al*, 1989).

A physiological response of many bacteria, which is of particular concern when this occurs in foodborne pathogens, is their ability to survive adverse environmental conditions after prior exposure to non-lethal stress such as cold, heat, acid, etc. This can induce expression of stress proteins by the cell, which can in turn enable them to survive adverse conditions or treatments that would otherwise be lethal. This cross-protection, whereby exposure to one stress factor can bring about enhanced resistance against another one, can result in greater heat resistance and acid tolerance of these bacteria. This phenomenon has been studied in many pathogens, including *E. coli* 0157:H7 which exhibits greater heat and acid resistance in foods after exposure to sub-lethal treatments and stresses (Ryu and Beuchat, 1998; Duffy and Garvey, 2001).

Temperature is one of the key points of the food chain process used to control the growth of microorganism, and also to kill them. The survival of *E. coli* and *L. monocytogenes* at their optimum growth temperature (37ºC and 30ºC) and room temperature (20ºC) on stainless steel is part of the focus of this study, prior to study of modified surfaces.

1.6.2. Equilibrium Relative Humidity (ERH)

Equilibrium relative humidity is the ratio of the mass of water vapour in a given volume of air to the maximum amount of vapour that the same volume of air can hold at the same temperature and pressure (Jensen, 2012). In food pathogen related literature, water activity a_w is often mentioned in the context of cell growth or survival. Water activity is a measurement of the energy status of the water in a system. It is defined as the vapour pressure of water above a sample divided by that of pure water at the same temperature (Mathlouthi, 2001). Therefore, pure distilled water has a water activity of exactly one. So, a_w is used when studying the water association with non-aqueous constituents and solids (e.g. bacteria) whereas RH is used to define the ratio of water and air.

E. coli survives best in high humidity levels (96% ERH) at normal room temperature, thus loses viability more quickly under desiccation than under high moisture. The absence of moisture exerts severe stress and pressure for survival whereas high humidity conditions and lower temperature (25°C) enhanced survival (Yang *et al*, 2009). During desiccation, water is removed from the lipid bilayer which forces the polar lipid head groups to get close together, thereby increasing lipid packing and the interactions between hydrocarbon acyl chains of neighbouring lipids, thus leading to a decrease in membrane fluidity (Scherber *et al*, 2009).

L. monocytogenes is capable of survival in dry conditions for up to three months, on clean or soiled stainless steel food grade contact surfaces (Vogel *et al*, 2010). The number of cells decreased in dry conditions but viable cells remained and were able to recover when encountering a favourable environment, thus presenting a considerable risk for cross-contamination. Different strains of *L. monocytogenes* were shown to have

a common ability to tolerate desiccation conditions when attached to stainless steel surfaces. The presence of soil during the incubation of the bacteria in dry environment was shown to have a “protective effect” which was also enhanced in the presence of salt (Vogel *et al*, 2010). An increase in desiccation resistance of *L. monocytogenes* due to shift in cell metabolism by production of significant amount of extracellular carbohydrates (Hansen and Vogel, 2011) has also been noted, relating to properties of the biofilm phenotype.

Gram-positive cocci became larger when near their humidity growth limits: for example staphylococcal species grew in “tetrad/cubical formations instead of their normal grape-like structure” (De Goffau *et al*, 2009). Gram-negative cells were also affected by low humidity levels and started to grow in wave-like patterns, instead of their original rod shape becoming more filament-like. This change in morphology may affect surface properties and cell retention on the surface – as well as the method used to assess adhesion: if a coverage method is used rather than cell count, then enlarged cells will contribute to coverage, but not cell count. Also, the smaller cells and features affect retention.

A change in room temperature may affect the equilibrium relative humidity (De Goffau et al, 2009). Thus it is important to control both temperature and humidity to ensure that survival of target microorganisms on surface is minimised. The novelty of the research conducted in the following experiments includes the use of different equilibrium relative humidities with different temperatures on stainless steel and surfaces coated with antimicrobial metal alloy using bacteria related to food outbreaks: Gram negative *E. coli* and Gram positive *L. monocytogenes*.

1.7. Aim

To define conditions that minimise survival of *E. coli* and *L. monocytogenes* on stainless steel and stainless steel coated with titanium nitride, alloyed with silver.

1.8. Objectives

1.8.1. Method developed:

- To characterise test surfaces: Atomic Force Microscopy (AFM), White Light Profilometry (WLP), Scanning Electron Microscopy (SEM) and Energy-Dispersive X-ray (EDX).
- To identify the methods that best reveal the presence of bacteria on surfaces: Tetrazolium blue chloride (TBC) metabolic dye assay, epifluorescence microscopy, swabbing and live-dead differential staining.
- To assess the efficiency of antimicrobial titanium nitride/silver alloys: Contact kill, Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) or ion release, Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Zone Of Inhibition (ZOI).

1.8.2. Novel investigations:

- To investigate survival of *E. coli* and *L. monocytogenes* on stainless steel surfaces in different humidities.

- To assess antimicrobial properties of titanium nitride silver thin film coating on stainless steel
- To investigate survival of *E. coli* on stainless steel and coated surfaces in the presence of organic food material.
- To examine the effect of re-using stainless steel and coated surfaces on the survival of microorganisms and the properties of the surfaces.

Chapter 2

Characterisation of putative antimicrobial surfaces

2.1. Introduction

2.1.1. Metals used in surface coatings

Copper and silver are the most commonly used elements amongst metals for their widely known antimicrobial properties (Ohsumi *et al*, 1988; Rodriguez-Montelongo *et al*, 1993; Nies, 1999; Dowling *et al*, 2003; Wang *et al*, 2006; Dong *et al*, 2008; Martínez-Castañón *et al*, 2008; Macomber and Imlay, 2009; Sharma *et al*, 2009; Rai *et al*, 2009; Grass *et al*, 2011). In this work, aspects of copper toxicity precluded its selection for use in development of food contact surfaces (Airey and Verran, 2007). To our knowledge, copper is not currently used as antimicrobial agent in surfaces coming in contact with food. However, copper is widely used as an antimicrobial in surface coatings in hospitals (Casey *et al*, 2009). Silver zeolites were used in food packaging as antimicrobial agent (Quintavalla and Vicini, 2002). Thus, silver was selected for this study because it was deemed to present a better and safer option.

Titanium nitride is widely used as a surface coating because of its low coefficient of friction, high hardness, resistance to corrosion and adhesive wear (Zhang and Zhu, 1993). Titanium nitride coatings deposited on cutting tools and other surfaces that are susceptible to wear help to extend the operating life and range of conditions for which they are used (Watmon and Ijeh, 2010). Silver is a soft metal when used pure, therefore if intended for a surface coating, it is necessary to combine it with a harder coating such as titanium nitride.

2.1.2. Preparation of the metal coatings

Stainless steel is the most commonly used food contact material because its properties make it robust and easy to clean (Holah and Thorpe, 1990; Boulange-Petermann, 1996; Boulange-Petermann *et al*, 1997; Boyd *et al*, 2001). Finishes applied to stainless steel will have a direct bearing on surface appearance, and the environmental performance of the material. Grade 304 stainless steel (SS304) is found throughout the food production chain, from manufacture to storage, in large scale catering, and domestic kitchens (Driessen *et al*, 1984; Lewis and Gilmour, 1987; Boulange-Petermann, 1996) and used as substrate for the deposition of the metal coatings (fine polished).

Magnetron sputtering (*Figure 2.1.*) is a powerful process which is used for the production of thin films and coatings by using a flow of electrons controlled by an external magnetic field to sputter deposit metals as layers (Slavcheva *et al*, 2006). Different power densities (in Watts) are used to control the quantity of deposition: the higher the power, the more deposition. The sputter deposition process operates under different physical conditions which make it possible to form films with new physical and functional properties e.g. alloy films (Wei *et al*, 2002; Gibson *et al*, 2008). The sputtering sources can also be scaled up into large industrial coaters. In addition, sputtering technology is continuously being improved not only through the improvement of existing sputtering systems, but also through the development of new systems (Musil, 2000). This makes it possible to form films with new properties and to develop new technological processes for their production, such as low-pressure sputtering, pulsed sputtering, coating material ions-assisted sputtering, high-rate sputtering and self-sputtering (Window, 1995; Musil, 1997; Musil 1998a; Musil 1998b).

The resultant coatings are characterised by a number of methods appropriate to intended use.

2.1.3. Surface analysis

Surface analysis methods are wide ranging. In scanning electron microscopy (SEM), a fine probe of electrons is focused on a specimen, and scanned along a pattern of parallel lines. Various signals are generated as a result of the impact of the incident electrons, which are collected to form an image or to analyse the sample surface. SEM provides high resolution in combination with good depth of field (Jensen, 2012), and with an energy dispersive X-ray spectrometer (EDX) attached, it provides information on chemical composition within the microstructure at one location (Paoletti *et al*, 2011).

White light profilometry (WLP) is used to perform surface measurements of three dimensional objects by projecting a white-light beam onto the surface (Gorecki *et al*, 1983). The atomic force microscope (AFM) can provide three dimensional images of surface topography in ambient liquid or gas environments. Unlike other techniques used to study microorganisms at high magnification, atomic force microscopy can use samples with minimal preparation over a range of temperatures and in repetitive studies. The high resolution (in the nanometre range) allows topographical imaging of samples such as DNA molecules (Lyubchenko *et al*, 1997), protein adsorption or crystal growth (Yip and Ward, 1996), and living cells adsorbed on biomaterials (Butt *et al*, 1990). In addition to topographical measurements, AFM is also capable of complementary techniques that provide information on other surface properties, such as stiffness, hardness, friction, or elasticity.

In order to analysis surfaces, the R_a value (the arithmetic average height parameter) is generally used as a parameter for comparison of surface roughness (Boulange-Petermann, 1996; Verran and Maryan, 1997; Flint *et al*, 2000; Hilbert *et al*, 2003; Whitehead *et al*, 2005).

2.2. Aim

The aim of this chapter was to characterise test surfaces: stainless steel 304, stainless steel coated with titanium nitride alloyed with different concentrations of silver.

2.3. Methods

2.3.1. Production of nanocomposite coatings

2.3.1.1. Surface characteristics and preparation of fine polished stainless steel

Fine polished stainless steel (SS 304) (Outo Kumpu, Sheffield, UK) was cut into coupons (75 mm x 35 mm) using a guillotine. Before use, the steel coupons were soaked and rinsed in acetone overnight, after which they were rinsed in 96% alcohol for 5 min. Finally, the steel coupons were rinsed with distilled water and air dried, standing on a table overnight.

2.3.1.2. Production of TiN and TiN/Ag surfaces

Magnetron sputtering was used to produce nanocomposite TiN and TiN/Ag films on the stainless steel surfaces in the surface engineering labs at MMU (acknowledgment to David Wickens and Dr. Kathryn Whitehead). To enable the effective analysis of the nanotopographies produced by the thin film nanocomposites to be evaluated, TiN and TiN/Ag were also sputtered onto polished silicon wafers (WRS materials, USA) for atomic force microscopy (AFM) analysis. Before magnetron sputtering, both stainless steel coupons and silicon wafers were cleaned using methanol and a clean fibre-free paper towel (Buehler, TEXMET® 1000, IL, USA), then dusted using a compressed air duster (Electrolube, Leicestershire, UK) to remove any remaining physical contaminants.

To produce the coatings a method was followed from a previously published article (Kelly *et al*, 2009). The sputtering rig used was a Teer Coatings UDP 350 magnetron sputtering system in a closed field unbalanced magnetic configuration (*Figures 2.1 & 2.2*). The chamber was pumped down until a vacuum below 1.1×10^{-3} Pa was achieved. Argon gas (99.99 % purity) was introduced into the chamber using a mass flow controller (MKS Instruments, Altrincham, UK). Different levels of power (in Watts) were used to obtain coatings with different concentrations of silver, the higher the power, the more silver was deposited. The substrates were sputter cleaned by increasing the chamber pressure to 0.4 Pa and applying power to the substrate holder; 150 W 150 kHz for 10 minutes. Throughout the sputtering procedure the titanium target (99.95 % purity) was driven with 1.5 kW pulse DC power with a 20 kHz pulse and the silver target (99.99 % purity) with powers of 120 W to get silver concentrations in the deposited films. The sputter coating procedure took place in a 0.24 Pa argon atmosphere

with the nitrogen delivered for the nitride coating at 60% in relation to full metal signal using a reactive sputter controller following the optical emission signal of the titanium plasma. The samples were attached to the magnetron substrate holder (18 cm x 8 cm) using kapton tape (Agar Scientific, Essex, England). The substrate holder was placed in the magnetron sputtering chamber with the samples facing away from the titanium target and rotated at 16 RPM (Rotation Per Minutes) throughout deposition.

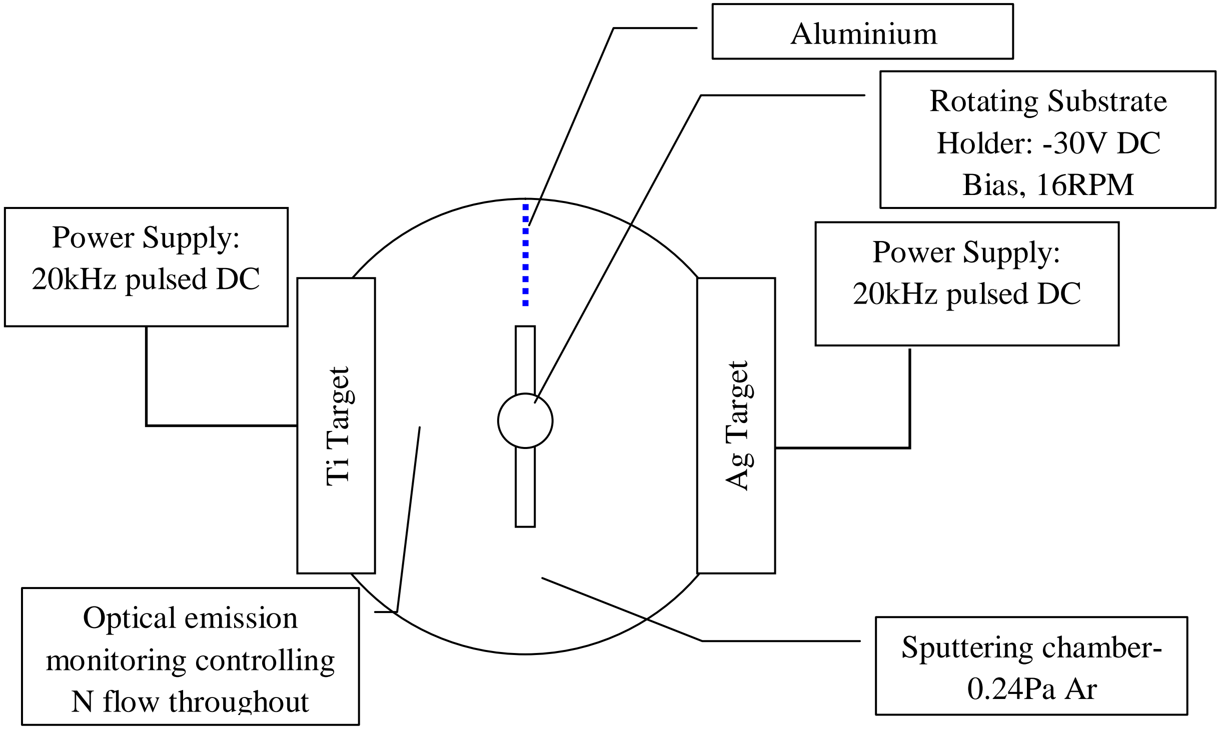


Figure 2.1. Simplified diagram of the Teer Coatings UDP 350 sputter coating rig for Ti, Ag and N. Both targets run simultaneously whilst the substrate holder is rotated at a constant speed. Reactive sputtering is undertaken via optical emissions monitoring to control the nitrogen flow to give a stoichiometric coating.

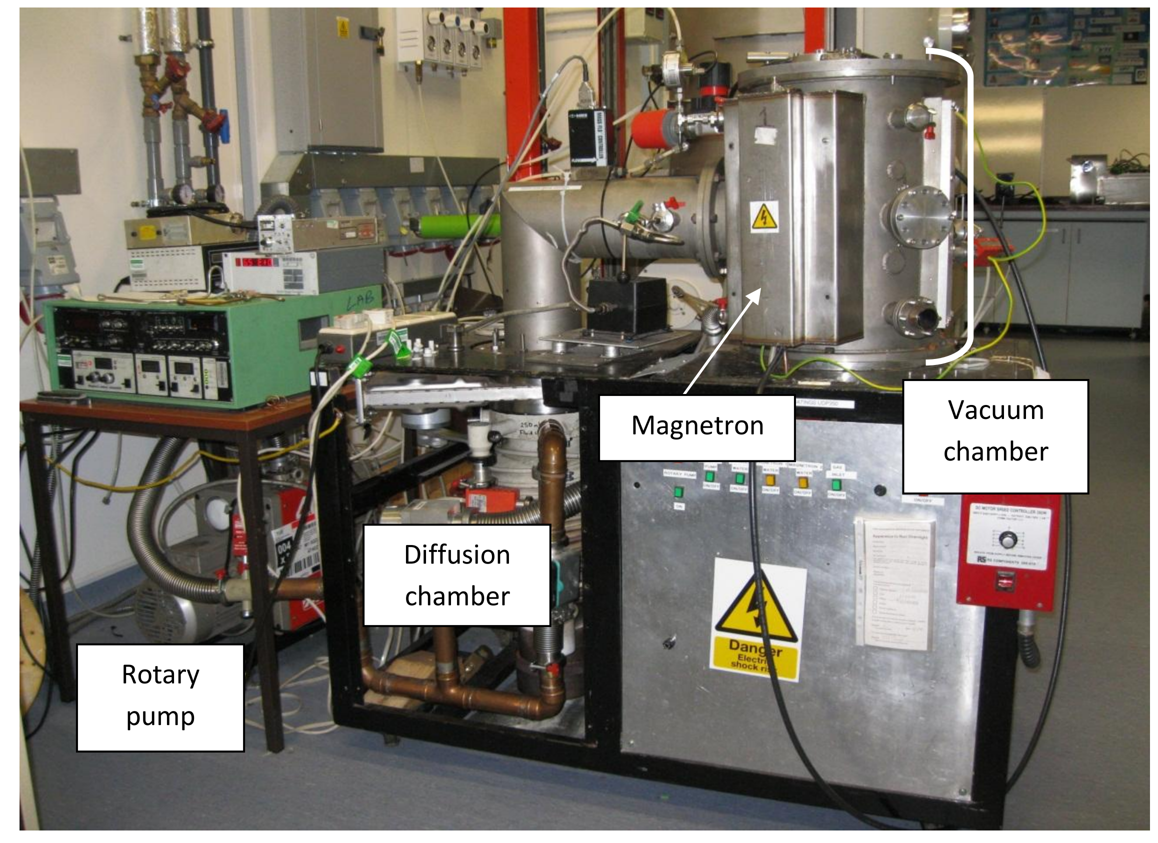


Figure 2.2. Teer coatings UDP 350 magnetron sputtering rig, displaying the chamber on the right, the pumps on the left (below) and monitoring equipment (above).

2.3.2. Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray analysis (EDX)

To determine the presence of an element in a sample the EDX spectra are compared with known characteristic x-ray energy values. Between approximately 0.1 to a few atoms percent can be detected using this method depending on the element and the sample matrix. Images were obtained using a JEOL JSM 5600LV scanning electron microscope (Jeol Ltd, Hertfordshire, UK). Three replicates were used and five areas per replicate were analysed. Chemical analysis of substrata was carried out to a 1µm depth using a Link Pentafet detector (Oxford Instruments, Buckinghamshire, UK), with Inca software (Oxford Instruments, Buckinghamshire, UK). Analysis used a windowless system with a resolution of 133eV.

2.3.3. White Light Profilometry (WLP)

Images of the surface topography of the coatings on stainless steel (x 101.61 magnification = 86.14µm x 64.07µm) were taken using a MicroXAM (phase shift) surface mapping microscope with an ADE phase shift XYZ 4400 mL system and an AD phase shift controller (Omniscan, Wrexham, UK). These images were used to calculate the S_a roughness values (*Table 2.1*). The roughness parameters were represented by R_a values when calculating profile roughness (in two dimensions: atomic force microscopy) and S_a values when calculating area roughness (in three dimensions: white light profilometre). The image analysis system used was Mapview AE 2.17 (Omniscan, Wrexham, UK). Analysis was carried out using EX mode on stainless steel, TiN, TiN/Ag 50W, 100W and 120W, so n = 5.

Table 2.1. Definitions of the roughness values used in this study (www.outokumpu.com)

R_a	Average length between the peaks and valleys and the deviation from the mean line on the entire surface within the sampling length
S_a	Arithmetical mean height of the surface
R_q	The root mean square average of the roughness profile
S_q	Root mean square height of the surface

2.3.4. Atomic Force Microscopy (AFM)

For coating characterisation, coatings were also deposited onto highly polished silicone (Si) wafers (WRS Materials, PA, USA), cut into 10mm x 10mm coupons. Roughness parameters, images and determination of grain sizes were obtained using an atomic force microscopy (AFM) (Explorer, Veeco Instruments, Cambridge, UK) operated in contact mode using silicon nitride pyramidal shaped tips with a force constant defined by the manufacturer as 0.05 $N \cdot m^{-1}$. Cantilever spring constants were taken before each experimental run. Averages of the roughness measurements were taken from replicate 1 x 1μm^2 samples using a scan rate of 20.03$\mu m \cdot s^{-1}$ with 300 pixels resolution (n = 5). Surfaces were analysed using image and linear profiles and roughness parameters.

2.3.5. Wettability of nanocomposite films

Contact angle measurements were determined at room temperature using the sessile drop technique for all substrata. Five microlitres of HPLC grade water (BDH, Poole, UK) were deposited onto the horizontal substrata using a syringe. Contact angle measurements were determined using a goniometer (KRUSS GMBH, Hamburg, Germany). Five measurements were taken on three different newly prepared samples, so for each liquid per sample n = 15.

2.3.6. Statistical tests

All statistical results were obtained by using Excel (Microsoft Office, version 2007). The p-value was calculated by using t-test and determined the probability of rejecting the null hypothesis (H_0). The results are reported as mean ± standard deviation represented by the bars on the graphs. Differences observed were considered significant at $P < 0.05$.

2.4. Results and discussion

2.4.1. SEM and EDX

EDX analysis of the surfaces revealed the chemical composition of the coatings (% atomic weight) and the concentration of the silver in the coatings (*Table 2.2.*). Unexpectedly, TiN/Ag coating prepared at 50W did not show any traces of silver, showing that 50W was too low to allow deposition of silver on the surface or that the silver present was below the limit of detection. TiN/Ag 100W showed a 2.44% and TiN/Ag 120W a 8.60% of silver present. Nevertheless, the topography differed from TiN coated stainless steel 304 fine polish, so the surface was included in subsequent studies. To investigate the differences in topography, the white light profilometer was used to obtain the surface roughness of the substrata.

The presence of silver particles in the coatings was confirmed by SEM images taken of the surfaces (*Figure 2.3.*) where silver was present as small white spheres (on average 0.35µm in diameter in *Figure 2.3.* d) and e). The presence of oxygen in fine polish stainless steel is due to the oxidised layer on the surface which protects it from corrosion.

Table 2.2. Percentage of chemical composition and silver of substrata: Fine Polish Stainless Steel (FPSS), Titanium nitride (TiN) and Titanium nitride/silver coatings (TiN/Ag 50W, 100W and 120W) with ± representing the standard deviation

		Percentage of chemical elements								
		C	O	Si	Cr	Fe	Ni	Ti	N	Ag
Surfaces	FPSS (Fine Polish Stainless Steel)	5.91 ± 0.54	2.86 ± 0.15	0.61 ± 0.06	16.60 ± 0.51	63.51 ± 0.75	10.51 ± 0.03			
	TiN							37.06 ± 0.74	62.94 ± 0.74	
	TiN/Ag 50W							37.45 ± 1.87	62.55 ± 3.13	
	TiN/Ag 100W							36.1 ± 1.81	61.46 ± 3.07	2.44 ± 0.12
	TiN/Ag 120W							33.00 ± 0.4	58.40 ± 0.7	8.60 ± 0.1

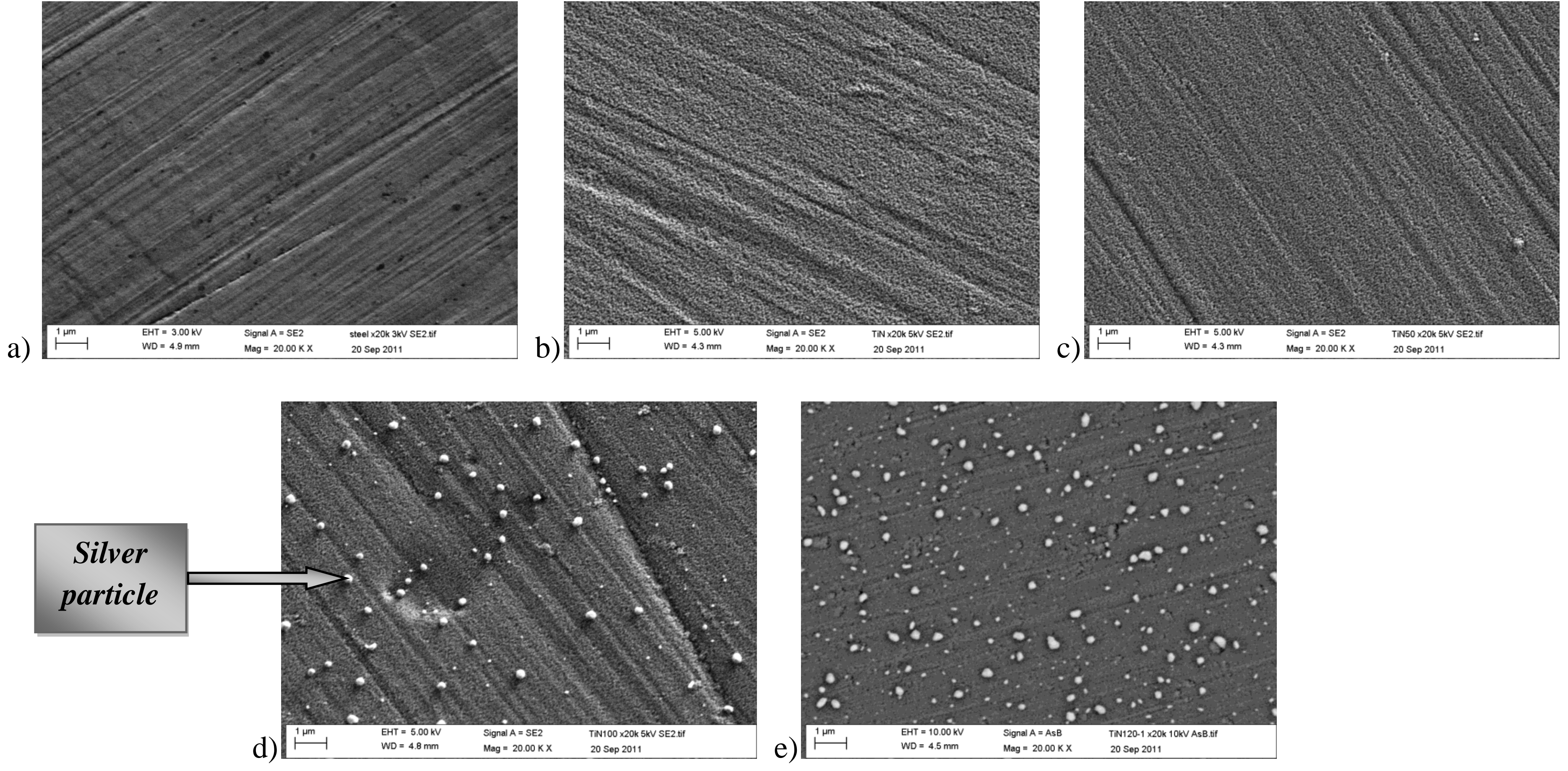


Figure 2.3. Scanning electron microscopy (SEM) images of a) fine polished stainless steel and fine polished stainless steel coated with b) TiN c) TiN/Ag 50W d) TiN/Ag 100W e) TiN/Ag 120W with silver particles (arrowed)

2.4.2. WLP

WLP images were obtained (*Figure 2.4.*) to enable comparison of topographies for all surfaces and to show any change in the underlying surface topographies of the stainless steel. The TiN surface appeared rougher than any other surfaces and the increase of silver concentration decreased roughness and smoothed out features.

Surface profiles of the substrata were measured from the recordings (*Figure 2.5.*) and the range of feature sizes were calculated to enable comparison between topographies of coatings and underlying stainless steel substrate. Profiles showed that there was no significant difference ($P > 0.05$) in height of peaks between substrata except between FPSS and TiN/Ag 50W. There were significant differences of valley values between TiN and all other surfaces and also between TiN/Ag 100W and TiN/Ag 120W ($P < 0.05$) which suggested that TiN had deeper valleys than other surfaces. Overall, the surfaces showed similar peak to valley results.

WLP data (*Figure 2.6.*) showed significant ($P < 0.05$) difference between S_a of FPSS and all of TiN surfaces, but not between TiN and TiN/Ag surfaces. Significant differences were also noted between S_q of FPSS and all of TiN surfaces except for TiN/Ag 120W and between TiN/Ag 100W and both TiN/Ag 50W and TiN/Ag 120W which suggested similar results for S_q and S_a. Generally, coatings reduced roughness S_a (*Figure 2.6.*).

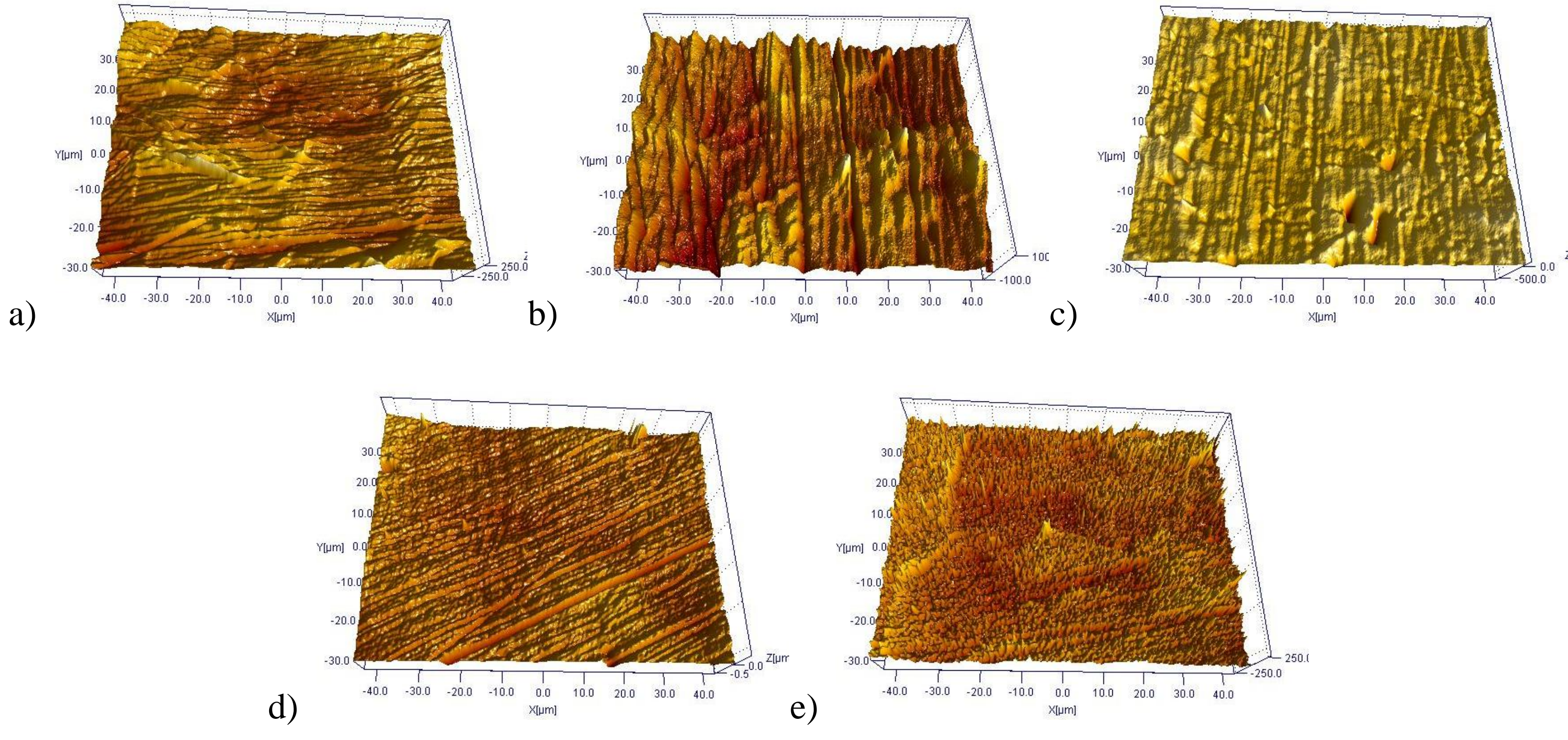


Figure 2.4. WLP images a) FPSS z axis (-250µm; 250µm) b) TiN z (-100µm; 100µm) c) TiN/Ag 50W z (-500µm; 0µm) d) TiN/Ag 100W z (-0.5µm; 0µm) and e) TiN/Ag 120W z (-250µm; 250µm); x axis (-40µm; 40µm); y axis (-30µm; 30µm)

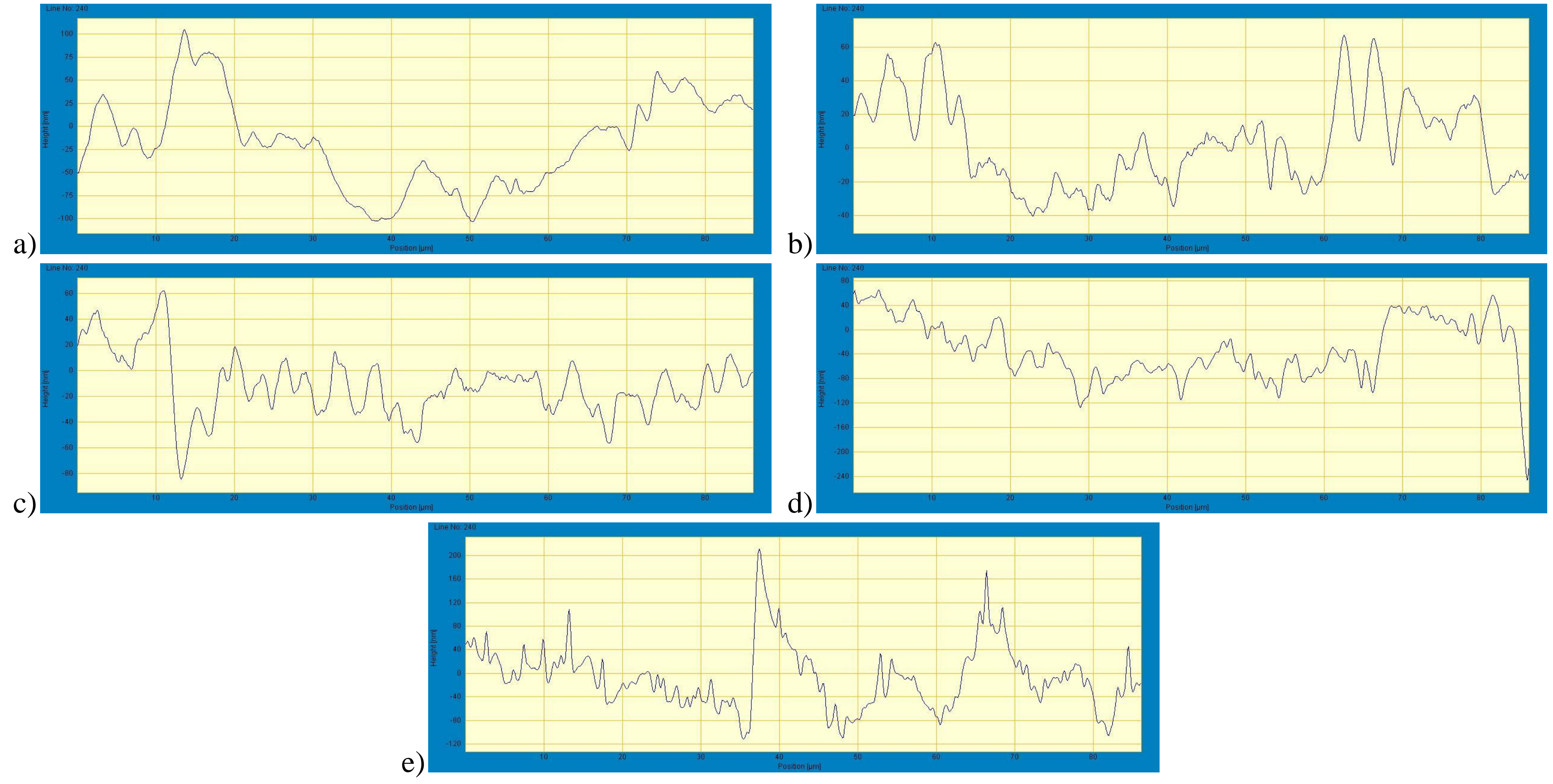


Figure 2.5. Surface profiles a) FPSS y axis (-100nm; 100nm) b) TiN y (-40nm; 60nm) c) TiN/Ag 50W y (-80nm; 60nm) d) TiN/Ag 100W y (-240nm; 80nm) and e) TiN/Ag 120W y (-120nm; 200nm) obtained by WLP; x axis (0nm; 80nm)

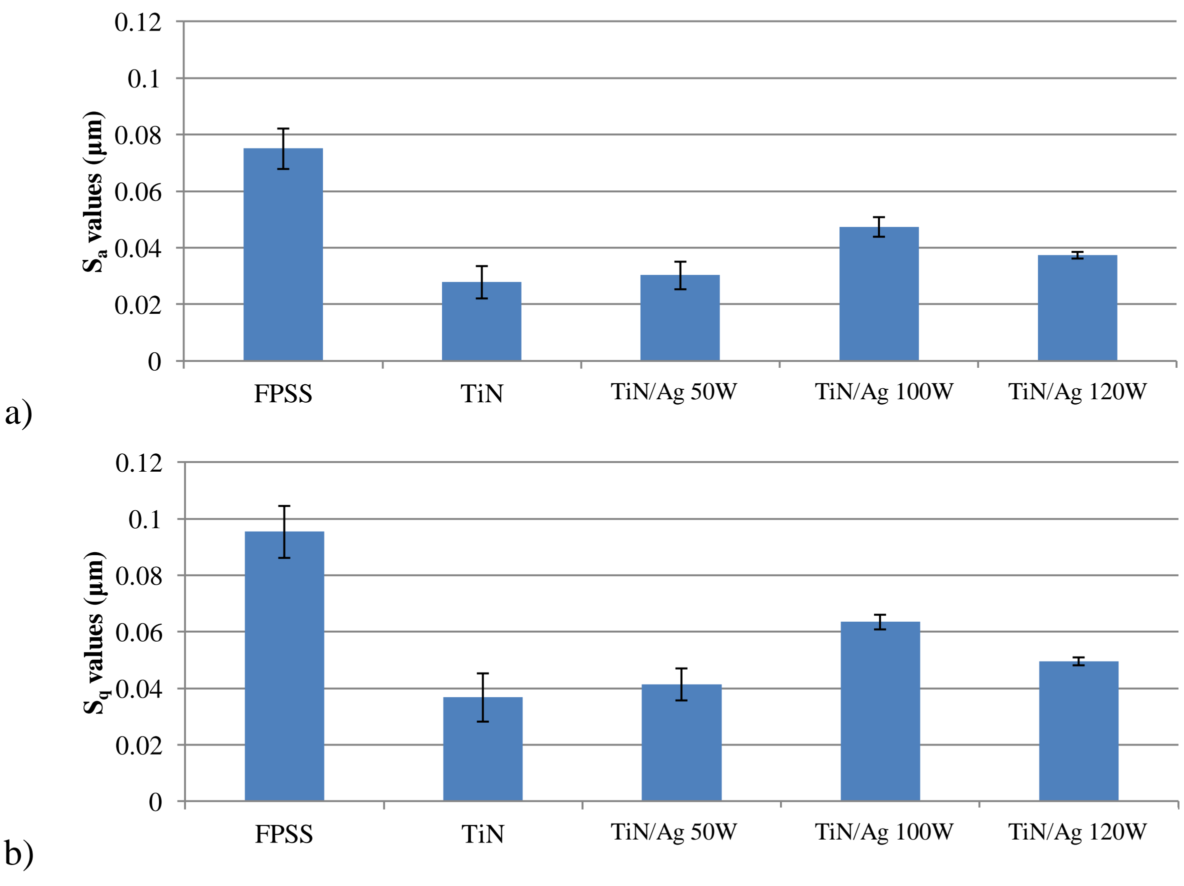


Figure 2.6. WLP data of a) S_a and b) S_q

2.4.3. AFM

Coatings may be qualified as nanocomposite since TiN and/or Ag make up a two or three dimensional phase of less than 100µm. AFM images of the nanocomposite coatings on silicon (*Figure 2.7.*) showed different surface topographies. Putative silver particles (in average 188nm width and 32nm height) are evident in images c) and d) *Figure 2.7.* as large circular shapes in lighter colour.

The profile of topographies were recorded (*Figure 2.8.*). R_a values and R_q values of nanocomposite coatings were calculated (*Figure 2.9.*) and demonstrate that at the nanoscale there were significant differences between the topographies of all the surfaces. Significant differences for R_a values were found between all surfaces except TiN/Ag 50W and TiN/Ag 120W. Similar R_a and R_q values were found for all surfaces.

Overall, S_a values of WLP values (µm scale) showed that TiN coated surfaces were smoother than FPSS and all TiN surfaces. The R_a values of AFM (nm scale) showed that TiN/Ag 50W and 120W were smoother than TiN and TiN/Ag 100W more rough than TiN.

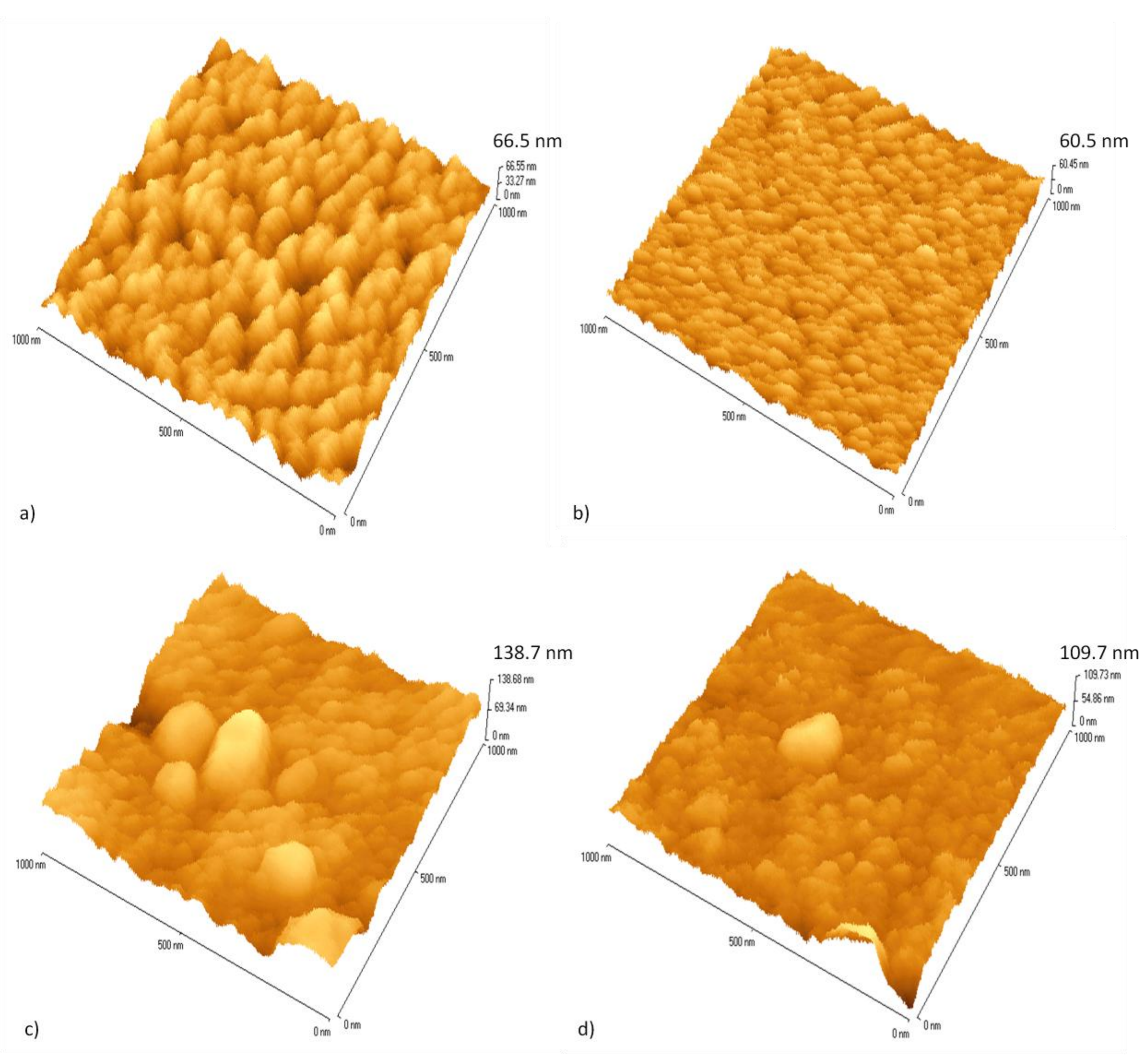


Figure 2.7. AFM images of nanocomposite coatings on silicon a) TiN b) TiN/Ag 50W c) TiN/Ag 100W and d) TiN/Ag 120W

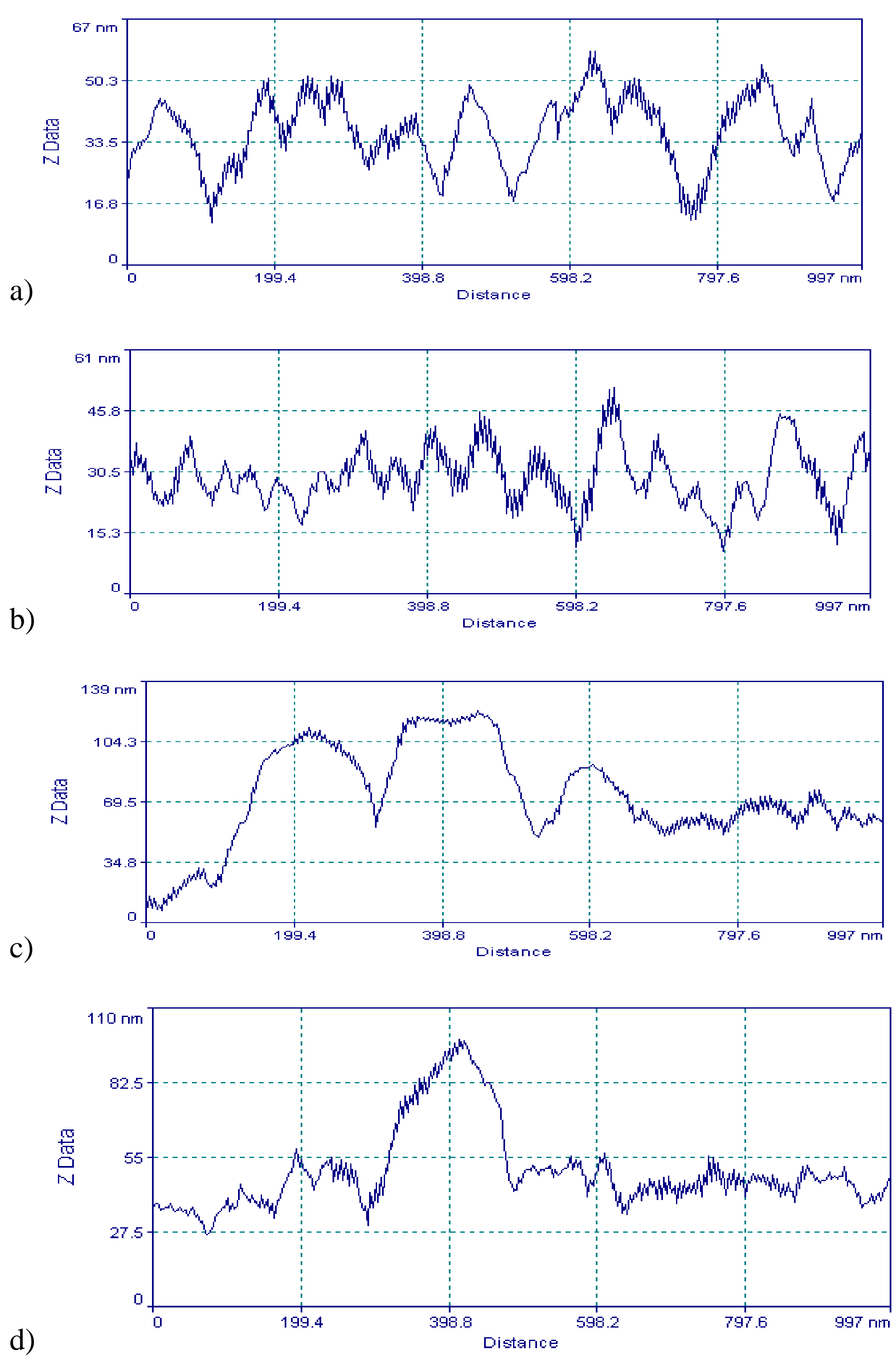
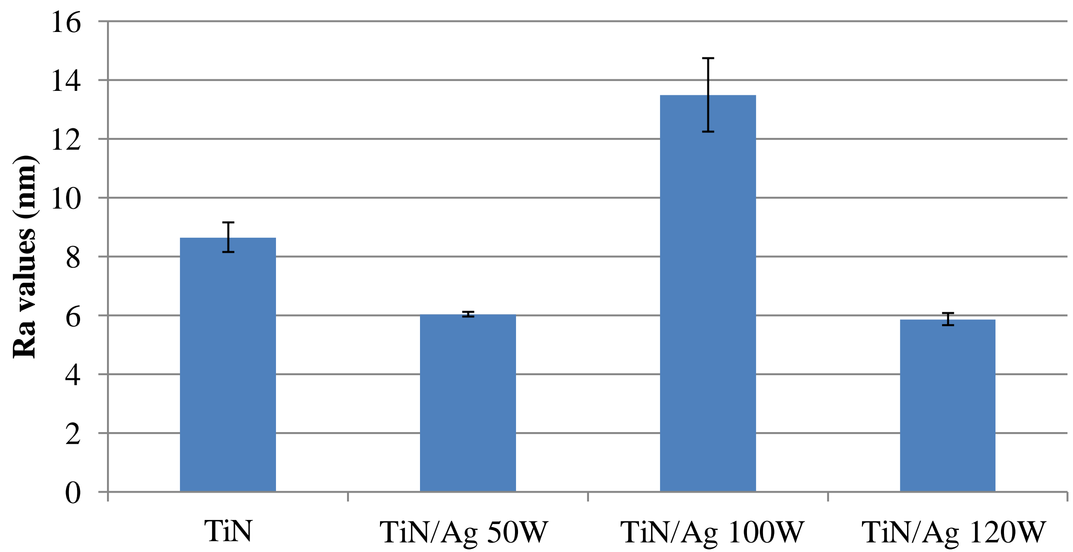


Figure 2.8. Size of topographies of a) TiN, b) TiN/Ag 50W, c) TiN/Ag 100W and d) TiN/Ag 120W on silicon

a)



b)

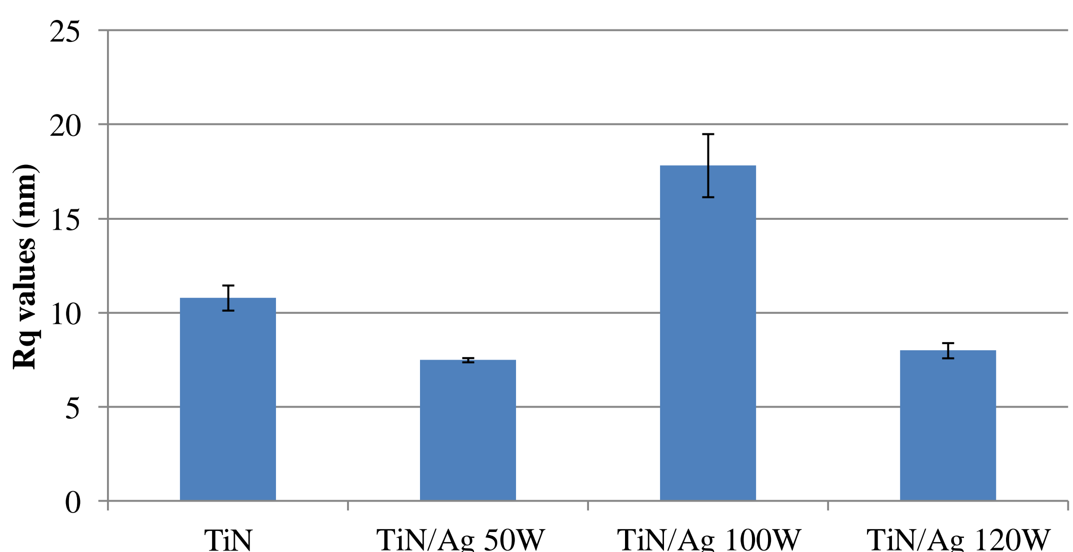


Figure 2.9. R_a values and R_q values of nanocomposite coatings

2.4.4. Wettability of nanocomposite films

Contact angle was used to determine the wettability of the surfaces by using the contact angle method (*Figure 2.10.*). The wettability is defined as: the higher the angle the poorer or dryer wetting, the lower the angle the better or complete wetting. Bonn *et al* (2009) defined the wetting of a surface as complete at 0º, good at 60º, moderate at 90º, and poor at 120º and none at 180º.

Stainless steel and TiN/Ag 100W demonstrated a poor wetting (>90º and >80º respectively), TiN/Ag 50W was classified as having a poor wetting with a contact angle of 51° whereas TiN, and TiN/Ag 120W showed a good wetting (< 30º). Thus, stainless steel and TiN/Ag 100W were hydrophobic. TiN/Ag 50W was at the limit of hydrophobic surface. TiN and TiN/Ag 120W were hydrophilic.

In previous results (AFM and WLP), stainless steel and TiN/Ag 100W was found to have bigger surface features than other surfaces which could explain the hydrophobicity whereas TiN, TiN/Ag 50W and TiN/Ag 120W had smaller typeface which could explain their hydrophilicity.

The hydrophobicity of the substrata may affect the survival of microorganisms. A droplet of water will spread on a hydrophilic surface and dry quicker than on a hydrophobic surface where the droplet will have less surface contact with the air, thus protecting for a longer time potential microorganisms trapped inside the droplet (Williams *et al*, 2008; Vogel *et al*, 2010). Surface hydrophobicity may also affect cell retention also depending on the bacterial cell hydrophobicity (Peng *et al*, 2001; Ukuku and Fett, 2002; Nguyen *et al*, 2011).

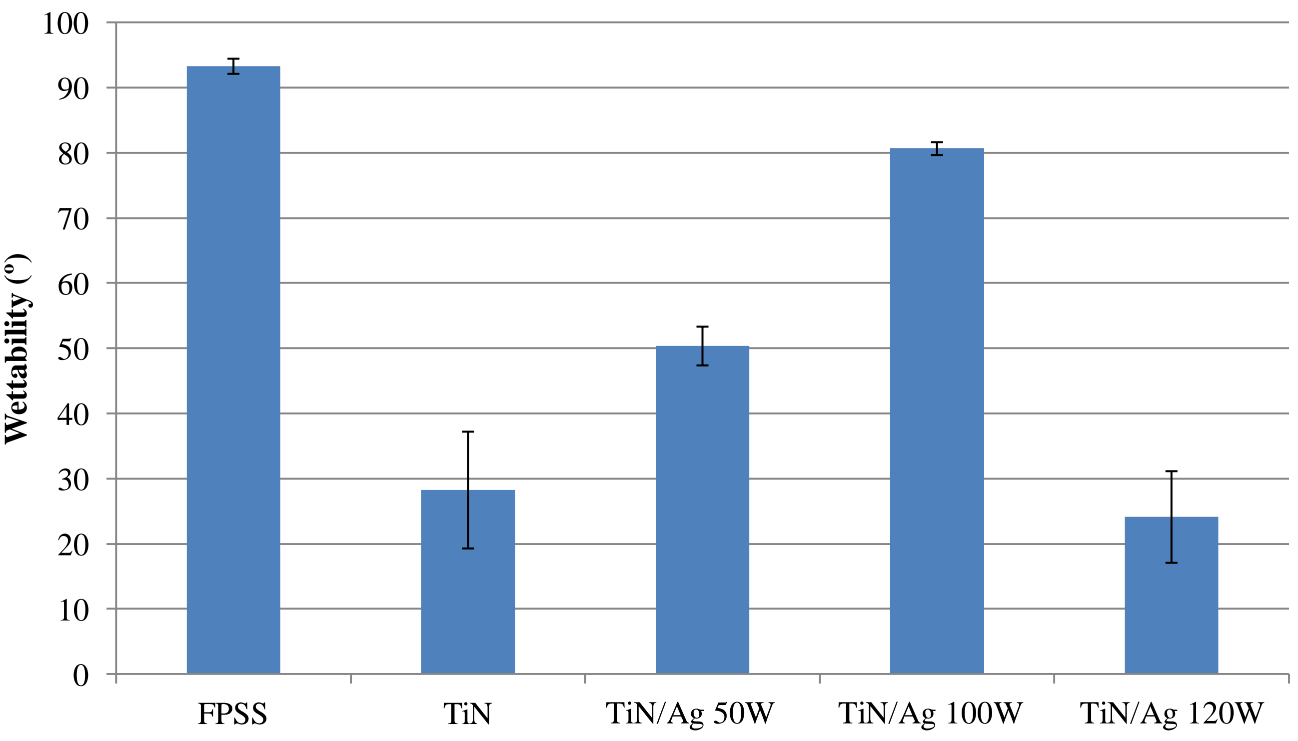


Figure 2.10. Wettability of the substrata by the use of contact angle method with poor wettability for FPSS and TiN/Ag 100W, good wettability for TiN and TiN/Ag 120W and TiN/Ag 50W in the limit of poor wettability

2.5. Conclusion

The test substrata presented different surface features where coatings decreased roughness and smoothed out features as there was significant difference between FPSS and all coatings. As previously described, FPSS is classified as highly hygienic due to its smooth surface features. Therefore, all coatings being smoother than FPSS are potentially highly hygienic too. However, further characterisation such as wear of the coatings is necessary to say so. FPSS, TiN/50W and TiN/Ag 100W presented hydrophobic characteristics whereas TiN and TiN/Ag 120W were hydrophilic. The hydrophobicity of a surface may affect the potential for cell retention which is also an important factor to consider for future use in food industry. Overall, the surfaces have similar topographies with varying hydrophobicity and concentrations of silver.

The substrata need to be tested *in situ* to investigate their effect on the retention and survival of potential pathogens which may increase the risk of contamination via transfer from substrata to food products.

Chapter 3

Antimicrobial properties of coated surfaces

3.1. Introduction

Having generated a series of putative antimicrobial surfaces, work progressed to evaluate their antimicrobial effectiveness in a range of conditions that mimic those encountered in the food industry.

3.1.1. Equilibrium relative humidity in the food industry

The equilibrium relative humidity can be measured by several means including wet and dry bulb, polymer sensors where humidity alters the current between two electrodes and hygrometers and hair hygrometers which use human hair contracting in the presence of humidity (Dyer, 2012). There is a correlation between rise in temperature and the capacity of the air to hold moisture: as the temperature increases, so does the capacity of the air to hold moisture (De Goffau *et al*, 2009). However, when the air comes in contact with a cooler surface, the condensation process takes effect. The deposition of water droplets may increase the survival and growth of microorganisms on surfaces and may also be responsible for other moisture related problems such as corrosion. Corrosion deteriorates every part of a food plant from surfaces in contact with food, machineries and storage facilities to food packaging such as cans (www.intechopen.com). Conversely, the water might help solubilise any antimicrobial component released from an “active” surface and enhance antimicrobial effect although, leaching of ions from an “active” surface may have adverse effects such as spoiling the food (e.g. copper) and thus be undesirable (Reilly, 2008).

Dry food products are more prone to moisture absorption in the presence of high equilibrium relative humidity, which can result in spoilage. Conversely, some food

products such as fresh vegetables require a high equilibrium relative humidity (90-95% ERH) (Anonymous, 1986; Hardenburg *et al*, 1986; Snowdon, 1990, 1992) because low equilibrium relative humidity would increase the water loss from the product occurring naturally post-harvest. In the meat industry, the air temperatures and equilibrium relative humidities range from 4 to 40ºC and 60 to 99% ERH (Robles Olvera *et al*, 1998). Overall, low temperature reduces spoilage and high humidity prevents drying of the product.

3.1.2. Effect of humidity on microorganisms

The presence of high humidity levels has often been shown to increase the survival of microorganisms and also create the right conditions for growth (Wilks *et al*, 2006, Yang *et al*, 2009). In the present study, the optimum growth temperatures of *E. coli* (37ºC) and *L. monocytogenes* (30ºC), and room temperature (20ºC) were used in combination with a low (11% ERH), medium (52% ERH) and high (86% ERH) humidity to monitor the effects of these conditions on the survival of microorganisms on test surfaces.

Gram-negative *E. coli* has thin peptidoglycan layers in its cell walls (Iida and Koike, 1974) in addition to an outer membrane layer less resistant to environment factors than Gram-positive *L. monocytogenes*, which has a thicker peptidoglycan layer in its cell wall (Edwards and Stevens, 1963). It has been suggested that low humidity may help the survival of some microorganisms at low concentration by triggering the cells' sensors to switch to a "dormant" state or, in the case of *L. monocytogenes*, to create an osmotic pressure which will keep the outer cell moist to survive for longer

periods in low humidity (Vogel *et al*, 2010). *E. coli* and *L. monocytogenes* survive better in high humidity (97.2% ERH: *E. coli* optimum) (Yang *et al*, 2009). There is little data in the literature about optimum equilibrium relative humidity for the survival of *L. monocytogenes*, but, low temperature and high equilibrium relative humidity have been noted to contribute to its survival (Palumbo and Williams, 1990).

These different resistances to humidity and the ambient conditions used for storage in a given environment should be taken into account when considering microbial survival. In addition, when including antimicrobial metal ions in the surface, moisture may be required to exert an antimicrobial effect (Chen *et al*, 2010; Kelly *et al*, 2010).

3.1.3. Antimicrobial properties of coatings in the presence of moisture

Recent methods implemented to reduce the survival of microorganisms and prevent the contamination of food products coming in contact with working surfaces include the use of antimicrobial metals in the surface coatings (Klibanov, 2007; Chen *et al*, 2010; Kelly *et al*, 2010). The most commonly used coatings include silver (Silver, 2003; Samuel and Guggenbichler, 2004), copper (Domek *et al*, 1984) and photocatalytic agents such as titanium dioxide (Huang *et al*, 2000; Asahi *et al*, 2001). Some metals such as silver proved to be toxic to microorganisms while being safe for humans, when used in low concentration. (Kelly *et al*, 2010).

Increased humidity enhanced the antimicrobial properties of silver in coated surfaces (Juan *et al*, 2010; Møretrø *et al*, 2012). The surface of a cell itself will also

contain some moisture which may enable the release of ions from the coated surfaces (Verran *et al*, 2008) and access into the cell.

3.1.4. Antimicrobial properties of silver

Silver was used very early in human history for its antimicrobial properties and researchers are now showing an increasing interest in the properties of silver and possible applications in the industrial systems as well as in medicine (Rupp *et al*, 2004; Lawrence and Turner, 2005; Lv *et al*, 2008; Kelly *et al*, 2009; Lv *et al*, 2009).

As described in "*Chapter 1: Introduction*", there is a significant literature on the antimicrobial properties of silver (Dowling *et al*, 2003; Wang *et al*, 2006; Dong *et al*, 2008; Martínez-Castañón *et al*, 2008; Rai *et al*, 2009; Sharma *et al*, 2009; Shrestha *et al*, 2009). However, there is still a debate as to exactly how the cell is affected by the ions released from the antimicrobial metals. In the particular case of silver ions, a study showed an increase in antimicrobial activity with increasing quantity of silver particles in a coating (Liao *et al*, 2010). It also indicated that when water came in contact with silver particle, dissolution occurred, thus releasing silver ions that inactivated attached cells.

Silver binds to the bacterial cell wall and cell membrane and inhibits the respiration process (Klasen, 2000). In the case of *E. coli*, silver acts by inhibiting the uptake of phosphate and releasing phosphate, mannitol, succinate, proline and glutamine from *E. coli* cells (Rosenkranz and Carr, 1972; Haefeli *et al*, 1984; Yamanaka *et al*, 2005). The effect of silver ions on bacteria may be observed by the structural and morphological changes. It is suggested that when DNA molecules are in relaxed state the replication of DNA can be effectively conducted, but when the DNA is in

condensed form it loses its replication ability. When silver ions penetrate the bacterial cell and react with the DNA molecule which condenses as a result and is unable to replicate, leading to cell death. Also, it has been reported that heavy metals react with proteins by attachment to thiol group hence inactivating proteins (Liau *et al*, 1997; Feng *et al*, 2000).

3.1.5. Detection of viable microorganisms in food soil directly on surfaces

The presence of viable, if not necessarily multiplying, cells on open surfaces possess a biotransfer potential. Transfer of microorganisms from an inert, non-nutritive surface to a different environment (e.g., food) may result in their multiplication and colonization. This may lead to an accompanying potential for contamination, spoilage, and/or disruption to quality assurance/quality control procedures. Thus, any viable cells that remain, that is, are retained, are of concern. Similarly, the presence of organic material can affect cell viability, and surface cleanability (Fuster-Valls *et al*, 2008), thus the differential response of these two components (microbial cell and organic soil) to cleaning and disinfection needs to be considered. On many food contact surfaces, the microorganisms will be present in combination with organic material such as fat, starch, protein, other food debris and foulants such as residues of cleaning or sanitizing agents. Retained organic material may assist in the survival of the microorganisms by providing an element of protection from cleaning procedures when other conditions are favourable for growth (Verran *et al,* 2002). Many methods have been used to evaluate the cleanability of stainless steel, including crude visual analysis with an incorporated stain, gravimetric studies, ellipsometry (McGuire *et al,* 1990), contact angle measurements

(Decker *et al,* 1999) and fluorescence microscopy (Whitehead *et al,* 2009). However, most of these techniques cannot differentiate between the components of an organic soil and cells.

A method has been developed whereby microbial cells can be differentiated and enumerated in the presence of an organic soil (Whitehead *et al*, 2010). However, the viability of the cells has to date not been determined in the presence of organic soil. The quantification of cells and soil following cleaning assays remains problematic. The viability of the remaining cells is also a major factor in the potential transfer, contamination and spoilage of food. The LIVE/DEAD™ stain enables the determination of cell viability on surfaces. The kits provide two-colour fluorescence staining on both live (green) and dead (red) cells. The green-fluorescent nucleic acid dye (SYTO 9) stains both live and dead bacteria with intact and damaged cell membranes while the red-fluorescent nucleic acid dye (propidium iodine) stains only dead bacteria with damaged cell membranes. However, difficulties may arise when using this staining method in the presence of a food soil. Although the food soil alone may not cause a pathogenic hazard, its ability to provide both protection from cleaning agents and nutrients for microorganisms makes the evaluation of both cells (with their viability) and soil following cleaning regimes on a surface of extreme importance. As far as the authors are aware, this is the first time that differential staining methods have been developed in order to detect the viability of the cells in different food soils as well as the soils themselves on surfaces.

3.2. Aim

The aim of the work described in this chapter is to investigate the survival of *E. coli* and *L. monocytogenes* on stainless steel, TiN and TiN/Ag coated surfaces.

3.3. Methods

3.3.1. Characterisation and maintenance of microorganisms and meat soil

3.3.1.1. Culture maintenance

Microorganisms were kept in freezer stocks in 1mL cryovials containing 10% glycerol at −80 °C. The culture was then prepared by leaving the tube containing microorganisms at room temperature to defrost for 5 minutes and a loopful was streaked onto solid Tryptone Soya agar (TSA) (Oxoid, Basingstoke, UK) for *Listeria monocytogenes* or Brain Heart Infusion agar (BHI) (Oxoid, Basingstoke, UK) for *Escherichia coli* and incubated overnight at the appropriate temperature (30ºC and 37ºC). The tube was placed back in the freezer for future use and stock plate cultures were kept for 4 weeks at 4°C.

In preparation for experiments, the identity of each bacterium was checked on a regular basis by bacterial colony morphology, growth on appropriate agar plates at specified temperatures (as described below) and Gram-stain.

3.3.1.2. Preparation of suspensions

3.3.1.2.1. *Escherichia coli*

E. coli CCL 410 (a non-pathogenic O157:H7 strain) was kindly provided by Brigitte Carpentier (Agence française de sécurité sanitaire des aliments (AFSSA) France). 100mL of sterile BHI broth (Oxoid, Basingstoke, UK), in a 250mL Erlenmeyer flask, was inoculated with a single colony of *E. coli* from stock plate and incubated at 35°C in an orbital incubator at 150 rpm for 18 hours (stationary phase) (Akerlund *et al*, 1995; Rodrigues *et al*, 2009). Following incubation, the culture was dispensed into 25mL plastic bottles and spun for 10 minutes at 3,226×g in IEC Centra-3C Centrifuge (22°C). The supernatant was discarded, the cells washed once by re-suspension in sterile distilled water, vortexed for 30 seconds, and then centrifuged again for 10 minutes. Cells were re-suspended to an O.D. (Optical Density) of 1.0 at 540 nm and serial dilutions of the inoculum (from 10^{-1} to 10^{-8}) were plated out in duplicate and incubated overnight at 35°C and the cfu mL^{-1} calculated (colony forming unit). The resultant standardised, cell suspension contained on average 1.7-8.0 x 10^{7} cfu mL^{-1}.

3.3.1.2.2. *Listeria monocytogenes*

L. monocytogenes EGDe, ATCC (American Type Culture Collection) was kindly provided by Nils Arneborg, Danish Technical, University of Copenhagen. Standardised cells suspensions were prepared following the same method as for *E. coli* but with incubation at 30°C in Tryptone Soya broth (TSB). The resultant standardised, cell suspension contained 1.1-8.0 x 10^{8} cfu mL^{-1}.

3.3.1.2.3. Characterisation of cultures by microbial adhesion to solvents (MATS)

The MATS assay is based on comparison of the affinity of microbial cells for monopolar and nonpolar solvents with the same Van Der Waals surface tension components, and was used to characterise test cultures. The following pairs of solvents were used: chloroform (Merck Millipore, Darmstadt, Germany), a polar electron acceptor solvent; hexadecane (Sigma-Aldricht, Dorset, UK), a non-polar solvent; ethyl acetate (Merck Millipore, Darmstadt, Germany), a polar electron donor solvent, and decane (Fluka, Gillingham, UK), a non-polar solvent. *L. monocytogenes* and *E. coli* were grown as previously described. Stationary phase cells were harvested (3,763×g, 5 min) and the supernatant discarded. The cells were washed twice in 0.85% NaCl (7,527×g, 5 min), and re-suspended in 0.85% NaCl to $OD_{400} \approx 0.8$ (A_0 value) (UV-1800 Shimadzu spectrophotometer, UK). A saline solution was used to decrease the concentration of water around the cells to enhance the migration of the cells towards the solvents. Two millilitres of cell suspension were added to a test tube with 0.4 mL of one of the solvents. The cell suspensions were vortexed with the solvent for 1 min. The emulsions were left to stand for 15 min to allow phase separation, and the OD_{400} (A value) of the aqueous phase was measured. Affinity of the cells for each solvent was calculated by use of equation 1:

Equation 1. $\% \text{ affinity} = 100 \times (1 - A/A_0)$

Where A and A_0 are the OD_{400} values of the cell suspension in the aqueous phase after and before mixing, respectively. Measurements were carried out in triplicate. Then, the results of the solvents were compared to determine the ability donate/accept electron and hydrophobicity/hydrophilicity of the cells.

3.3.1.3. Preparation of food soil: meat extract

The meat extract was prepared according to Whitehead *et al* (2008). One kilogram of fresh rolled beef brisket (CO-OPERATIVE, UK) was cut into 10 mm x 10 mm pieces. The meat pieces were put into a stainless steel tray and covered with aluminium foil. The meat was covered by another tray and weighed down with 8.4 kg of stainless steel sheets and frozen at -20°C for 24 h. The meat was defrosted at room temperature. The meat exudate was poured off and the meat squeezed between two sterile plastic plates to recover additional exudate. The meat exudate was stored in 20 mL aliquots at -20°C until needed. The extract was sterilised by filtration with Acrodisc Syringe Filter, PALL® Acrodisc® 32mm, Syringe Filter 0.2µm Supor® Membrane. Using a PolyEtherSulphone membrane, the filter was screwed to the end of a 5mL syringe and replaced every time the filter was full. The process was repeated until all the meat extract was filtered sterile and maintained at -10ºC.

In order to weigh the food soil preparation to enable standardisation, three sterile paper filters were left in the oven at 60ºC to dry for two days before use. They were placed in a desiccator for 5 to 10 minutes to cool and avoid any humidity absorption. The three filters were weighed then returned to the oven for an hour and weighed again several times to constant weight. Then 500µL of filtered meat extract was applied onto each filter then returned to the oven. The weight of each sample was checked every day until stabilised (seven days). The average dry weight obtained for 500µL of filtered meat was 2.8×10^{-3}g.

3.3.2. Release of silver ions and evaluation of antimicrobial properties

3.3.2.1. Substrata and re-use

Stainless steel 304 fine polished coupons of 1cm x 1cm were used as a control to compare to coated surfaces. As previously (*Chapter 2*), the coated surfaces on stainless steel were: titanium nitride (TiN) and titanium nitride silver with different concentrations of silver: TiN/Ag 0% atomic weight of silver (TiN/Ag 50W), TiN/Ag 2.44% atomic weight of silver (TiN/Ag 100W) and TiN/Ag 8.60% atomic weight of silver (TiN/Ag 120W). As the energy (in Watt) increased, so did the concentration of silver deposited on the surface. In the experiments presented hereafter, the silver contained substrata were described as TiN/Ag followed by the energy used (50W, 100W and 120W) to deposit silver.

The surfaces were used when new then re-used a 1st time and a 2nd time to investigate the effect of re-use of coupons on the antimicrobial activity. After each use, the coupons were placed in 70% ethanol to soak for 10 minutes, rinsed with sterile distilled water for 3 seconds and left to air dry before use. Duran bottles were labelled to store clean and used coupons for each substratum. The bottles were stored at room temperature. All experiments were repeated. Coupons were re-used in the order of experiments, so re-used coupons did not contain any soil when soil was not used.

3.3.2.2. ICP-AES: Inductively Coupled Plasma Atomic Emission Spectroscopy

ICP-AES is an analytical technique that uses emission spectrophotometry in order to detect traces of metals (Asami and Hashimoto, 2003). It comprises exciting electrons from metals which emit energy at specific wavelengths. This technique was used to quantify the amount of metal ions released in water from the surfaces used in this project. Metal elements that would potentially leach out of the substrata were identified: Ag (silver), Al (aluminium), Cr (chromium), Fe (iron), Ni (nickel), Si (silicon) and Ti (titanium). Surfaces tested were as follows: A: stainless steel; B: TiN; C: TiN/Ag 50W; D: TiN/Ag 100W; E: TiN/Ag 120W. 600mL of sterile distilled water and 3 coupons of one substratum were placed into 1L flask. For each substratum, each flask was placed in an orbital flask shaker at 120 rpm at room temperature (≈20ºC). Three samples of 20mL were taken from a sterile distilled water control and placed in universal plastic bottles and stored at room temperature. Three 20mL samples were also removed from each test flask every hour for 10 hours. All samples collected were sent to the Analytical Sciences Department of Manchester Metropolitan University for analysis. The analysis was done using a Varian Vista AX CCD (Palo Alto, USA) inductively coupled plasma atomic emission spectrometer. Standard wavelengths are used for each metal element. The intensity of the energy emitted at the chosen wavelength is proportional to the amount (concentration) of that element in the sample.

3.3.2.3. MIC: Minimum Inhibitory Concentration

The purpose of this experiment is to test the antimicrobial activity of known concentrations of silver to enable comparison with the effect of different quantities present in the coating on the stainless steel surfaces , where different levels of ions may be released. A silver standard for Atomic adsorption spectrophotoscopy (AAS) (*Trace*CERT) (Fluka, Gillingham, UK) of 1000 mg/L Ag in 2% nitric acid, prepared with high purity silver metal, HNO_3 *Trace*SELECT and water *Trace*SELECT Ultra was used. Dilutions of silver standard in sterile broth were made in glass universal bottles (BHI for *E. coli* and TSB for *L. monocytogenes*) according to *Table 3.1.*. Since 1mg/L is equal to 1 part per million (ppm), the concentration given in the table is the same for both.

Table 3.1. Method used to obtain concentrations of silver standard mixed with sterile broth.

Ratios	Silver standard	Broth	Concentration of silver in mg/L or ppm
1:1	5mL	5mL	500
1:2	3mL	6mL	333
1:3	3mL	9mL	250
1:4	3mL	12mL	200
1:5	1mL	5mL	167
1:6	2mL	12mL	143
1:7	2mL	14mL	125
1:8	1mL	8mL	111
1:16	0.5mL	8mL	59
1:32	0.5mL	16mL	30
1:99	5mL from row above mix	10mL	10
1:300	5mL from row above mix	10mL	3

Following the preparation of the different concentrations, 1mL of each mixture was added to 1mL of washed standardised cell suspension in glass bijou bottles and incubated overnight at 37°C for *E. coli* and 30 °C for *L. monocytogenes*. 100µL of each mixture was dispensed into a microtiter plate to compare turbidity visually. Four replicates were produced for each concentration, thus producing four rows on the microtiter plate. Controls were made to monitor different parts of the experiment. A

mixture of broth with cells was used as a positive control to ensure the growth of the cells in the same conditions without silver. The silver solution was in 2% nitric acid, thus 2% nitric acid was used as second positive control to ensure that the inhibition or death of the cells was not caused by the presence of nitric acid. Here, when cells suspensions were standardised, they were re-suspended in 2% nitric acid instead of sterile distilled water. Finally, silver standard for AAS was used to re-suspend cell and assumed to be a negative control (no growth). All controls were checked for microbial growth by inoculating 100µL of mixture onto appropriate agar plates and incubating overnight at appropriate temperatures, followed by a visual confirmation of the presence or absence of growth on plates.

3.3.2.4. MBC: Minimum Bactericidal Concentration

The MIC tests for turbidity, thus indicating growth of microorganisms. However, it is possible that viable cells remain which are unable to grow. From the MIC test, 100µL of all incubated mixtures (showing growth or no growth) were spread onto agar plates (BHI for *E. coli* and TSA for *L. monocytogenes*) in duplicate to test for culturable cells, thus differentiating inhibition from kill depending on the concentration of silver. Following the MIC test, *E. coli* did not show any turbidity up to dilution 1:16. In order to detect if the absence of turbidity was due to a kill of the bacteria or a reduction of cells by inhibition, ratios 1:8 (clear), 1:16 (clear), 1:32 (turbid) and 1:99 (turbid) were plated above and below MIC and incubated overnight to record the presence of colonies. *L. monocytogenes* did not show any turbidity at 1:8, so the same method was used with the concentrations: 1:7; 1:8; 1:16 and 1:32. This experiment was used to determine at which concentration of silver the bacteria would be killed.

3.3.2.5. ZOI: Zone Of Inhibition

3.3.2.5.1. Effect of silver ion solutions on cells

The standard silver concentration mixtures described in *Section 3.3.3.2.* were used. Petri dishes containing 20mL of appropriate agar were inoculated with 100µL of washed standardised cell suspension and evenly spread with a glass spreader. Two wells of 3mm deep and 5mm wide were made in the agar with a cork borer 25mm apart. Each well was filled with 50µL of broth and silver mixtures (thus giving different concentrations of silver) before overnight incubation at 37ºC for *E. coli* and 30ºC for *L. monocytogenes*. The same controls were used as previously described in *Section 3.3.3.2.*. After incubation, data were recorded by measuring the diameter of the zones of inhibition where necessary.

3.3.2.5.2. Direct contact of cells with coatings

Zone of inhibition by direct contact of the coatings with agar was used to determine if any antimicrobial agent would leach out to create a zone of inhibition around the substrata. 20mL of TSA for *L. monocytogenes* and BHI agar for *E. coli* were poured in Petri dishes and 100µL of washed standardised cell suspension was evenly spread over the surface with a glass spreader and left to dry for 5 minutes. The substrata (stainless steel and coatings: TiN, TiN/Ag 50W, TiN/Ag 100W and TiN/Ag 120W) were placed with the coated side facing down in direct contact with the cells on the agar plates. Two duplicates were made and incubated overnight at 37ºC for *E. coli* and 30ºC for *L. monocytogenes*. The experiment was repeated once (n=20). Only the zone of

inhibition around the coupons was recorded not the absence or presence of growth beneath the coupon.

3.3.3. Indicator of metabolic activity (Tetrazolium blue chloride assay: TBC)

Metabolic activity assay with TBC (Sigma-Aldricht, Dorset, UK) is an alternative method for the detection of residual viable cells on the surface, and as an indication of viability (Verran *et al*, 2008). TBC solution was prepared using sterile distilled water to give a concentration of 1 $g \cdot L^{-1}$. The TBC solution was filter sterilised before use using a 10 mL Luer-Lok™ syringe and a 0.2 µm Acrodisc® filter. Preliminary work was required to establish appropriate experimental conditions. All types of surfaces were used in this experiment: stainless steel, TiN, TiN/Ag 50W, TiN/Ag 100W and TiN/Ag 120W. Standard cell suspensions of *E. coli* and *L. monocytogenes* were diluted to 10^{-8} and inoculated onto appropriate solid media to enable calculation of $cfu \cdot mL^{-1}$. 10µL of washed cells at a concentration of approximately 10^7, 10^6 and 10^5 cfu $\cdot mL^{-1}$ were spread on the surfaces and dried in a microbiological class 2 laminar flow hood. Preliminary research indicated that this range of concentrations would give more accurate results in terms of countable cells. After 2 hours, coupons with dried inocula were placed into sterile plastic Petri dishes. 25mL of cooled (50°C) molten BHI agar/TSA was poured gently over the top of the coupon and allowed to set at room temperature. Following drying, the respective agar plates (BHI for *E. coli* and TSA for *L. monocytogenes*) were incubated overnight at 37°C for *E. coli* and 30°C for *L. monocytogenes*. The surface of the agar was then flooded with 2 mL of 1 $g \cdot L^{-1}$ of the TBC solution. The principle is that viable cells

remaining on the surface of the substrata will produce colonies after incubation beneath the agar overlay. The metabolic dye diffuses through the agar, stains the colonies and makes them easier to visualise and enumerate. When possible, colony forming units were counted within 5 hours of application of TBC. In all cases, three replicate surfaces were prepared and the experiment was repeated once.

3.3.4. Survival of *Escherichia coli* and *Listeria monocytogenes* on surfaces

3.3.4.1. Swabbing assay

In order to expose cells to conditions of defined humidity (Moretro *et al*, 2010) saturated salt solutions were prepared to obtain the humidity levels required. Saturated solutions in sterile distilled water of lithium chloride (11% ERH), magnesium nitrate (52% ERH) and potassium sulphate (86% ERH) were placed in separate square Petri dishes (24cm × 24cm). A psychrometer (Extech Instruments HD500; Extech Instrument, Nashua, USA) measuring temperature and humidity was placed inside each container to monitor the conditions. The highest point of humidity was attained after 20 minutes for all three salts.

The standard cell suspension of *E. coli* and *L. monocytogenes* was diluted to 10^{-8} and enumerated on BHI and TSA as described previously. 10µL of standard cell suspension of *L. monocytogenes* and *E. coli* were spread onto the test substrata (1cm x 1cm), using the pipette tip to assist spreading. The coupons were placed onto the base of inverted Petri dishes (without lid) which were in turn fixed to the bottom of a large square Petri dish (24cm × 24cm) with Blue Tack (Bostik, Leicester, UK) (see *Figure*

3.1.). For each incubation time, humidity level and bacterium, three replicates were used. Controls with no applied microorganisms were tested under the same conditions to monitor contamination. During the experiment, coupons were kept in a microbiological class 2 laminar flow hoods for safety. All experiments were repeated.

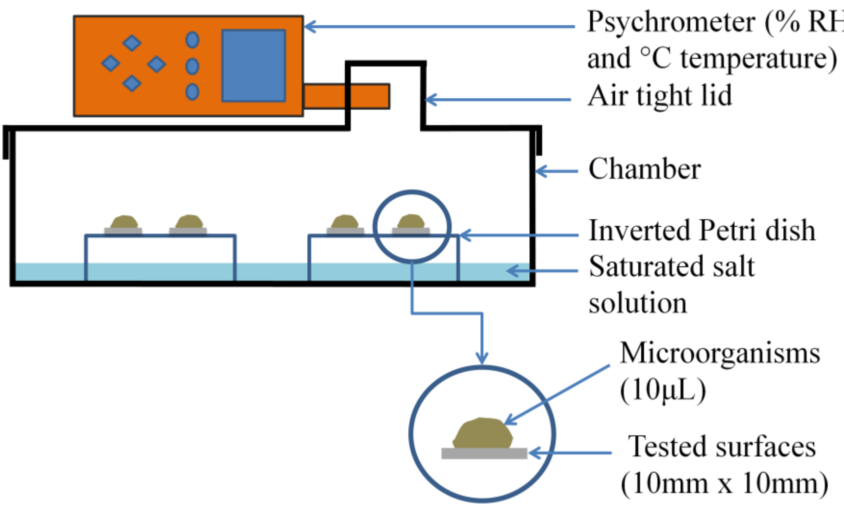


Figure 3.1. Apparatus devised to investigate the effect of humidity on the survival of *E. coli* and *L. monocytogenes*.

The sequence of experiments carried out was as follows:

1. Survival of the bacteria on stainless steel surfaces at optimum growth temperature and three humidity levels (11%ERH, 52%ERH and 86%ERH). Each system (*Figure 3.1.*) was placed in an incubator at 37°C for *E. coli* and 30°C for *L. monocytogenes*. Different incubation times (in hours) were used: T_0 (where samples were immediately swabbed after spreading), T_1, T_5 and T_{24}.

2. Survival on coated surfaces and stainless steel at 20ºC, thereby simulating room temperature, under the three humidity levels with different sampling times (identified according to results from a series of preliminary experiments (*Table 3.2.*) where *L. monocytogenes* had been shown to survive for considerably longer than *E. coli*.

Table 3.2. Exposure times (T_x in hours) used when testing *E. coli* and *L. monocytogenes* at 20ºC.

E. coli		***L. monocytogenes***		
Stainless steel	**Coated surfaces**	**Stainless steel**	**Coated surfaces**	**Equilibrium Relative Humidity**
T_0, T_1, T_2, T_3, T_4, T_5 & T_{24}	T_0 & T_2	T_0, T_1, T_5, T_6, T_7, T_8, T_9, T_{10}, T_{11} & T_{24}	T_0 & T_5	86%
	T_0, T_1 & T_2		T_0, T_5 & T_7	52%
	T_0, T_1 & T_2		T_0, T_5 & T_7	11%

After incubation, surfaces were swabbed for 10 seconds with a sterile cotton bud moistened from a 1mL aliquot of sterile distilled water. After swabbing, the cotton buds were broken into plastic bijou bottles (maximum volume: 5mL) containing the remainder of the 1mL of sterile distilled water, vortexed and diluted to 10^{-3} before being plated in duplicate on TSA for *L. monocytogenes* and BHI agar for *E. coli*, and incubated for 18 hours at the appropriate temperature (30ºC for *L. monocytogenes* and 37ºC for *E. coli*). After incubation, colony forming units were calculated. It is then possible to calculate the percentage of cells removed as proportion of number of cells applied. All experiments were repeated.

3.3.4.2. Effectiveness of swabbing

Epifluorescence microscopy was used to detect any cells remaining on the stainless steel coupons after swabbing (Whitehead *et al*, 2010). Once the stainless steel coupons were swabbed, the surface were stained for 2 min with 0.03% acridine orange (Sigma-Aldricht, Dorset, UK) in 2% glacial acetic acid (Sigma-Aldricht, Dorset, UK). Surfaces were rinsed for 5 seconds with sterile distilled water and air dried in a microbiological class 2 hood with the lights switched off. If necessary, stained coupons could be stored at 4°C in Petri dishes wrapped in foil for up to a week prior to examination. The surfaces were visualised using epifluorescence microscopy (Nikon Eclipse E600, Surrey, UK) at magnification x 400 with an excitation/emission of 495/522nm. The microscope was mounted with an F-View II black and white digital camera (Soft Imaging System Ltd, Helperby, UK, supplied by Olympus, Hertfordshire, UK). This system used a Cell F image analysis package (Olympus, Hertfordshire, UK), but due to the low number of cells remaining on the surfaces, only a cell count was necessary rather than a measure of coverage. The number of cells per coupon was estimated by counting remaining cells in ten random fields per coupon.

3.3.5. Viability and retention of *Escherichia coli* and *Listeria monocytogenes* on surfaces

3.3.5.1. Effect of substrata and re-use on cell retention

The aim of the retention assay was to assess the number of remaining cells left on the surfaces after rinsing, thus, testing the cleanability of the surfaces. 20mL of

washed standardised cell suspension was placed in a sterile glass Petri dish. Three stainless steel and coated coupons were placed in the cell suspension with the test side facing upwards, and incubated with the lid closed at room temperature for one hour without agitation. Following incubation, the test pieces were removed and rinsed once, gently with 5cm^3 of sterile distilled water with a water bottle (with a 3mm nozzle) held at a 45° angle for approximately 3 seconds. The substrata were air dried in a laminar flow hood and stained with acridine orange for 2 min before rinsing and drying in a dark laminar flow hood (*Section 3.3.4.2.*) or stained with live/dead solution (*Section 3.3.5.2.*). Each coupon was examined under an epifluorescence microscope and the amount of cells per coupon was estimated by measuring the percentage of surface covered by cells for ten random fields per coupon (*Section 3.3.5.2.*). Each type of substratum was re-used three times (*Section 3.3.2.1.*) and all experiments were repeated.

3.3.5.2. Cell viability on surfaces and re-use of surface

LIVE/DEAD *Bac*Light Bacterial Viability Kit for microscopy and quantitative assays (Invitrogen, Paisley, UK) was used to monitor the viability of the bacterial population on the surface. This kit used two dyes: SYTO 9 green-fluorescent nucleic acid stain with an excitation/emission of 490/500nm and propidium iodide red-fluorescent nucleic acid stain with an excitation/emission of 490/635nm. The two dyes have different spectral characteristics and behaviours regarding the way they stain the cells. SYTO 9 stain labels all bacteria without discrimination. However, propidium iodide penetrates damaged bacteria only, thus resulting in a reduction in the SYTO 9 dye. Under epifluorescence microscopy, viable undamaged cells will fluoresce green whereas damaged or dead cells will appear red. For the purpose of this experiment, the

method of Whitehead *et al* (2008) was used, where each stain was diluted 1 µL in 39 µL of sterile distilled water and both stains were mixed to a ratio 3:1 (SYTO 9: propidium iodide). 10µL of washed cells were deposited on stainless steel and coated surfaces and left to dry (approximately 20ºC and 45% ERH laboratory room conditions) for 1& 2 hours for *E. coli* and 1& 7 hours for *L. monocytogenes* (times determined from results of numerous preliminary studies) in a microbiological class 2 laminar flow hoods before being stained with 2µL of differential staining solution. Stainless steel and coated surfaces were tested by using 3 samples for each condition and repeated. Each sample was examined under epifluorescence microscope and differential counts of live and dead cells made for ten random fields per coupon.

Each surface was used three times to investigate effect of re-use of substrata (*Section 3.3.2.1.*) on antimicrobial activity. All experiments were repeated.

3.3.6. Effect of soiling on *Escherichia coli* survival on surfaces and re-use of surface

3.3.6.1. Swabbing assay

From the results obtained in *Section 3.3.4.1.*, the following conditions were used for this experiment: saturated salt solution with magnesium nitrate (52% ERH) and temperature of 20ºC. The same swabbing protocol was followed (*Section 3.3.4.1.*). *E. coli* standard cell suspension (*Section 3.3.1.2.1.*) was mixed (50:50) with meat extract (*Section 3.3.1.3.*) prior to loading onto the surfaces. In previous swabbing method, 10µL

of standard cell suspension was deposited, so 20µL of the mixture was deposited to have similar concentrations for more comparable results.

For each incubation time and humidity level and bacterium, three replicates were used. Controls with no applied microorganisms were tested under the same conditions to monitor contamination. Data from previous experiments, with cells alone, were used as control. During the experiment, coupons were kept in a microbiological class 2 laminar flow hood for safety. Coupons were re-used as previously described and all experiments were repeated.

3.3.6.2. Effectiveness of swabbing

As described in *Section 3.3.4.2.*, the surfaces used were stained with acridine orange post-swabbing in order to monitor the effectiveness of swabbing in presence of food soil.

3.3.6.3. Retention of *Escherichia coli* on surfaces in the presence of meat soil

The same method as described in *Section 3.3.5.1.* was followed. 20mL of meat extract mixed with 20mL *E. coli* standard cell suspension (50:50) mixture was poured in glass Petri dishes containing test surfaces.

3.3.7. Detection of viable and non-viable *E. coli* cells in the presence of meat extract

In order to assess the viability of *Escherichia coli* in the presence of conditioning film on the surfaces, a method was developed to distinguish viable, non-viable cells and food material using differential staining. Fluorescein isothiocyanate (FITC) (Sigma-Aldricht UK) at 0.01g/L in 0.1M Tris pH 8.0 (Fisher, Loughborough, UK) and 4',6-diamidino-2-phenylindole (DAPI) dissolved in water (0.01g/L) were used as potential stains for meat extract. These fluorescent stains have an excitation/emission of 490nm/514nm for FITC and 340nm/488nm for DAPI (www.invitrogen.com).

LIVE/DEAD *Bac*Light Bacterial Viability Kit for microscopy and quantitative assays (Invitrogen, Paisley, UK) was used to potentially detect viable and non-viable cells using the method described in *Section 3.3.5.2.*.

10µL of specified component A (*E. coli*), B (meat extract), C (*E. coli* and meat extract mixture, ratio 50:50), D (FITC stain), E (live/dead stain) & F (DAPI stain) listed in *Table 3.3.* was deposited in a series of sequential combinations onto 10mm x 10mm stainless steel coupon. After each component was applied and spread, the substrata were air dried in a laminar flow hood, for approximately 15 minutes. Each coupon was viewed under the epifluorescence microscope at magnification x 1000 directly after drying. Three coupons were used per experiment per combination *Figure 3.34.* to *3.42.* and were repeated once.

A subsequent modification involved smaller volumes (2µL) of each component being used. The combination N°13 (meat extract, drying, FITC stain, drying, *E. coli*, incubation time, live/dead stain and drying) and N°14 (meat extract, drying, DAPI stain,

drying, *E. coli*, incubation time T_x, live/dead stain and drying) of *Table 3.3.* on stainless steel and TiN/Ag 120W were used, and the substrata were incubated at room temperature for 0, 2 and 4 hours (T_0, T_2 and T_4) before live-dead stain stage of the combination to monitor damages over time. Stainless steel was used as a control to assess whether the stains were affected or not by the different coatings. TiN/Ag 120W was used for its high concentration of silver in order to see if there would be a visual difference between the number of live and dead cells covering the surface. At times, some images required different microscope filters revealing different parts of the image (cells and soil), therefore the same image with different microscope filters was referenced as Nº X a) and Nº X b) on *Figures 3.34.* to *3.42.*

Table 3.3. Combination of components used to investigate differential staining technique. After application of each component a drying period was employed before adding the next component as indicated in *Table 3.3*.b) enabling a range of permutations to be investigated

a)

Characters	Stains/soil/bacteria
A	*E. coli*
B	Meat extract
C	*E. coli* + Meat extract mixture (50:50)
D	FITC
E	Live/dead
F	DAPI

b)

Nº	Combinations using characters
1	A + B + D + E
2	A + B + E + D
3	C + D + E
4	C + E + D
5	B + A + D + E
6	B + A + E + D
7	A + B + E + F
8	A + B + F + E
9	C + E + F
10	C + F + E
11	B + A + E + F
12	B + A + F + E
13	B + D + A + E
14	B + F + A + E
15	C + E
16	B + A + E

3.3.8. Visualisation of surfaces via reflective light microscopy

The surfaces were visualised using reflective light microscopy (Olympus Vanox-T, Hertfordshire, UK) at magnification x 1000 in order to allow visual assessment of the condition of the coatings (scratches and wear). The microscope was mounted with a Panasonic WV-CL350 black and white digital camera (Panasonic, Bracknell, UK). This system used an HLImage ++ 97 image analysis package (Olympus Vanox-T, Hertfordshire, UK). For each coating, images of three coupons for each re-use were recorded.

3.3.9. Statistical analysis

All statistical results were obtained by using Excel (Microsoft Office, version 2007). The p-value was calculated using t-test and determined the probability of rejecting the null hypothesis (H_0). A level of 5% was chosen to set the confidence interval limit at 95% when conducting the analysis.

3.4. Results and discussion

3.4.1. Hydrophobicity of cells

The role of electron-donor/electron acceptor, i.e. Lewis acid-base properties, in the interaction between two materials has been widely studied (Van Oss, 1992). Several studies (Boulangé-Petermann *et al*, 1993; Van Oss, 1993) have reported that the electron-donor/electron acceptor plays a crucial role in the microbial adhesion

phenomenon. Cells adhesion result from interactions caused by the surface charge (Hogt *et al*, 1985; Dickson and Koohamaraie, 1989), the hydrophobicity (Dahlback *et al*, 1981; Van Loosdrecht *et al*, 1987) and electron acceptor and electron donor (Van Oss, 1993) of the interacting surfaces.

Both bacteria were found to have hydrophobic surfaces with *L. monocytogenes* being more hydrophobic than *E. coli.* In *Chapter 2*, *Section 2.4.4.* stainless steel, TiN/Ag 100W, 50W were found to be hydrophobic. Substrata TiN and TiN/Ag 120W were hydrophilic. One might expect hydrophobic bacteria to be less well retained or have a weaker contact with substrata with hydrophilic surfaces TiN and TiN/Ag 120W. However, all test surfaces were very smooth which will reduce the retention and increase the hygienic status of the substrata, so it will not have much effect.

In this MATS analysis, two approaches were taken: Lewis acid/base and van der Waals. Chloroform and ethyl acetate were chosen as polar electron donor/acceptor solvent for the estimation of the Lewis acid/base character, while two non-polar solvents, hexadecane and decane, were employed to estimate the hydrophobic/hydrophilic cell surface properties also known as van der Waals properties (Szlavik *et al*, 2012). The electron donor character was estimated by the difference between affinity to chloroform and hexadecane (Bellon- Fontaine *et al*, 1996). This technique appeared to be more useful than contact angle method (Van Oss *et al*, 1988), which requires specific and elaborate equipment.

As presented in *Figure 3.2., L. monocytogenes* showed an equal, high, relative affinity for both chloroform and decane solvents (86%). There was a small adherence to ethyl acetate (26%) and a relative affinity for hexadecane (67%) lower than that of decane. Thus, *L. monocytogenes* exhibited characteristics of an electron donor with a

moderate electron accepting character as well as a hydrophobic nature. The affinity of *E. coli* cells was similar to that of *L. monocytogenes* with a high adherence to chloroform and decane solvents (74% and 85%) (*Figure 3.2.*). A low affinity for ethyl acetate (17%) and hexadecane (23%) was also noted. These results implied the hydrophobicity nature of cell surface with a mild electron accepting nature and strong electron donor nature.

In this study, results indicated that *E. coli* and *L. monocytogenes* showed ability to donate electrons due to high affinity to electron acceptor solvent (chloroform). *L. monocytogenes* presented a higher surface hydrophobicity than *E. coli* which was shown by high affinity to non polar solvents (*Figure 3.2.*).

Fletcher (1996) reported a general trend indicating that hydrophobic cells attach more readily than hydrophilic cells to biotic or abiotic surfaces, presumably due to their reduced stability in the bulk aqueous medium. Zita and Hermansson (1997) found that *Escherichia coli* with high affinity for hydrophobic activated sludge flocs were more hydrophobic than less-adherent cells. A study on food contact surfaces demonstrated how cell adhesion occurred mostly on hydrophobic surfaces such as stainless steel 304 (Silva *et al*, 2008). It is also important to take in consideration other factors such as the growth temperature and the phase of growth which were reported to influence the cell wall composition and thereby modify the surface electrical properties, hydrophobicity, and electron donor or electron acceptor character of the bacteria (Smoot and Pierson, 1998 a, b; Giovannacci *et al*, 2000).

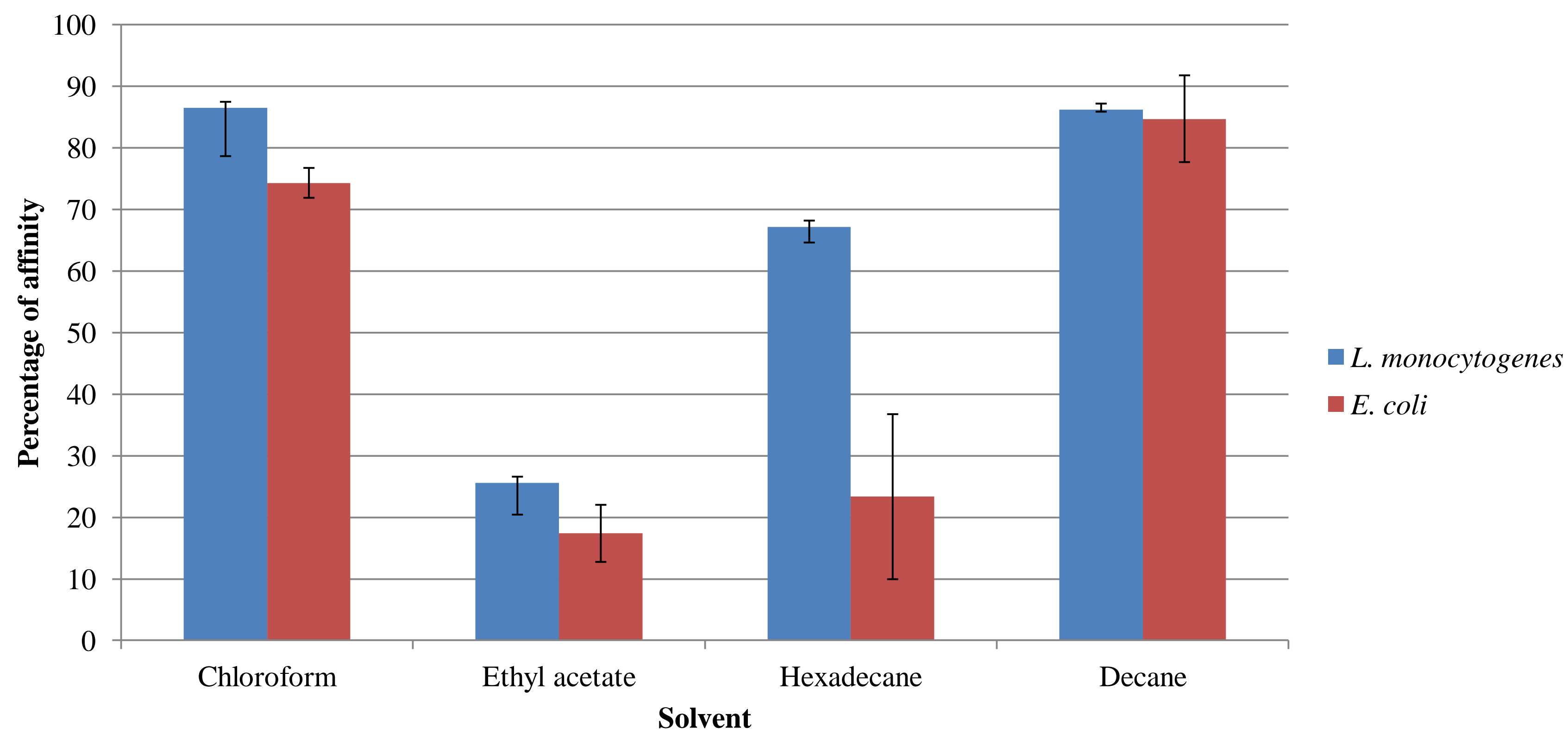


Figure 3.2. Partition of *E. coli* and *L. monocytogenes* cells into four solvents as an indicator of electron donor/acceptor (chloroform/ethyl acetate) and hydrophobicity/hydrophilicity (hexadecane/decane)

3.4.2. Characterisation of surfaces

3.4.2.1. Ions released from metals contained in surfaces and coatings (ICP-AES)

ICP-AES is a method which has been extensively used to monitor trace elements of metals in air, water and solid wastes (Rubio *et al*, 1984; Alimonti *et al*, 1988; Boevski *et al*, 2000). In this study, metals ions released from test substrata were recorded. These may affect the survival of bacteria coming in contact with the surfaces. Ions present in sterile distilled water were subtracted from the results recorded for all substrata. Stainless steel results were subtracted from those obtained for the coated surfaces assuming the same metals would be released through the coatings.

Figure 3.3. represents ions released in the water from stainless steel 304 with a general decrease over time. Traces of silver and aluminium were recorded which are not part of the listed elements composing stainless steel 304: this may be due to minor fluctuations in the water tested (*Chapter 1*, *Section 1.1.*). However, higher traces of nickel than chromium were detected, with iron being released in the smallest quantity (*Figure 3.3.*). Traces of silver were detected only at the highest concentration from the coatings after one hour and no trace of Ti or N were detected. The ability of coatings such as titanium nitride to protect underlying metal against corrosion is related to their chemical composition and microstructures, as well as their adhesion to the substrate. TiN coatings are now regularly applied to prolong tool lifetime in conventional machining processes (Sproul, 1985; Randhawa, 1988).

Overall, the TiN coating was very stable and did not leach Ti or N ions in the water. There was little release of silver ions from any surface which meant that coatings

were stable with a potential use in food industry. Metals such as silver exhibit antifungal and antibacterial properties, which have been widely utilised in advanced coating technologies (Avery *et al*, 1996; Zoroddu *et al*, 1996; Yonehara *et al*, 2001; Mann *et al*, 2002; Stoimenov *et al*, 2002).

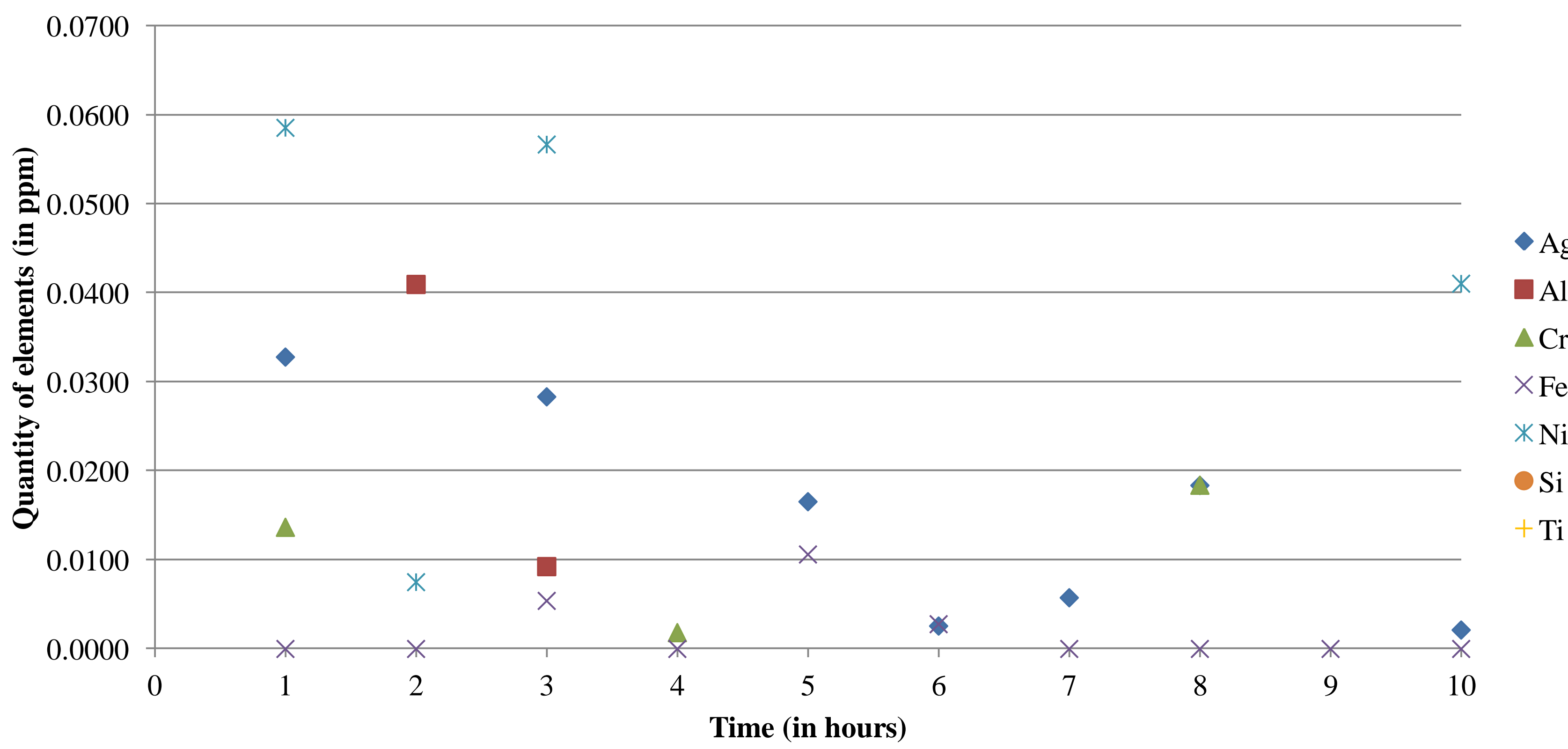


Figure 3.3. Selected metal elements for release detection from stainless steel 304 during ten hours in sterile distilled water

3.4.2.2. Minimum concentration of silver ions to inhibit growth of *E. coli* and *L. monocytogenes* cells (MIC)

MIC is often used to determine the minimum inhibitory concentrations of antibiotics in hospitals (Buffet-Bataillon *et al*, 2011) and was used here to test the antimicrobial effect of silver, a silver standard solution containing 2% nitric acid was used. However, the presence of 2% nitric acid could have itself killed the microorganisms. Thus separate controls were employed, confirming that 2% nitric acid had no apparent effect on the growth of either culture. The broth control was positive (growth) and silver standard negative (no growth).

Results showed that *E. coli* was not inhibited at and below 30mg/L concentration of silver. Bacterial growth was inhibited at and above 59mg/L of silver. The minimum inhibitory concentration for *E. coli* was 30mg/L of silver (*Figure 3.4.*). *L. monocytogenes* showed more resistance to silver than *E. coli*. Growth of its cells was observed up to and including 59mg/L. Minimum inhibitory concentration for Gram positive *L. monocytogenes* was 59mg/L of silver.

In Malaysia, a study showed that Nano Colloidal Silver emerged as a viable treatment option for infections encountered in burns, open wounds and ulcers (Petrus *et al*, 2011). They found that the silver concentration needed to inhibit *L. monocytogenes* was higher than for *E. coli*. Similar results were found in this study, thus showing a higher resistance from Gram positive *L. monocytogenes* to silver than Gram negative *E. coli*.

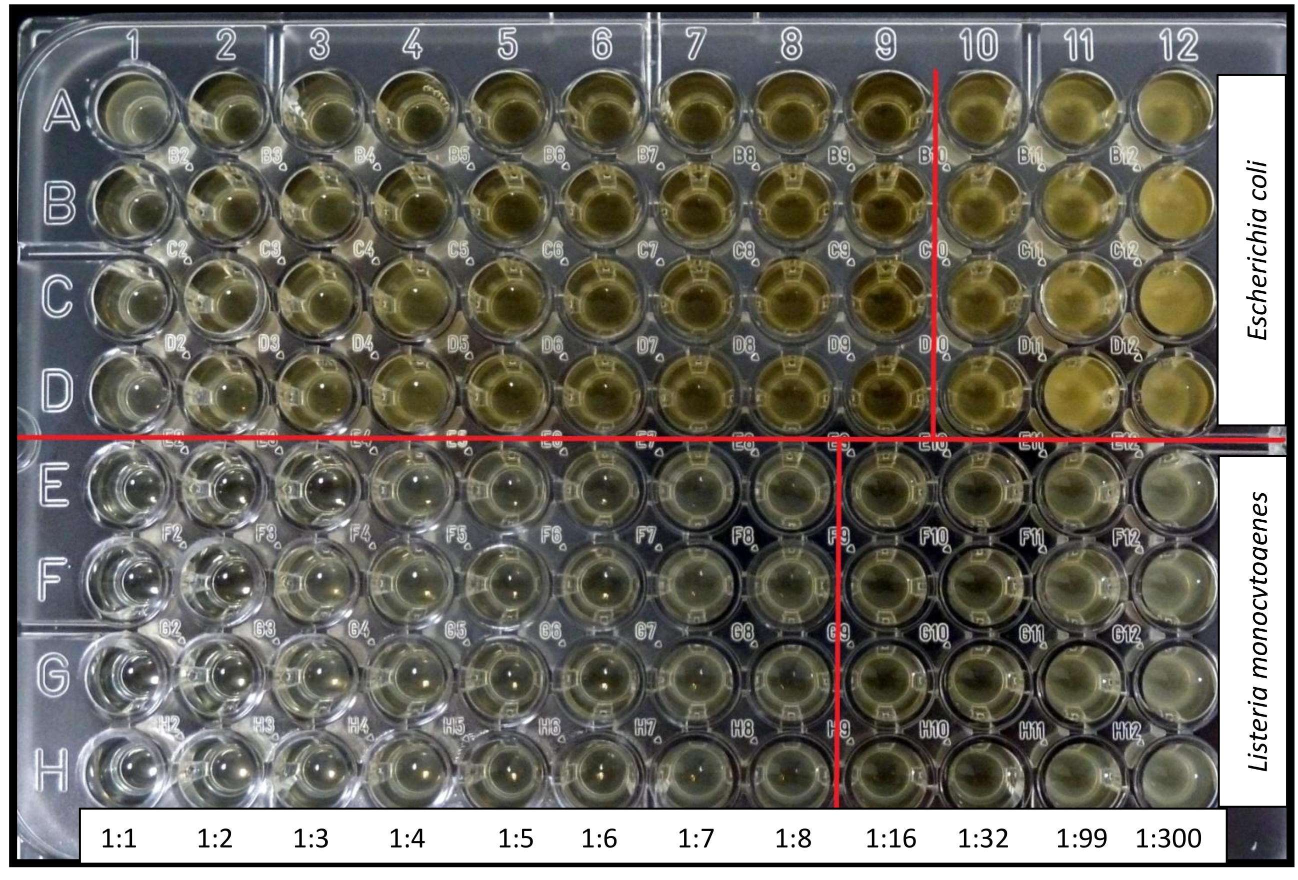


Figure 3.4. Effect of different silver standard concentrations on the growth of *E. coli* and *L. monocytogenes* (red line separating the two bacteria and demarking the visible turbidity on the right from the clear liquid on the left side of the line)

3.4.2.3. Minimum concentration of silver to kill *E. coli* and *L. monocytogenes* cells (MBC)

The controls from the MIC were subcultured in duplicate and showed growth of colonies for both positive controls (broth and cells), and 2% nitric acid control (with cells) and no growth for the negative control of silver standard with cells.

Table 3.4. a) showed growth of *E. coli* at 59mg/L of silver which indicates that although there was a growth inhibition, there was no killing at this concentration. There was no viable *E. coli* detected at 111mg/L of silver which means the minimum bactericidal concentration is 59mg/L.

The same result was observed for *L. monocytogenes* (*Table 3.4. b*) with an MBC of 59mg/L, thus showing the same results for both MIC and MBC. So the two microorganisms only differed in MIC which only showed the concentration of silver needed to visually inhibit microorganisms (no turbidity).

MBC is an extension of the MIC test where the lowest concentration of drug for which no growth occurs in the subculture is the minimum bactericidal concentration (Petrus *et al*, 2011). These results showed that the concentration of silver needed to kill both tested microorganisms was consequent. Therefore, the coated surfaces would be likely to be most efficient at the highest concentration of silver (TiN/Ag 120W). The concentration of silver is only indicatory to the kill of the microorganisms as the results are not comparable to those found in ICP-AES. ICP-AES results only indicated the release of silver ions at the highest concentration.

Table 3.4. MBC assay: growth of a) *E. coli* and b) *L. monocytogenes* from microtiter plate rows A, B, C and D showing same results

a)

Ratios / ***E. coli* samples**	**1:8**	**1:16**	**1:32**	**1:64**
Row A	Ø	X	X	X
Row B	Ø	X	X	X
Row C	Ø	X	X	X
Row D	Ø	X	X	X

b)

Ratios / ***L. monocytogenes* samples**	**1:7**	**1:8**	**1:16**	**1:32**
Row A	Ø	Ø	X	X
Row B	Ø	Ø	X	X
Row C	Ø	Ø	X	X
Row D	Ø	Ø	X	X

Legend:
Ø no growth on agar
X growth on agar

3.4.2.4. Zone of Inhibition

3.4.2.4.1. Effect of silver ions on cells

Increasing concentration of silver ions increased inhibition of *E. coli*. Zones of inhibition (ZOI) were observed for *E. coli* at 59mg/L (*Figure 3.6.*).

The diameter of ZOI for *L. monocytogenes* (n=24) reduced more rapidly than *E. coli* (n=24) as the concentration of silver decreased. However, there was no statistical significant difference in diameter of ZOI between *E. coli* and *L. monocytogenes* ($P > 0.05$). Inhibition occurred at 125mg/L of silver for *L. monocytogenes* (*Figure 3.6.*). All controls performed appropriately (*Figure 3.5.*).

ZOI is used in medicine to compare antimicrobial agents by measuring the size of the zone of inhibition which depends on the rate of diffusion through agar. Skov *et al* (2005) showed in their study that variations between ZOI and MIC/MBC results occurred. This is due to the fact that the same agent has to travel through different media which may alter the results. Overall, in this study, the results found for both microorganisms between MIC/MBC and ZOI were similar.

3.4.2.4.2. Direct contact with substrata

There was no ZOI around the substrata tested for *E. coli* and *L. monocytogenes*. Silver had been shown to inhibit Gram negative *E. coli* at 59mg/L and Gram positive *L. monocytogenes* at 125mg/L. Similar results were found with MBC assay with a concentration of 59mg/L of silver. Results from zone of inhibition assay were similar for *E. coli* with inhibition at silver concentration of 59mg/L and higher for *L.*

monocytogenes at 125mg/L than in previous experiences. So, test surfaces were not releasing silver ions in concentrations high enough to kill cells away from the surface. However, this does not indicate whether cells were killed or not on the surface in contact.

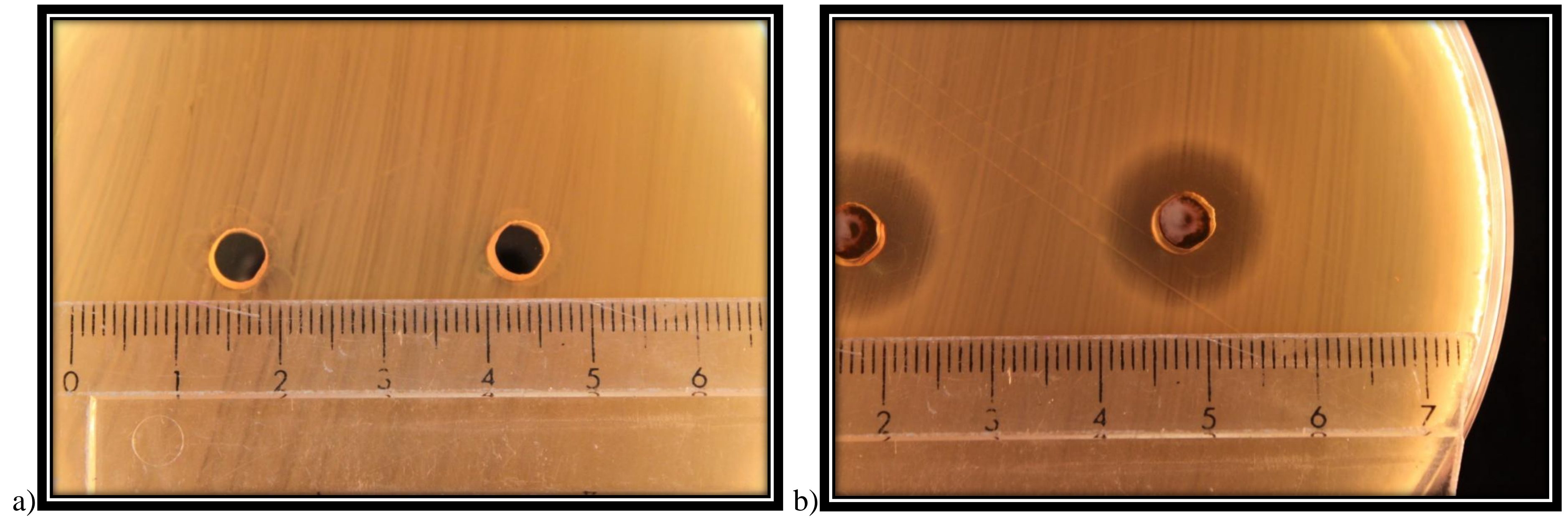


Figure 3.5. ZOI of *E. coli* with a) positive control (n=4) and b) negative control (n=4) with a zone of 1.5cm diameter

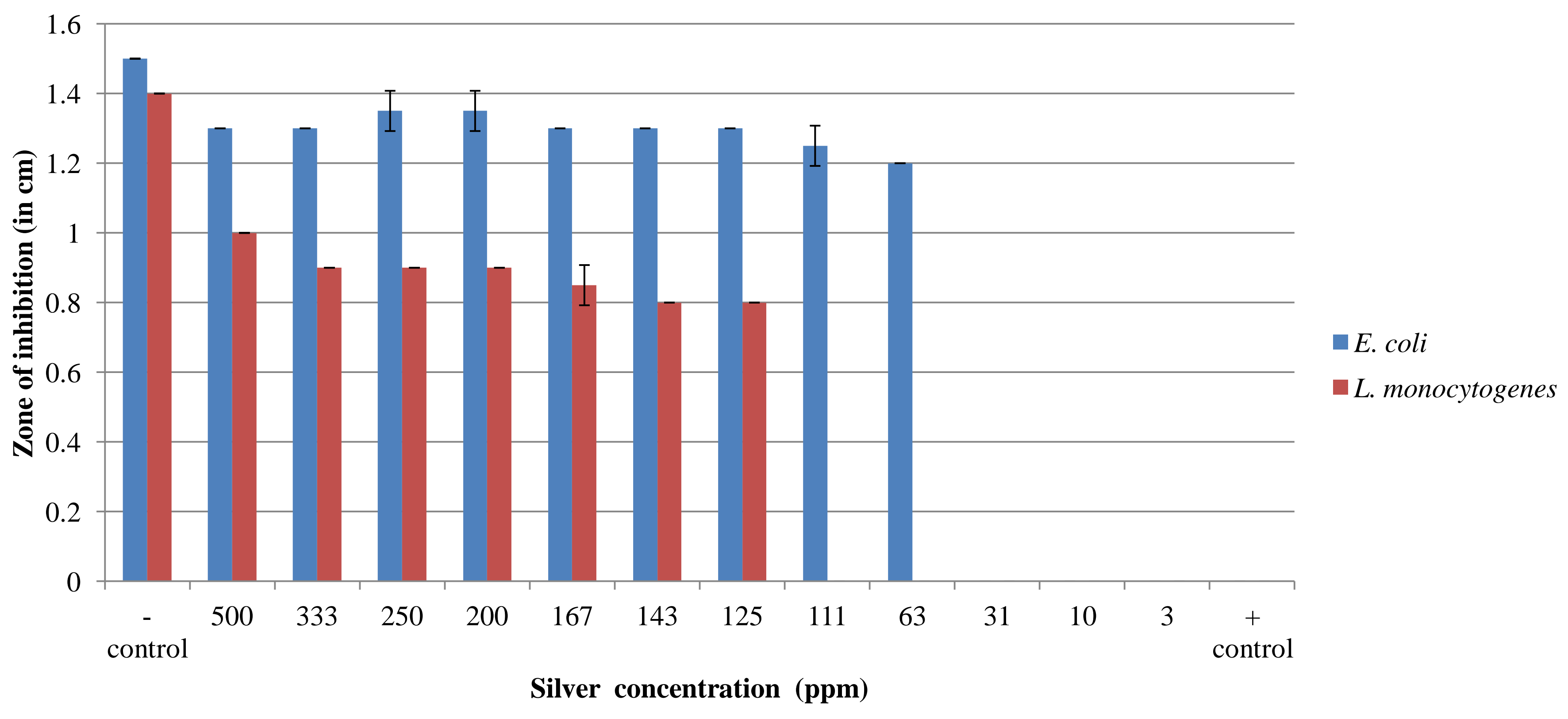


Figure 3.6. ZOI of *E. coli* and *L. monocytogenes* from the reaction with silver standard

3.4.3. Viability of cells on surfaces via metabolic assay

The use of tetrazolium redox dyes for direct visualisation of metabolic activity in bacterial cells has gained much interest in recent decades. Tetrazolium redox dyes scavenge electrons from oxidation/reduction reactions and are reduced intracellularly to brightly coloured or fluorescent formazan precipitates by the electron transport system components or dehydrogenases in metabolically active cells (Bhupathiraju *et al*, 1999a,b). Overall, there is consensus that surface binding and damage to membrane function are the most important mechanisms for the inhibition of bacteria by silver nanoparticles (Clement and Jarrett, 1994), so TBC would show effect.

In the TBC assay, the cells dried on a surface are covered with molten agar which is left to set before incubation. Thus, viable cells on the surface grow in the medium and the resultant colonies are stained with TBC to become visible to the naked eye. This method was used to determine the antimicrobial effectiveness of a surface (if the antimicrobial agent does not diffuse into the agar) as well as providing a viable count of cells on the surface itself (Verran *et al*, 2008).

With an initial inoculum at 10^{-1} and 10^{-2} dilutions of standard cell suspension, colonies were too numerous to count (TNTC) for both microorganisms on all the surfaces, thus the 10^{-3} dilution (10µL of 10^{-3} inoculum $\approx 5 \times 10^{2}$ cells) was chosen as inoculum with individual colonies visible and countable. When too many cells were present on the surface, the stain formed large patches (see TNTC 1, *Figure 3.7.*) or stained individual colonies which were too numerous to count (see TNTC 2, *Figure 3.7.*). At an ideal dilution (10^{-3} in this experiment), distinct colonies grew (see Countable colonies, *Figure 3.7.*). When antimicrobial surfaces inhibited growth or

killed the cells, no stained colonies were visible on the surface (see "No growth" *Section 3.4.3*, *Figure 3.7.*).

Attached cells of *E. coli* and *L. monocytogenes* grew on stainless steel and TiN (*Figure 3.8.*). There was no statistical significance between the two substrata or between *L. monocytogenes* and *E. coli*. The growth of both microorganisms was inhibited significantly ($P < 0.01$) on TiN/Ag 50W, TiN/Ag 100W and TiN/Ag 120W compared with the silver-free stainless steel and TiN because no growth was recorded.

TBC assays were included to investigate the capacity of viable cells to produce colonies on the different substrata. TiN/Ag 50W, 100W and 120W inhibited the growth of both microorganisms. Therefore, although no silver was released from the surface, antimicrobial effects resulted from direct contact between cells and surface. It would have been interesting to repeat the experiment with meat soil to compare the results.

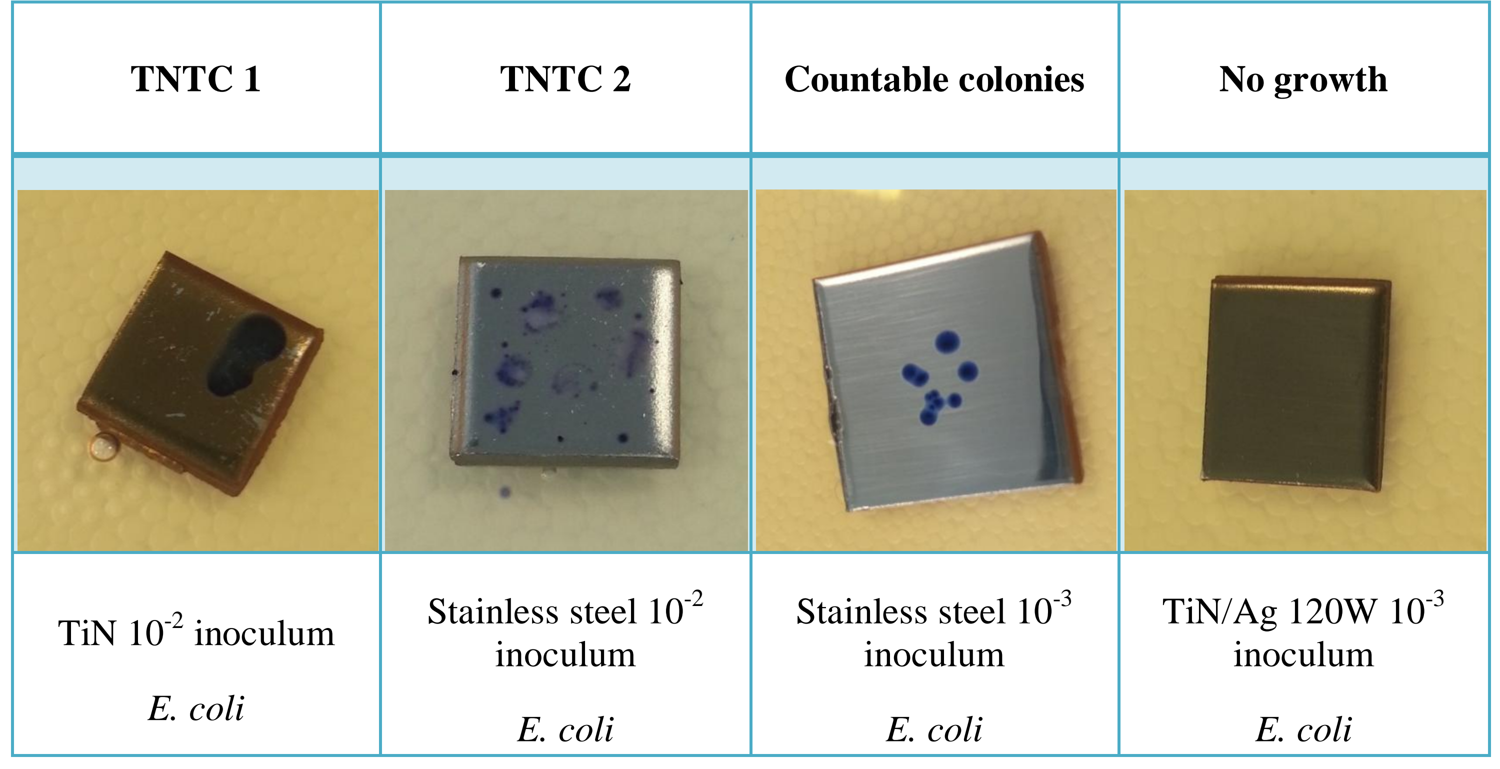


Figure 3.7. Representative photographs of metabolic assay

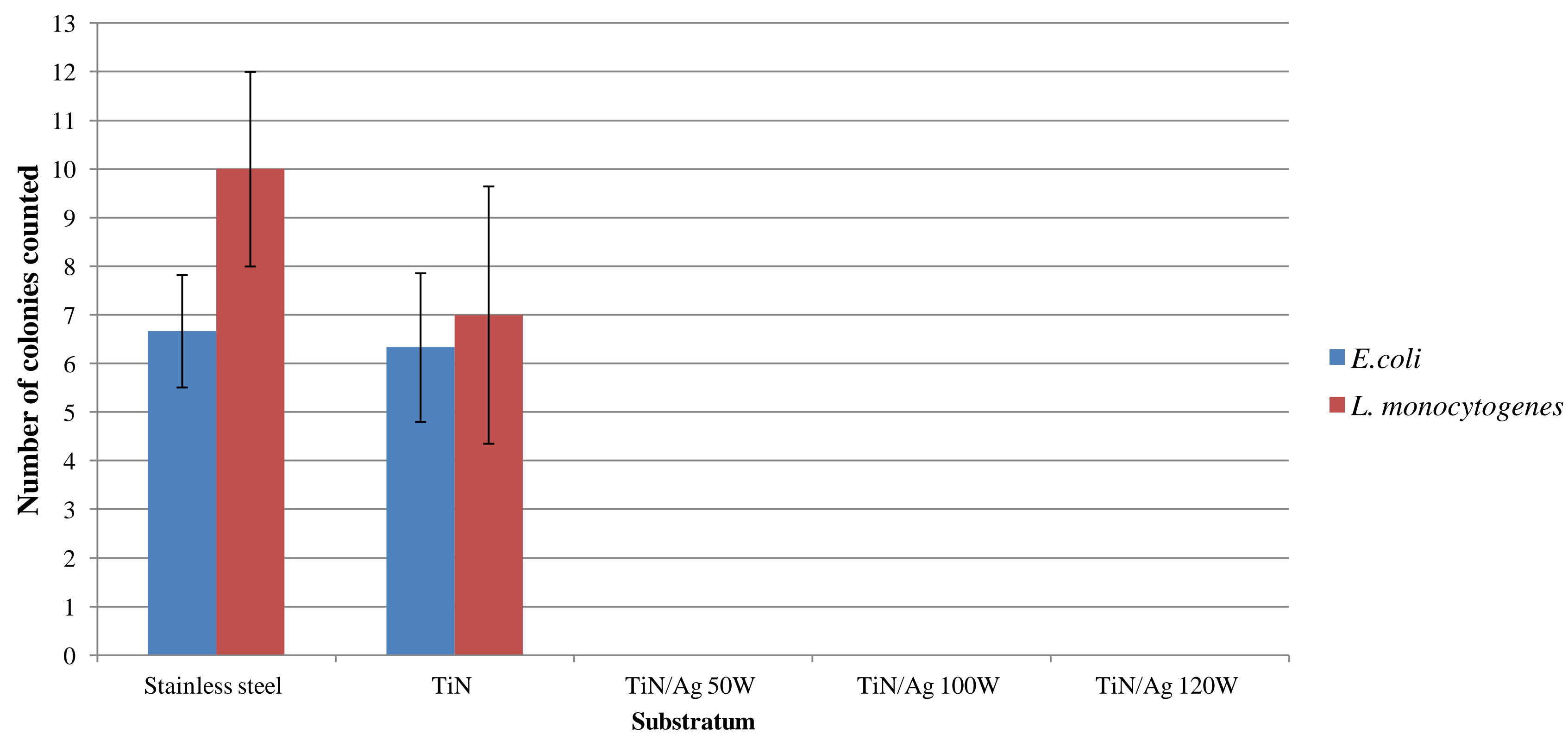


Figure 3.8. Survival and growth of *E. coli* and *L. monocytogenes* colonies on test surfaces from TBC assay (n=60) with inhibition on TiN/Ag 50W, 100W and 120W

3.4.4. Survival of *Escherichia coli* and *Listeria monocytogenes* on surfaces

3.4.4.1. Swabbing assay

Since the antimicrobial surfaces demonstrated a contact kill effect, the impact of different levels of humidity on survival was investigated. In preliminary work, methods were developed to assess the effects of different humidities on *E. coli* and *L. monocytogenes* survival on stainless steel. Subsequently these methods were used for surfaces with putative antimicrobial coatings. The methods which have been developed involve deliberately inoculating known numbers of *E. coli* and *L. monocytogenes* on stainless steel surfaces. Different methods were used to assess the amount of viable cells left on the surfaces. Epifluorescence microscopy was used to assess the efficiency of the swabbing technique (Moore and Griffith, 2007).

Overall, *L. monocytogenes* survived better than *E. coli*. *E. coli* and *L. monocytogenes* survival (at 37ºC for *E. coli* and 30ºC for *L. monocytogenes*) decreased at low humidity but survival was enhanced at high (86% ERH) and medium (52% ERH) humidity level (*Figure 3.9.* and *3.10.*). At 20ºC, no increase in antimicrobial effect was observed in the presence of high humidity, only the increase in the survival of the cells as previously noted. Nevertheless, all coatings significantly reduced the survival of both bacteria when compared to stainless steel at 20ºC. There was no increase of antimicrobial activity noted with the increase of silver concentration (*Figure 3.11.* to *3.18.*). These results correlated with those found with the ICP-AES experiment where silver did not leach from the coated surfaces. So, the postulate need for higher humidity to increase antimicrobial effect is not necessary as it increases the survival of the

microorganisms. The presence of silver in the coated surfaces decreased the bacterial survival by contact kill.

At optimum growth temperature (37ºC for *E. coli* and 30ºC for *L. monocytogenes*), *E. coli* survived best on stainless steel at 52% ERH for at least 5 hours and 1 hour at 86% and 11% ERH (*Figure 3.9.*). *L. monocytogenes* survived better at 86% ERH for 5 hours and less well at 52% and 11% ERH for 1 hour (*Figure 3.10.*).

At room temperature (20ºC), *E. coli* numbers reduced more rapidly at low humidity (1 hour at 11% ERH) (*Figure 3.11.*) than at medium and high humidity levels (4 hours at 86% and 52% ERH). Thus, *E. coli* survived for a longer time with higher humidity and lower temperature, i.e. at 86% ERH at 20ºC (4 hours) than at 37ºC (1 hour). The same experiment was repeated with *L. monocytogenes* (*Figure 3.12.*). *L. monocytogenes* survived for a statistically highly significant (P=0.013) longer period at 20ºC than at 30ºC. The bacterium survived up to 10 hours at 86% ERH and 8 hours at 52% and 11% ERH. This showed a longer period of survival of *L. monocytogenes* than *E. coli* at 20ºC on stainless steel. At 20ºC, *L. monocytogenes* survived much longer and better with high humidity. *L. monocytogenes* had been shown by others to survive better than *E. coli* in different conditions. For example, Nissen and Holck (1998) reported that *L. monocytogenes* survived storage better than *E. coli* O157:H7 in Norwegian fermented dry sausage at 4 and 20ºC. This significance represents an important factor to consider when storing cooked or chilled food in order to prevent potential outbreaks. Potential future work could investigate the effect of lower temperatures used in cool storage on the survival of the microorganisms on the test surfaces.

Four coated surfaces, TiN, TiN/Ag 50W, TiN/Ag 100W and TiN/Ag 120W were used under different humidities at 20ºC to investigate the use of silver as

antimicrobial agent. As previously, higher humidity increased survival ($P < 0.01$). Cells survived better at 86% ERH than at 52-11% ERH (*Figure 3.13., 3.15. and 3.17.*). There was no statistical difference between 52% and 11% ERH ($P > 0.1$) between each coated surfaces. At 20ºC, there was no significant decrease in *E. coli* survival between the highest concentration of silver (TiN/Ag 120W) and stainless steel ($P > 0.1$). At 20 ºC, humidity and increased silver concentration in coatings (TiN/Ag 50W, 100W and 120W) did not decrease the recovery in number of colonies of *L. monocytogenes* (*Figure 3.14., 3.16. and 3.18.*). There was a statistical significant decrease of *L. monocytogenes* survival ($P < 0.05$) between TiN and stainless steel. This may be due to the fact that stainless steel was found to be a hydrophobic surface while TiN was hydrophilic. The bacteria used being hydrophobic, this may have affect their removal an adhesion. The significance decrease was even higher when comparing stainless steel with TiN/Ag 120W ($P < 0.01$), as expected due to antimicrobial properties of silver.

In preliminary experiments, *E. coli* and *L. monocytogenes* survived better when high concentration of cells were applied to the surface, perhaps due to some protective effect as noted by Chmielewski and Frank (2003) or to lower cell numbers being below limit of detection. The experimental method selected informed by (not presented) preliminary work was as follows: standardised cell suspension was applied to the surface; the cells needed to be dry on surfaces prior to incubation; inoculated surfaces were left in different humidities for 24 hours at room temperature to simulate a daily clean. There was an overall decrease in number of colonies recovered of 3 Log_{10} during the drying period (Wilks *et al*, 2006) which is the reason for selecting the standardised cell suspension at a high initial concentration of cells for the experiment in this thesis.

The effect of different temperatures was also explored (Mai and Conner, 2007). *E. coli* did not survive as well as *L. monocytogenes* at 20ºC, but similar results in terms of survival were found for both bacteria at their respective optimum growth temperatures (37ºC for *E. coli* and 30ºC for *L. monocytogenes*). Gram-negative *E. coli* has thin peptidoglycan layers in their cell walls (Iida and Koike, 1974) in addition to an outer membrane layer, which in this particular case proved to be less resistant to environment factors than Gram-positive *L. monocytogenes* which has a thicker peptidoglycan layer in its cell wall (Edwards and Stevens, 1963). *E. coli* proved to be more sensitive to the silver in thin film coatings than *L. monocytogenes*. TiN coatings reduced the number of colonies recovered for both bacteria when compared to stainless steel results, which may be due to the good wetting of the surface (*Chapter 2, Figure 2.10.*) allowing the aliquot of cells to dry quicker.

The response of microorganisms to osmotic stress (which may be caused by changes in humidity levels) involves both physiological changes and variations of gene expression patterns and is called osmoadaptation (Hill *et al*, 2002). Desiccation tolerance is the ability of cells to undergo nearly absolute dehydration through air-drying, without being killed (Billi and Potts, 2002). The experiments testing both bacteria under different equilibrium relative humidities did not show any increase in antimicrobial effect of the surfaces when increasing equilibrium relative humidity, although it had been hypothesised that high moisture might increase release and death; high moisture actually increased the survival of microorganisms.

Overall, *L. monocytogenes* survived for a longer period than *E. coli* on both stainless steel and coated surfaces. There was a reduction in recovery of viable cells compared to the inoculum, of approximately 3 Log_{10}. The number of bacteria left on

surfaces was negligible according to acridine orange examination (*Section 3.4.4.2.*). The reduction might be due to cells being retained on the swab, as well as overall inefficiency of methods and cell death.

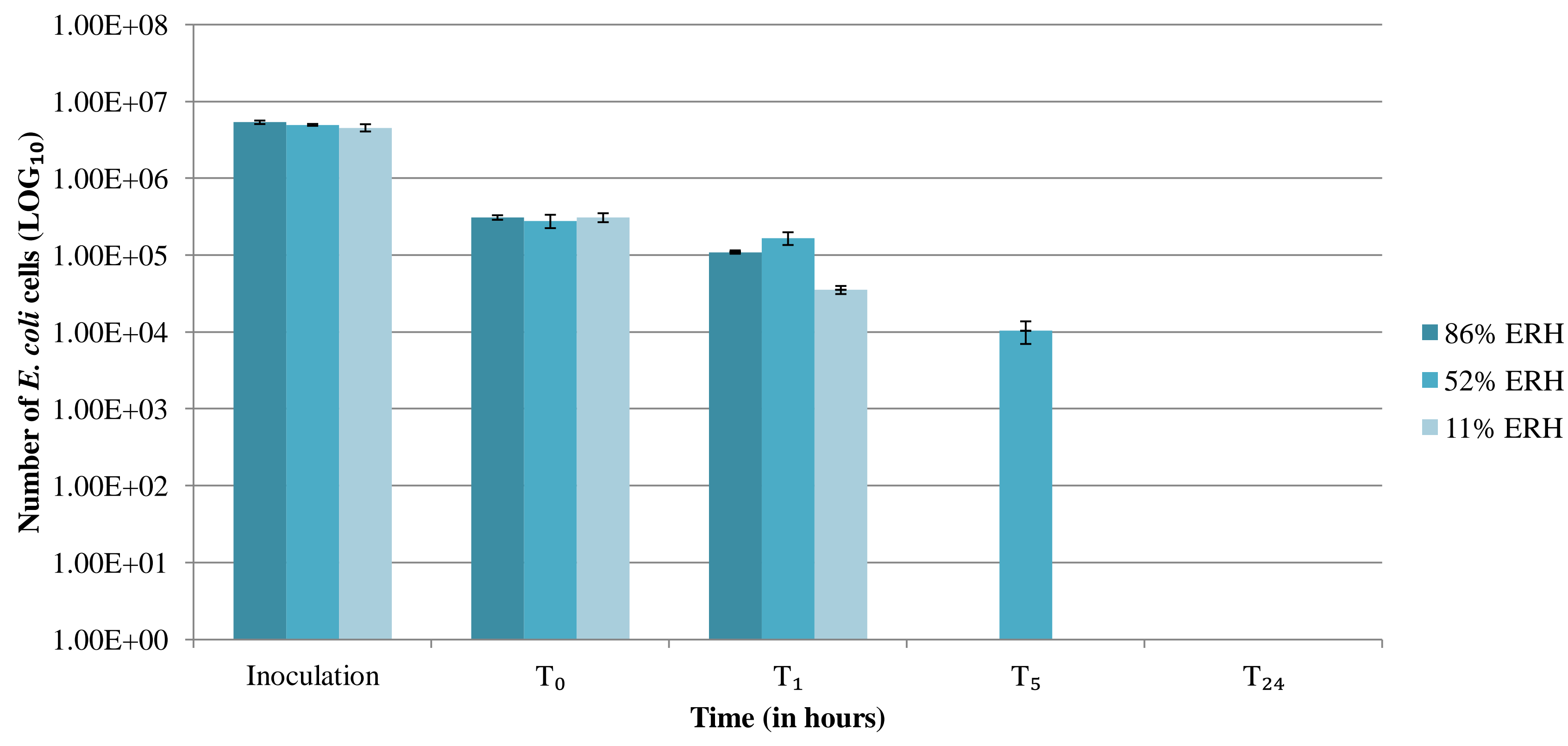


Figure 3.9. E. coli cells recovered after 0, 1, 5 and 24 hours of incubation in different equilibrium relative humidities (86%ERH, 52%ERH and 11%ERH) at 37ºC on stainless steel

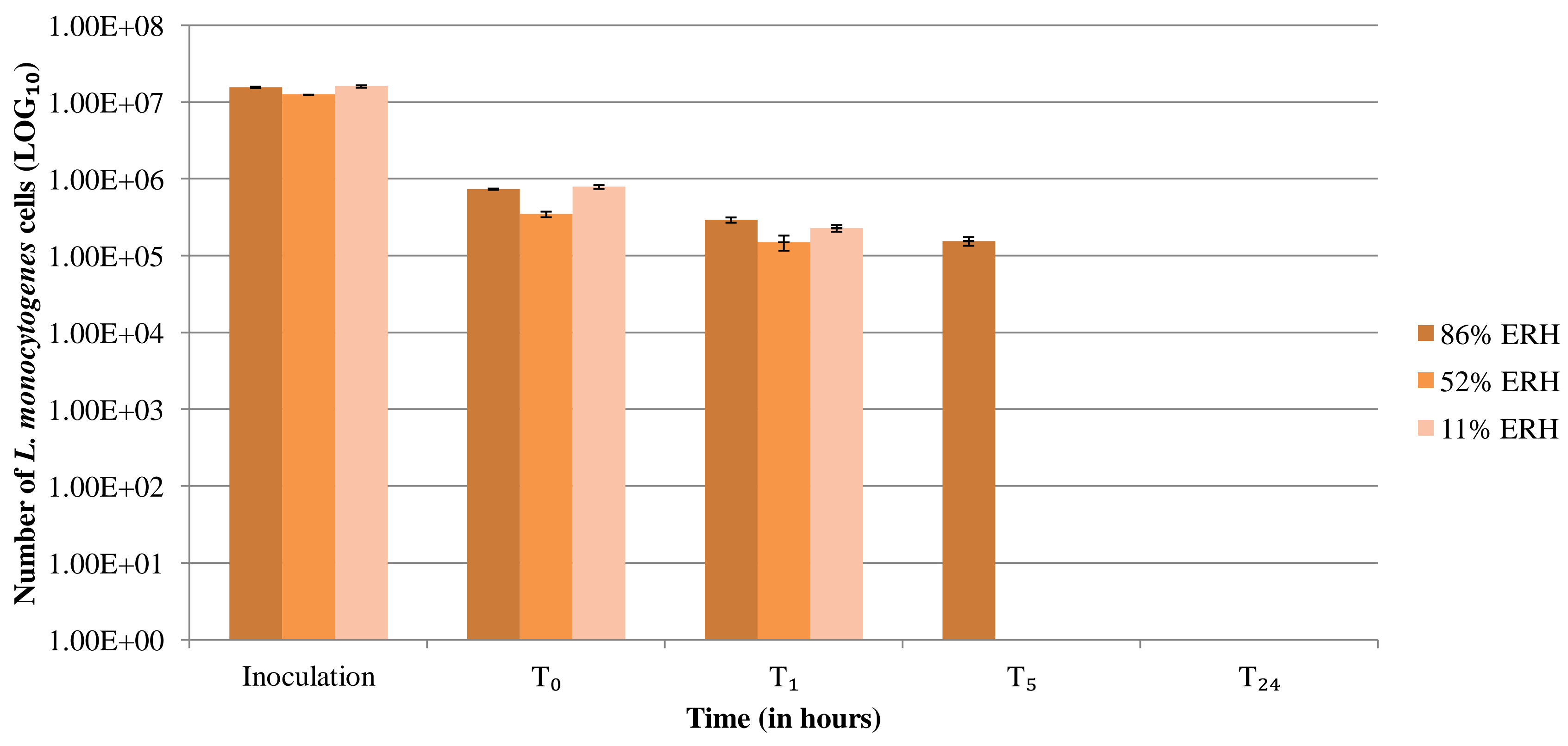


Figure 3.10. L. monocytogenes cells recovered after 0, 1, 5 and 24 hours of incubation in different equilibrium relative humidities (86%ERH, 52%ERH and 11%ERH) at 30ºC on stainless steel

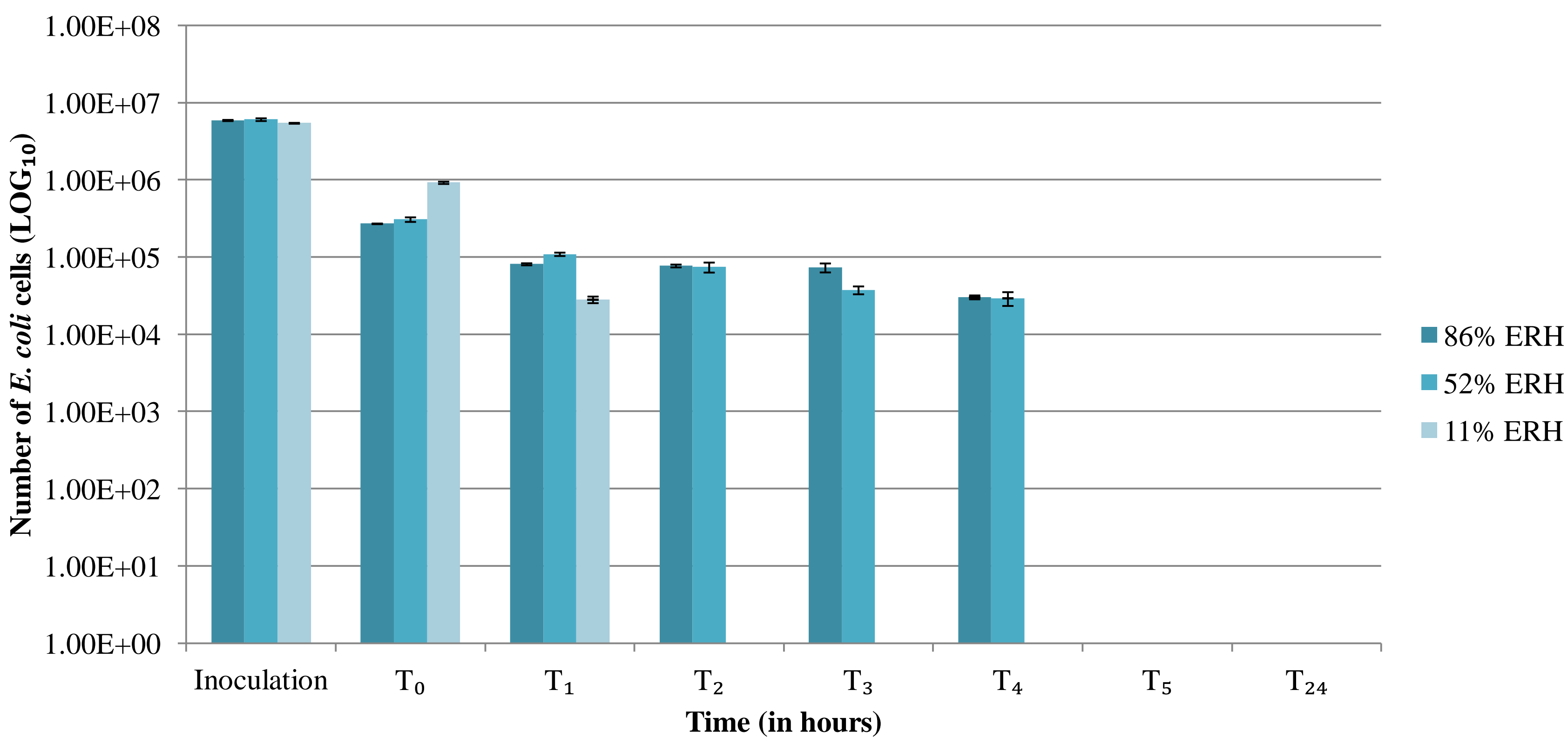


Figure 3.11. E. coli cells recovered after 0, 1, 2, 3, 4, 5 and 24 hours of incubation in different equilibrium relative humidities (86%ERH, 52%ERH and 11%ERH) at 20ºC on stainless steel

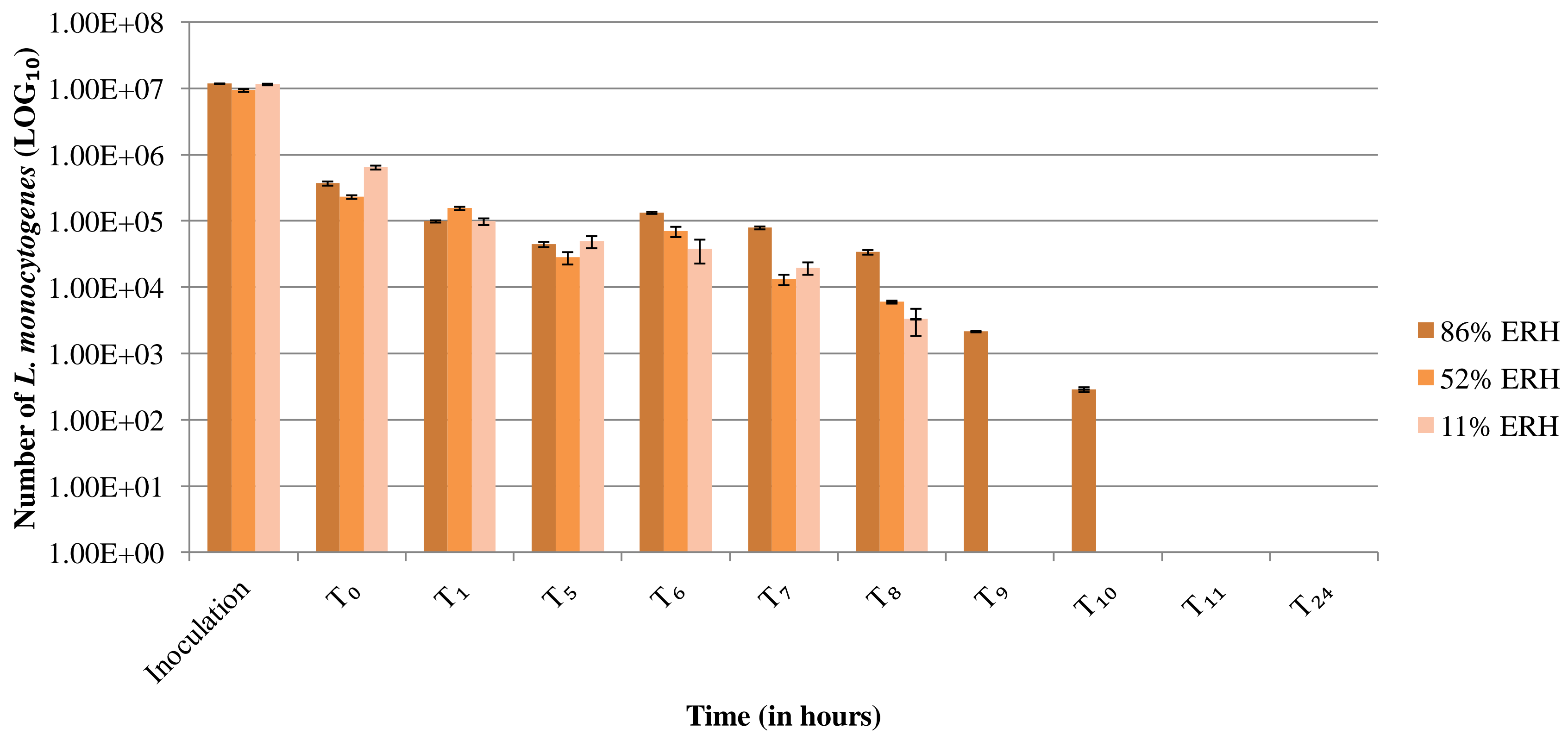


Figure 3.12. L. monocytogenes cells recovered after 0, 1, 5, 6, 7, 8, 9, 10, 11 and 24 hours of incubation in different equilibrium relative humidities (86%ERH, 52%ERH and 11%ERH) at 20ºC on stainless steel

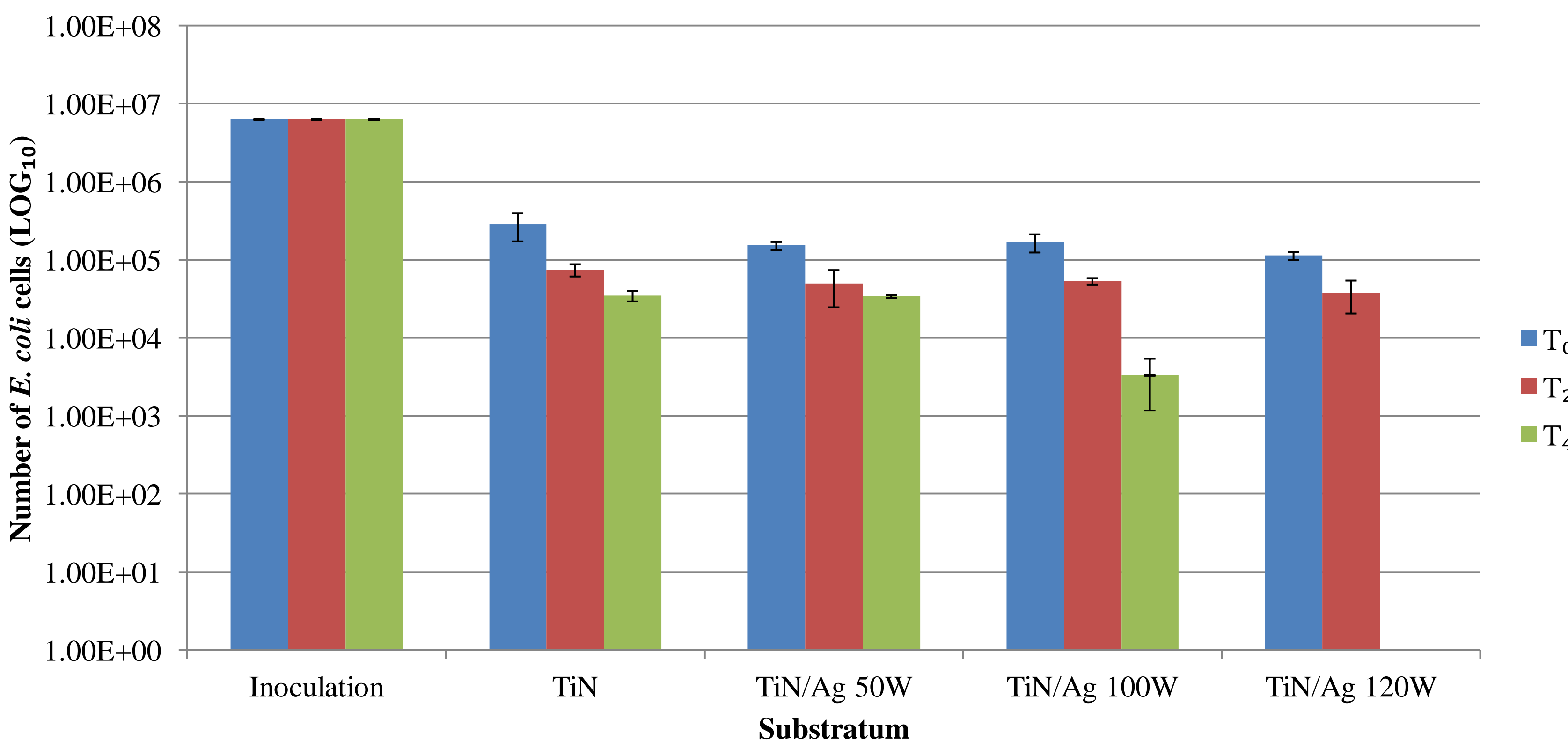


Figure 3.13. E. coli cells recovered after 0, 2 and 4 hours of incubation at 20ºC, 86% ERH, on coated surfaces

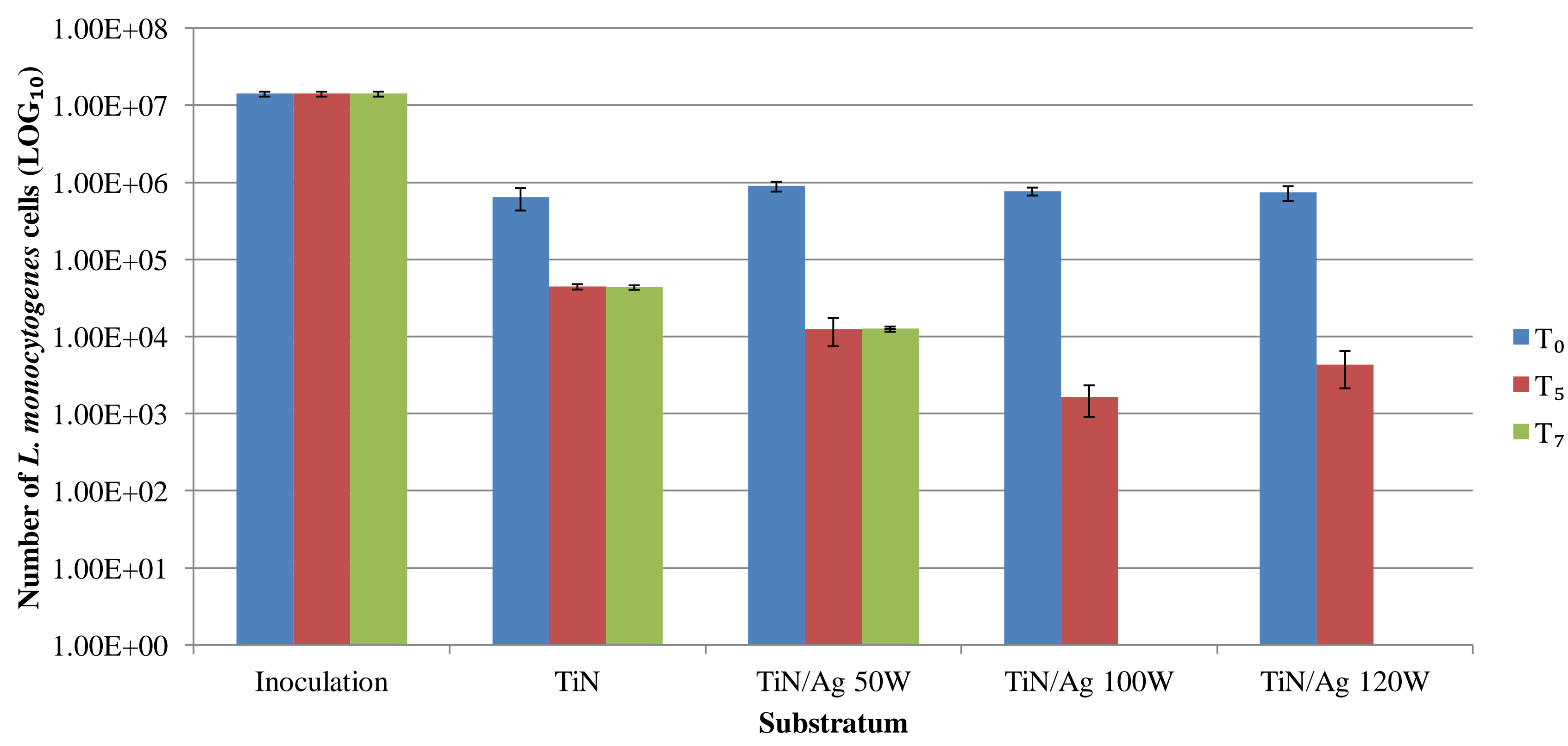


Figure 3.14. L. monocytogenes cells recovered after 0, 5 and 7 hours of incubation at 20ºC, 86% ERH, on coated surfaces

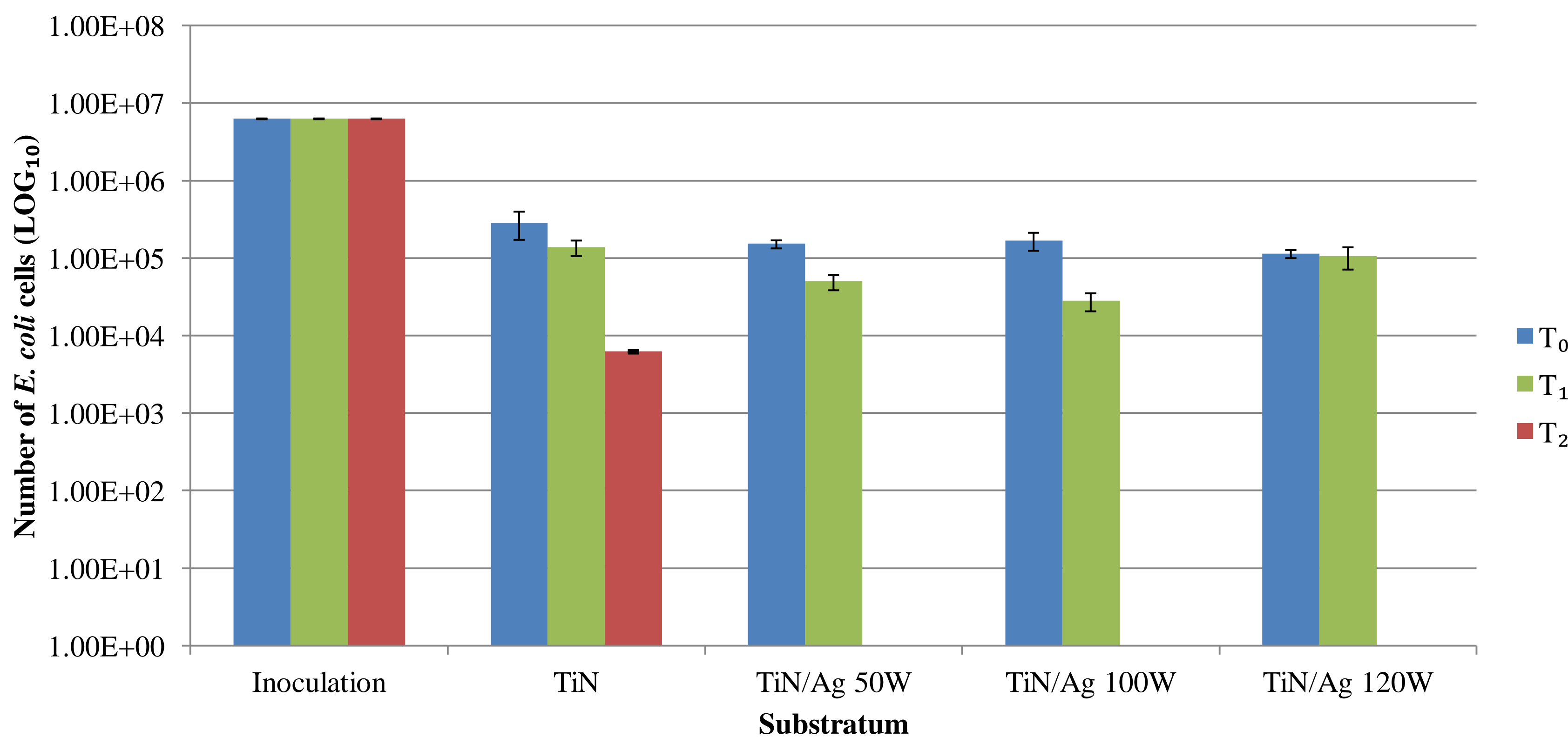


Figure 3.15. E. coli cells recovered after 0, 1 and 2 hours of incubation at 20ºC, 52% ERH, on coated surfaces

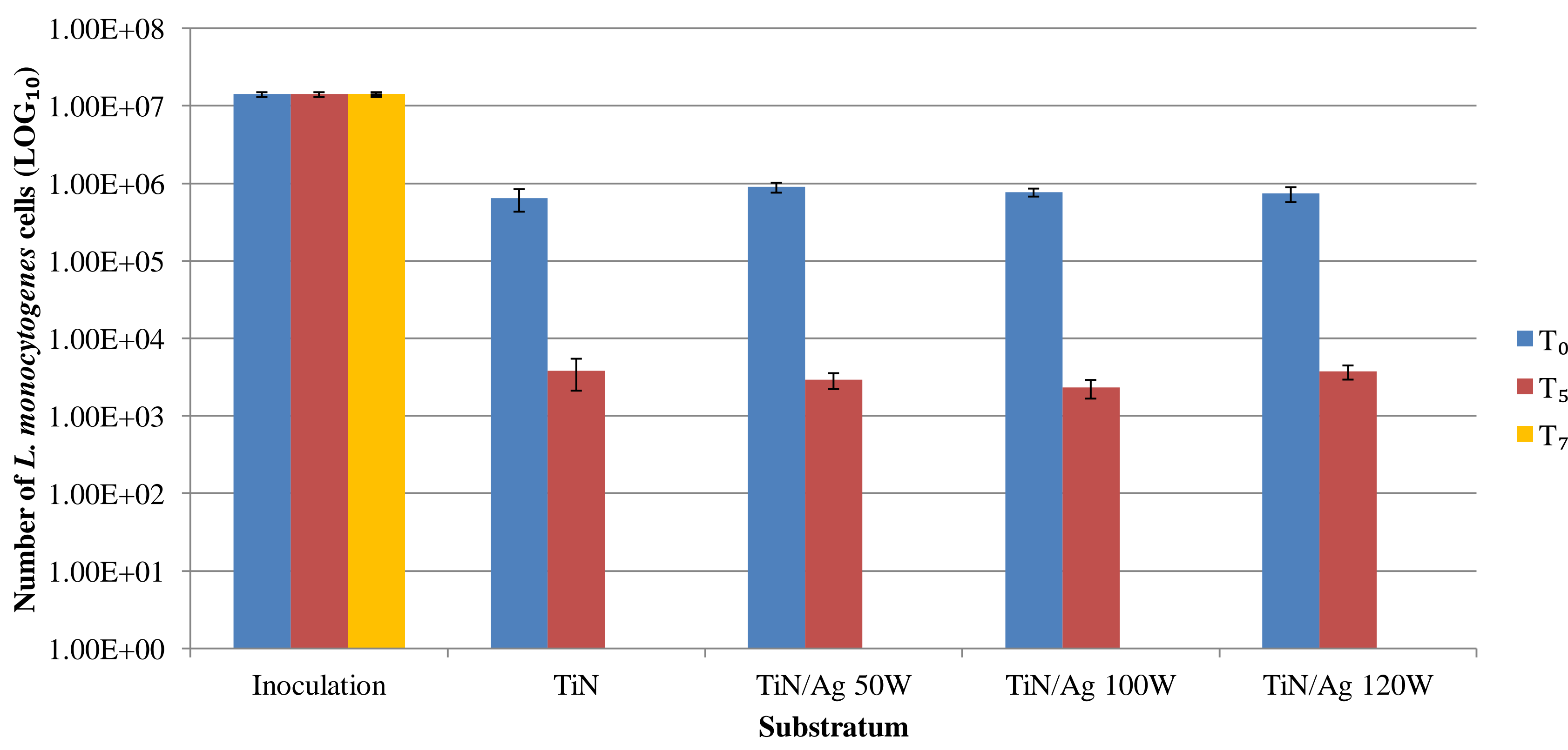


Figure 3.16. L. monocytogenes cells recovered after 0, 5 and 7 hours of incubation at 20ºC, 52% ERH, on coated surfaces

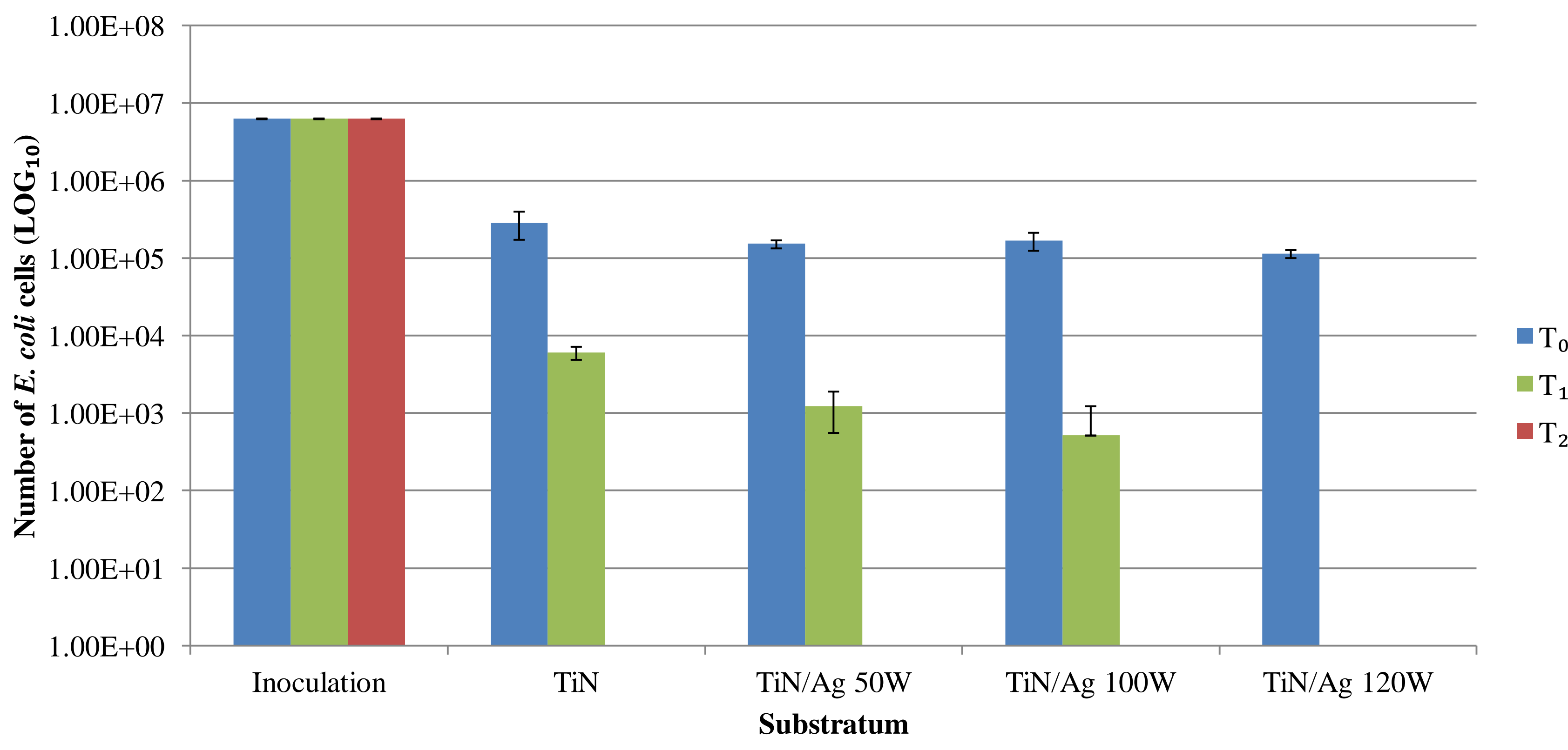


Figure 3.17. E. coli cells recovered after 0, 1 and 2 hours of incubation at 20ºC, 11% ERH, on coated surfaces

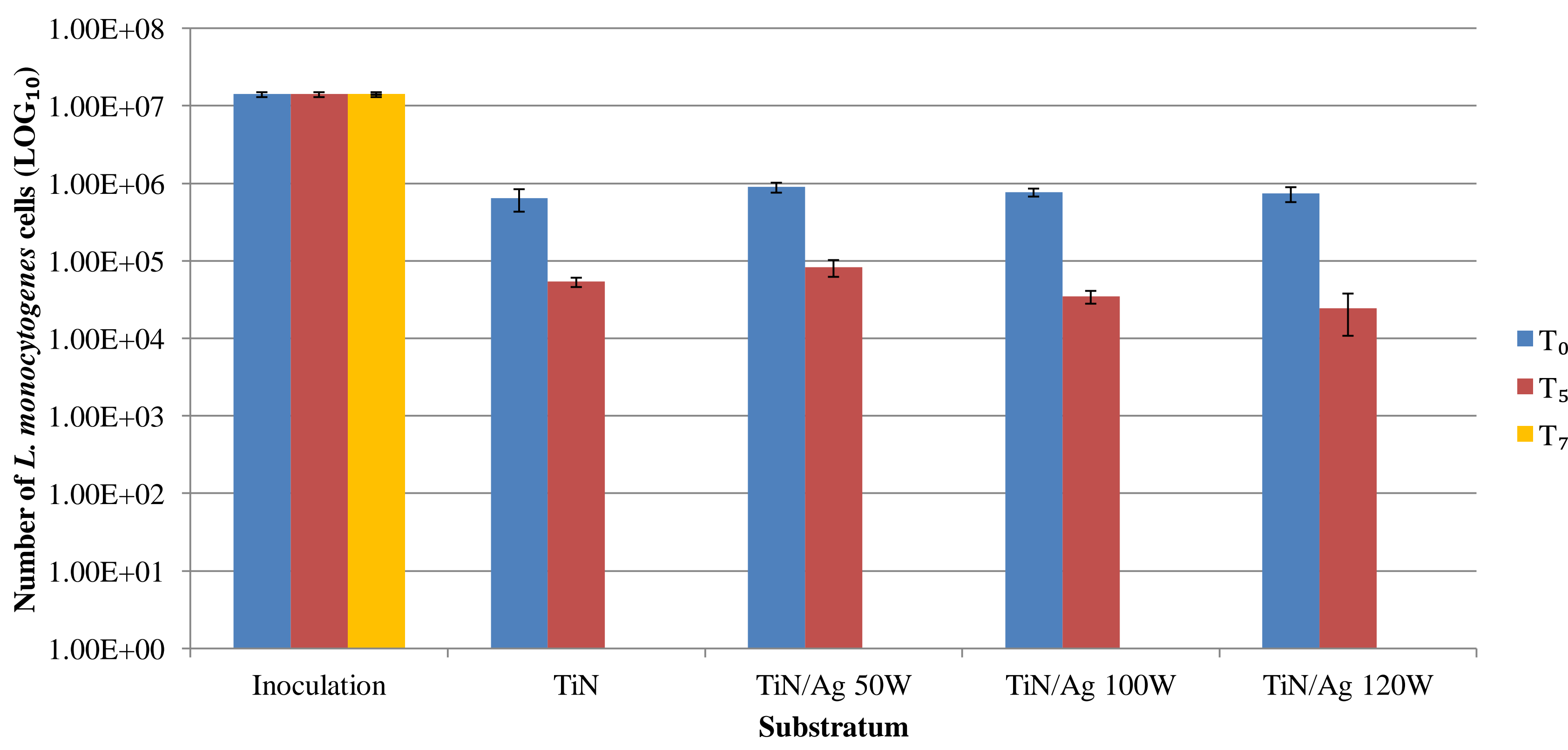


Figure 3.18. L. monocytogenes cells recovered after 0, 5 and 7 hours of incubation at 20ºC, 11% ERH, on coated surfaces

3.4.4.2. Effectiveness of swabbing

After each surface was swabbed, they were stained with acridine orange and examined under epifluorescence microscopy to monitor the efficiency of swabbing on the used surfaces because *Figure 3.19.* illustrates examples of images taken at random from all the surfaces in ten different areas for both microorganisms. Some debris may, at times, be observed but microorganisms were visually absent.

There was a reduction in recovery of viable cells compared to the inoculum, of approximately 3 Log_{10} during the equilibrium relative humidity assay. It may be suggested that a constant number of cells were retained on the swab since no drying period was allowed at time zero and no cells were observed on the surfaces post-swabbing (Salmela *et al*, 2013). Some studies have noted that swabbing may have variable efficiencies depending on the surfaces and microorganisms with or without the presence of inorganic compounds (Rose *et al*, 2004; Valentine *et al*, 2008; Verran *et al*, 2010). However, in this study, reproducibility was high and variation low, despite the 3 Log_{10} drop.

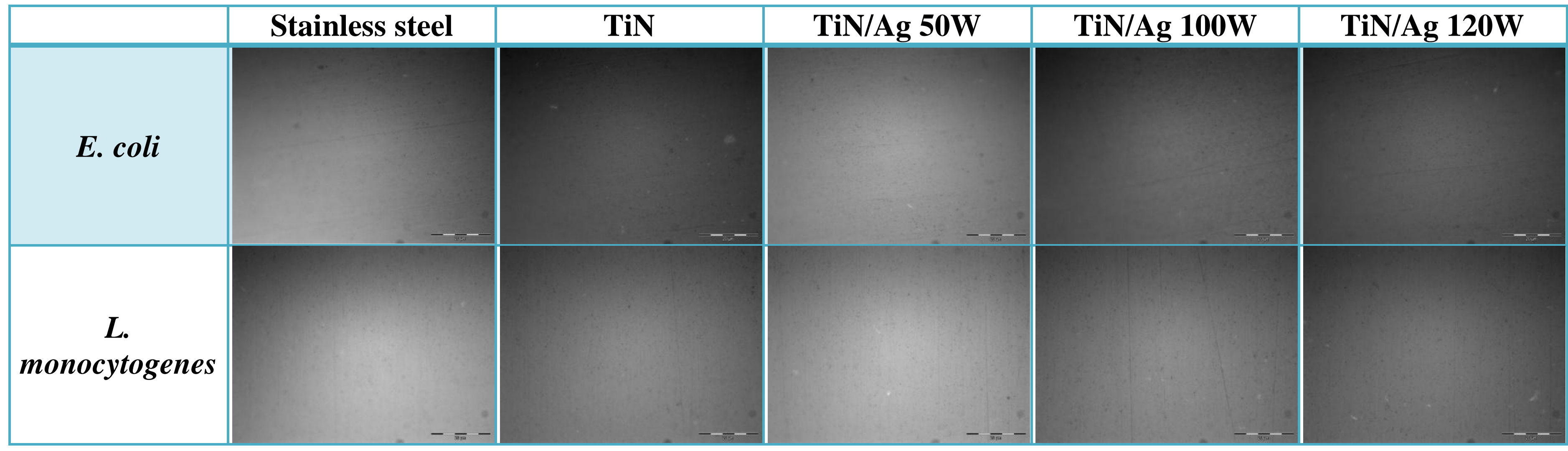


Figure 3.19. Epifluorescence microscopy images of *E. coli* and *L. monocytogenes* taken randomly across surfaces after swabbing to monitor the efficiency of cells removal (magnification x 1000, scale bar 20µm)

3.4.5. Viability and retention of *Escherichia coli* and *Listeria monocytogenes* on surfaces

3.4.5.1. Effect of substrata and re-use on cell retention

The cell retention assays aimed to quantify the number of cells retained by the surfaces after a rinsing procedure and also to monitor the effect of the re-use of the surfaces on the retention. When re-using the surfaces (*Section 3.3.2.1.*), features related to wear were previously observed on occasion which may affect the cell retention and a visual assessment of the used surfaces recorded later in this study. In the swabbing assay, the same number of cells was applied to the surfaces, whereas in the retention assay, active cell adhesion and retention was monitored; therefore, different results were expected. Differences in substratum topography and hydrophobicity might affect retention over and above any differences anticipated due to antimicrobial properties. Thus, all the substrata were tested against *E. coli* and *L. monocytogenes*.

There was no significant difference between retention of cell and re-use of the surfaces (*Figure 3.21. & 3.22.*). Thus, it was of value to see whether the viability of retained cells was affected by the chemical properties of the surfaces (silver) using live-dead stain, since any changes in the topography and wettability of the surfaces had no effect on the cell retention. *L. monocytogenes* was retained in higher numbers than *E. coli* ($P < 0.001$) on the first use of the substrata (*Figure 3.20.*). However, there was no difference in retention between all the substrata for each microorganism. Similar results were found by Verran *et al* (2010) where retention of *E. coli* was below 0.20% surface coverage on TiN coated surface and below 0.80% surface coverage on stainless steel.

Standard deviations on *Figure 3.20.* to *3.22.* increased on a few substrata as the surfaces were re-used. This is due to uneven bacterial coverage and possibly reflecting changes in underlying topography due to wear when recording results, although, the standard deviation was low and had no significant difference ($P > 0.05$).

There was no difference in cells retention on stainless steel and silver coatings, showing equal cleanability and no effect of topography. The smooth surface of all substrata (*Chapter 2*) and low levels of retention confirmed that these surfaces were hygienic in terms of ease of bacterial removal.

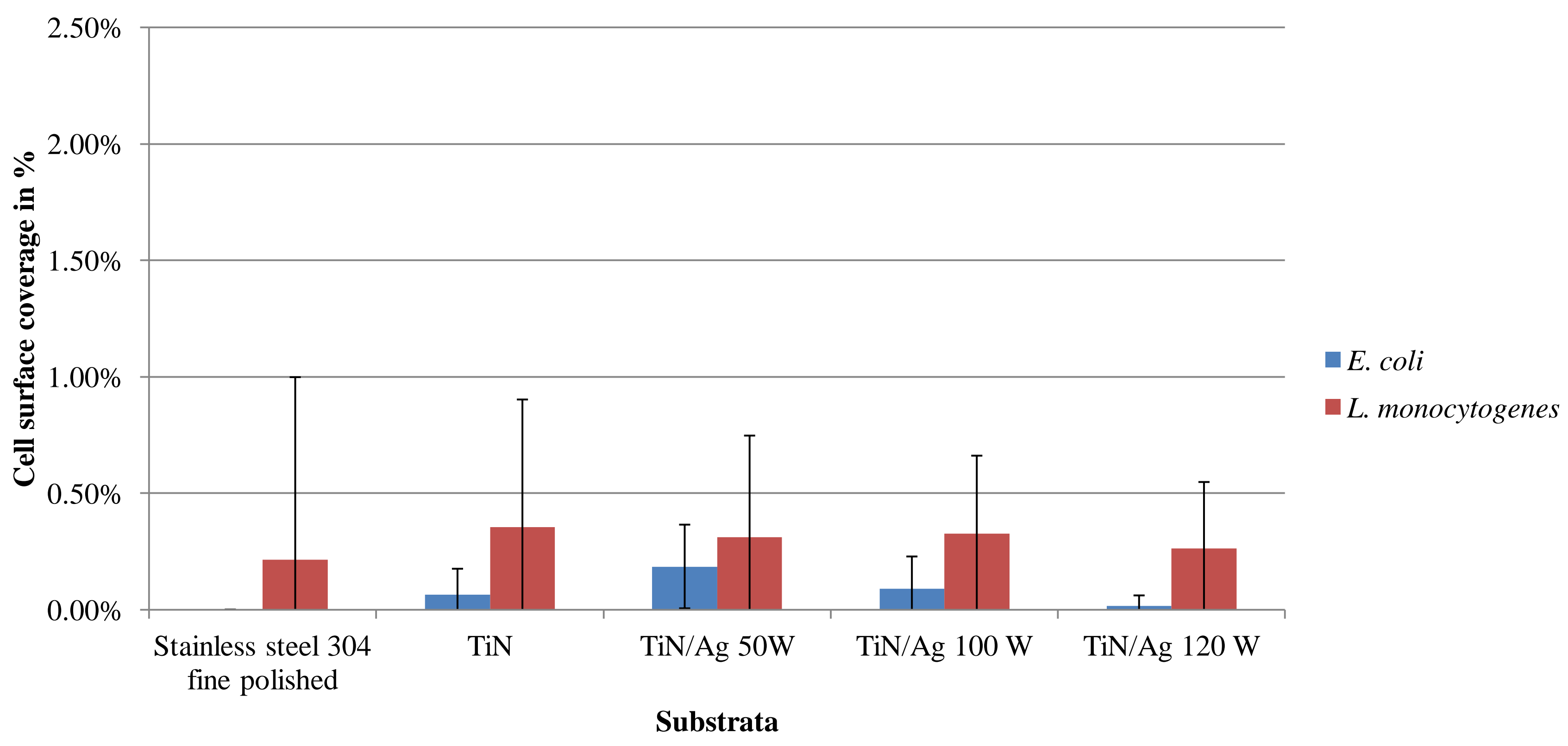


Figure 3.20. Retention of cells on test substrata 1st time use with results below 0.50% surface coverage

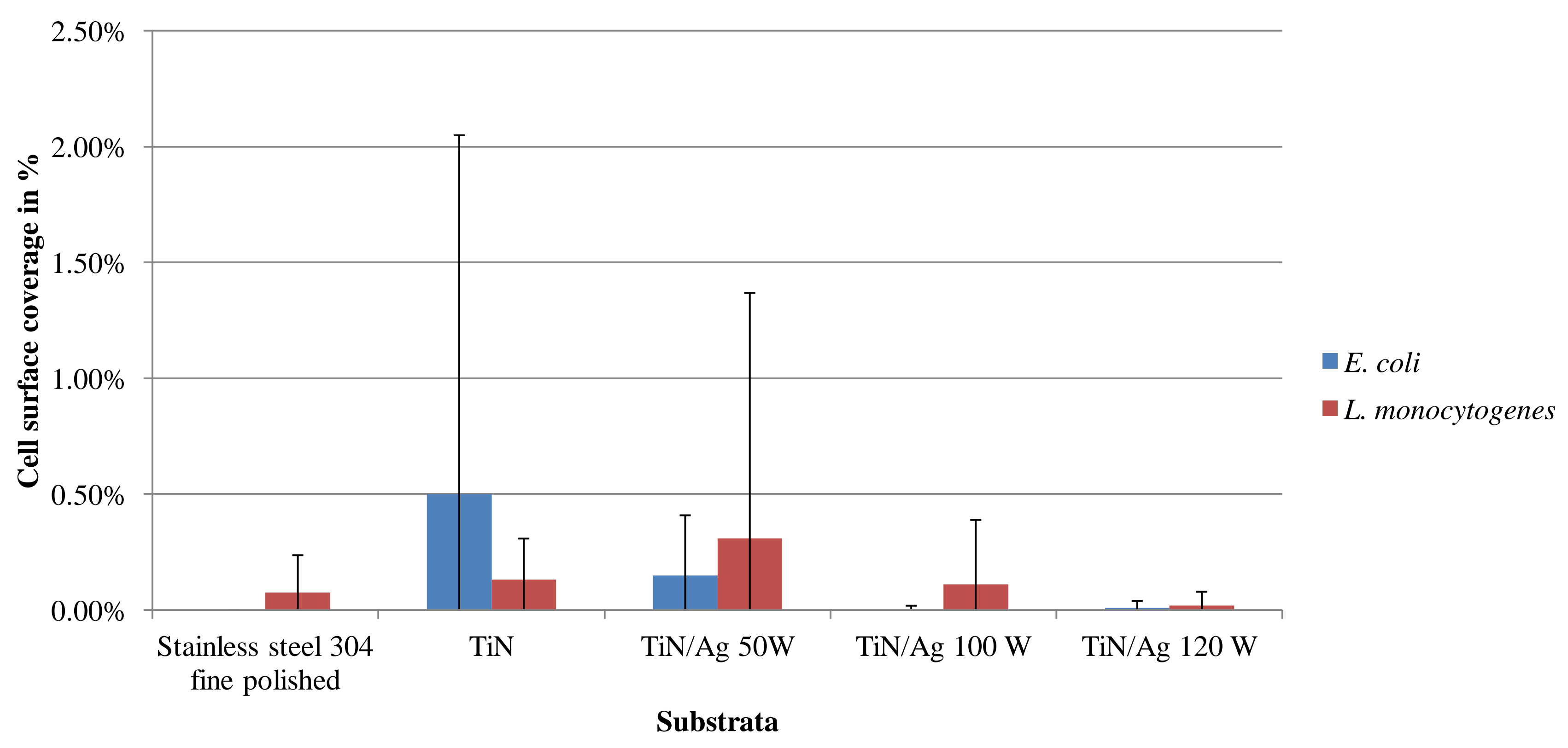


Figure 3.21. Retention of cells on test substrata 2nd use with results below 0.50% surface coverage but higher standard deviations

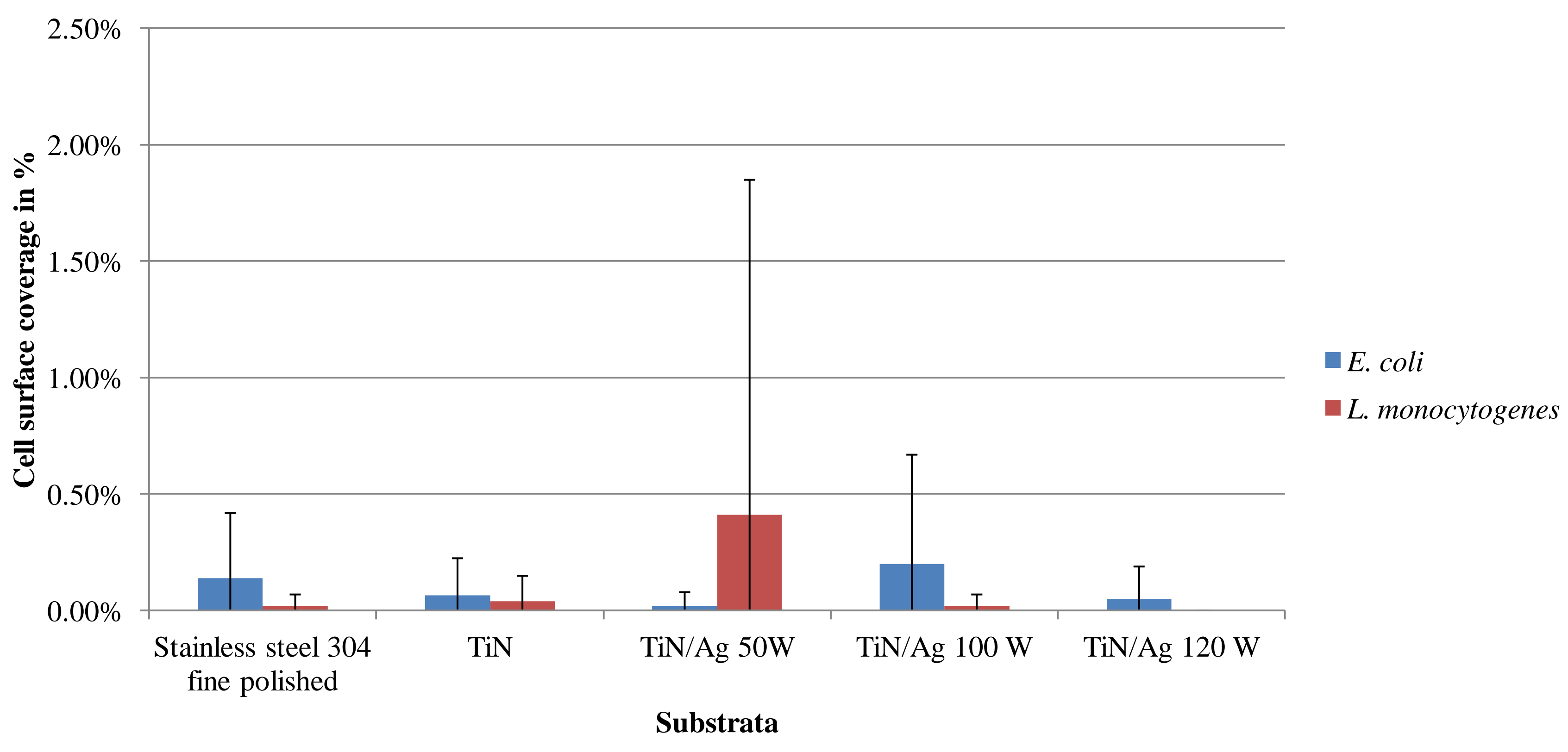


Figure 3.22. Retention of cells on substrata 3rd use with results below 0.50% surface coverage and few substrata for which standard deviations increase.

3.4.5.2. Viability of bacteria directly on surfaces and effect of re-use

Previously, the viability of bacteria was monitored via culture of cells removed by swabbing (*Section 3.4.4.1.*). This method assessed the potential number of viable bacteria which may be removed/transferred by contact with food products. However, it is important to also assess the viability of cells directly on the surfaces in order to monitor any direct effects of silver from the coated surfaces on the survival of both bacteria, since it appears that silver ions are not released. From the surface, LIVE-DEAD differentiate staining was used to monitor viable (green) and damaged cells (red). Although dead cells do not pose a biotransfer potential, if retained they could provide a substratum for subsequent adhesion of viable cells.

L. monocytogenes retained viability on surface for longer than *E. coli*. An increase in numbers of damaged (red) cells was observed over time and with increasing concentration of silver (*Figure 3.23.*). These visual observations were confirmed by cell counts (*Figure 3.24.* & *3.25.*). Approximately 75% of the cells remained viable after one hour on stainless steel. *E. coli* cells stained red on all coated surface (*Figure 3.24.*). However, this does not correlate with the results noted previously (*Figure 7*) where growth of *E. coli* colonies was recorded on TiN during the metabolic assay. This may be due to potential low number of individual viable cells which were not directly detected with live/dead on the surface, or this may have been a malfunction of the dyes on TiN for *E. coli*. Whitehead *et al* (2010) describes the importance of standardising dye concentration, cells and surfaces which means that further investigation is needed regarding the live-dead staining of *E. coli* on TiN. On stainless steel, after 2 hours incubation, there was a significant decrease of *E. coli* cells ($P < 0.05$) on stainless steel

with about 60% remaining alive (*Figure 3.25.*) and one might anticipate an enhanced effect with silver as indicated in previous assays. For *L. monocytogenes*, survival decreased most between 1 hour and 7 hours (P < 0.01). The number of cells remaining viable was still high after 7 hours of incubation when compared to *E. coli* after only 2 hours. In this assay, *L. monocytogenes* survived better than *E. coli* but its survival decreased with increased concentration of silver. However, it is not possible to comment on the survival of *E. coli* on TiN in this assay as in a previous assay (*Section 3.4.4.1.*) *E. coli* colonies recovered from TiN were viable. The antimicrobial properties of silver were evident, apparently proving a contact kill.

When re-using the substrata, similar results were recorded after one hour of incubation (*Figure 3.26.* & *3.28.*). *L. monocytogenes* survival appeared to increase over time increase as the surfaces were re-used, with the highest change observed on TiN/Ag 100W (*Figure 3,24.* to *3.29.*). However, survival on the highest concentration of silver (TiN/Ag 120W) was not significantly affected. Again, *E. coli* cells stained red throughout the re-uses of the substrata (*Figure 3.26.* to *3.29.*). *L. monocytogenes* cells decreased in viability over time on stainless steel and the proportion of damaged cells was increased in the presence of silver.

The silver containing substrata showed some antimicrobial effect in comparison with stainless steel when assessed on direct contact of cells with the substratum. *L. monocytogenes* survived better than *E. coli,* which did not survive on any of the coated surfaces (TiN coating results not included). The number of undamaged *L. monocytogenes* cells present on the surfaces decreased as the concentration of silver increased. Viable cells may survive and multiply, given appropriate conditions

(Chmielewski and Frank, 2003; Verran et al, 2008), but these antimicrobial surfaces would retard this colonisation by reducing the number of viable cells attached.

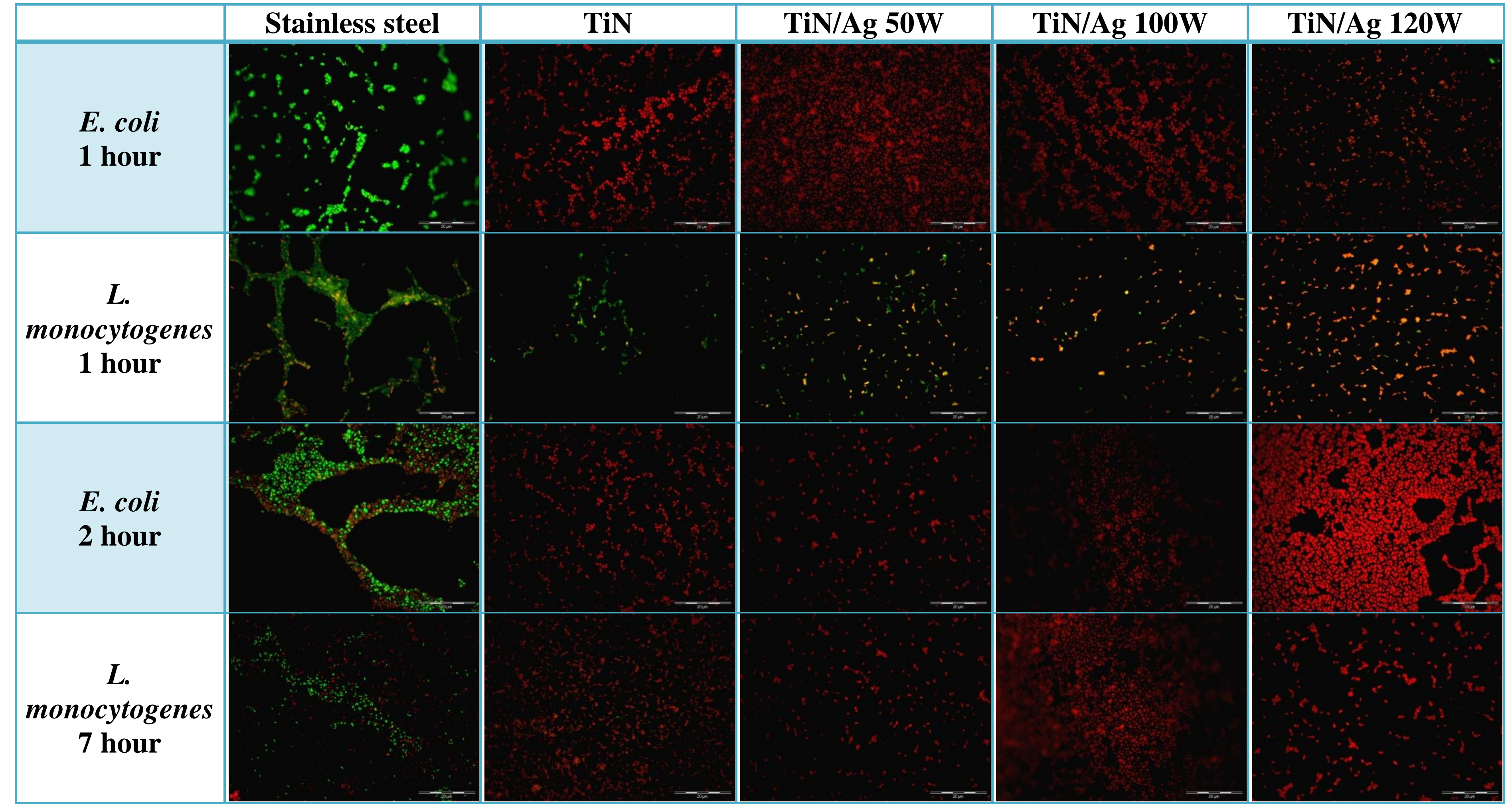


Figure 3.23. Live/dead epifluorescence microscopy images of *E. coli* and *L. monocytogenes* cells - first use of the surface

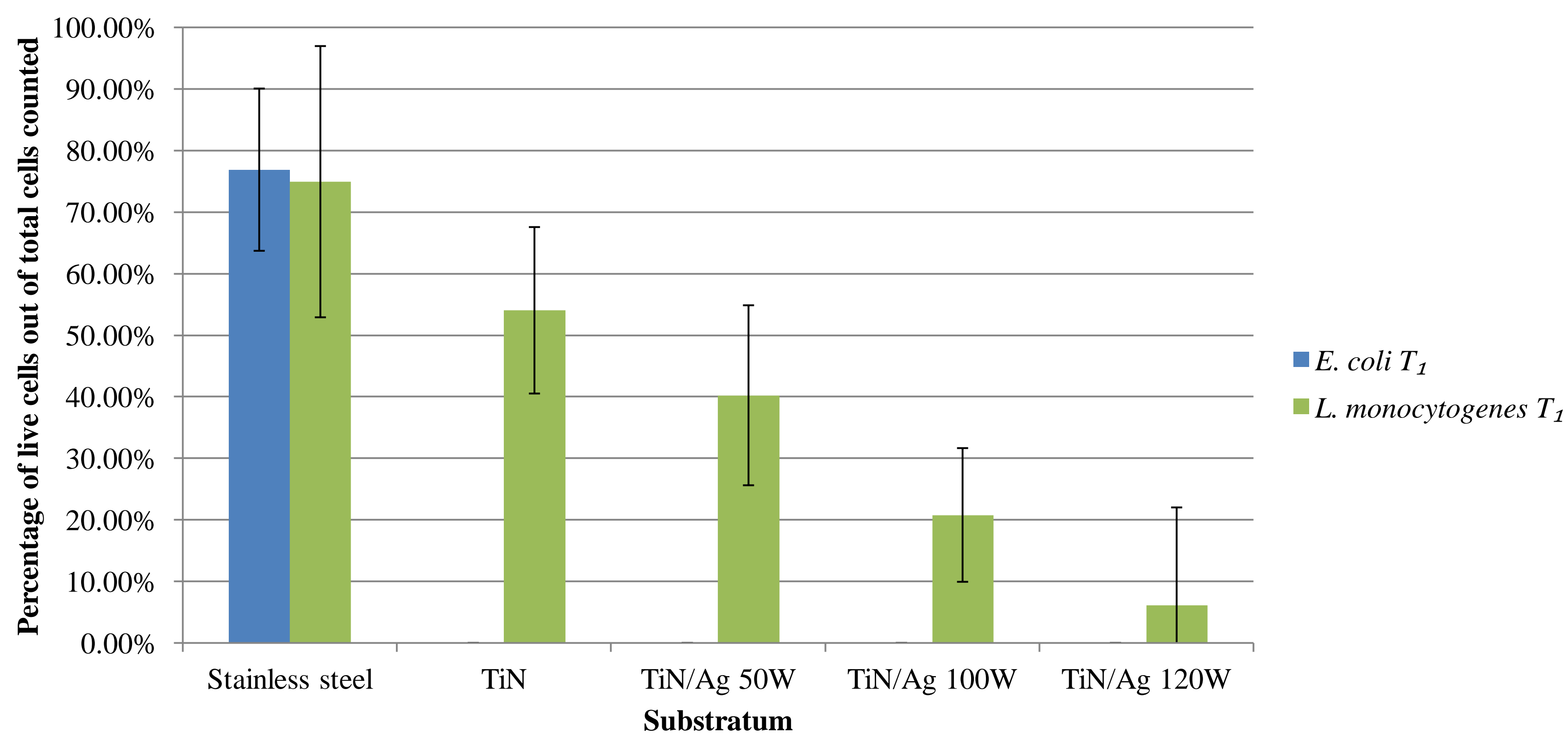


Figure 3.24. Viability of cells after for 1 hour of drying for *E. coli* and *L. monocytogenes* – 1st use of substrata

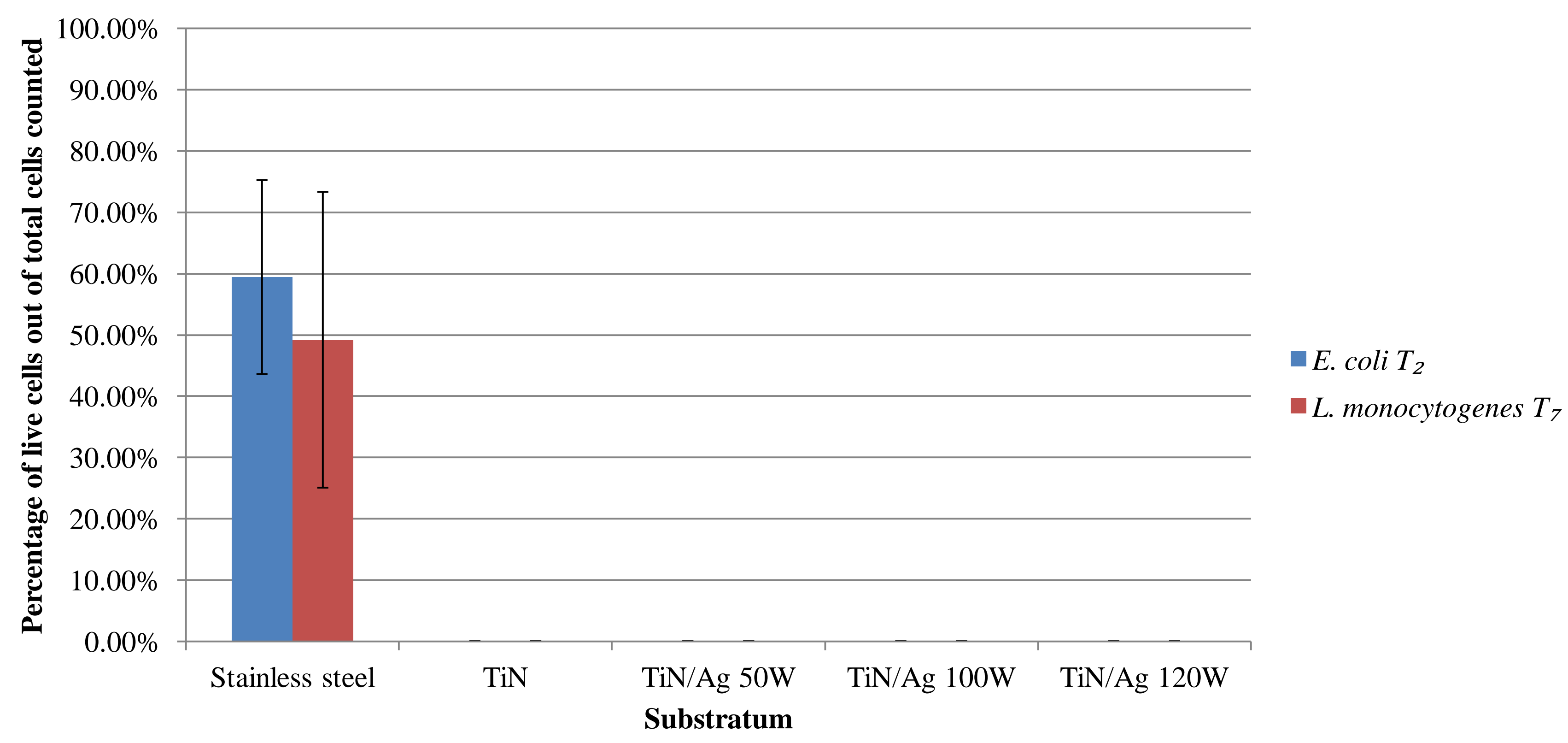


Figure 3.25. Viability of cells after for 2 hour of drying for *E. coli* and 7 hours for *L. monocytogenes* – 1st use of substrata

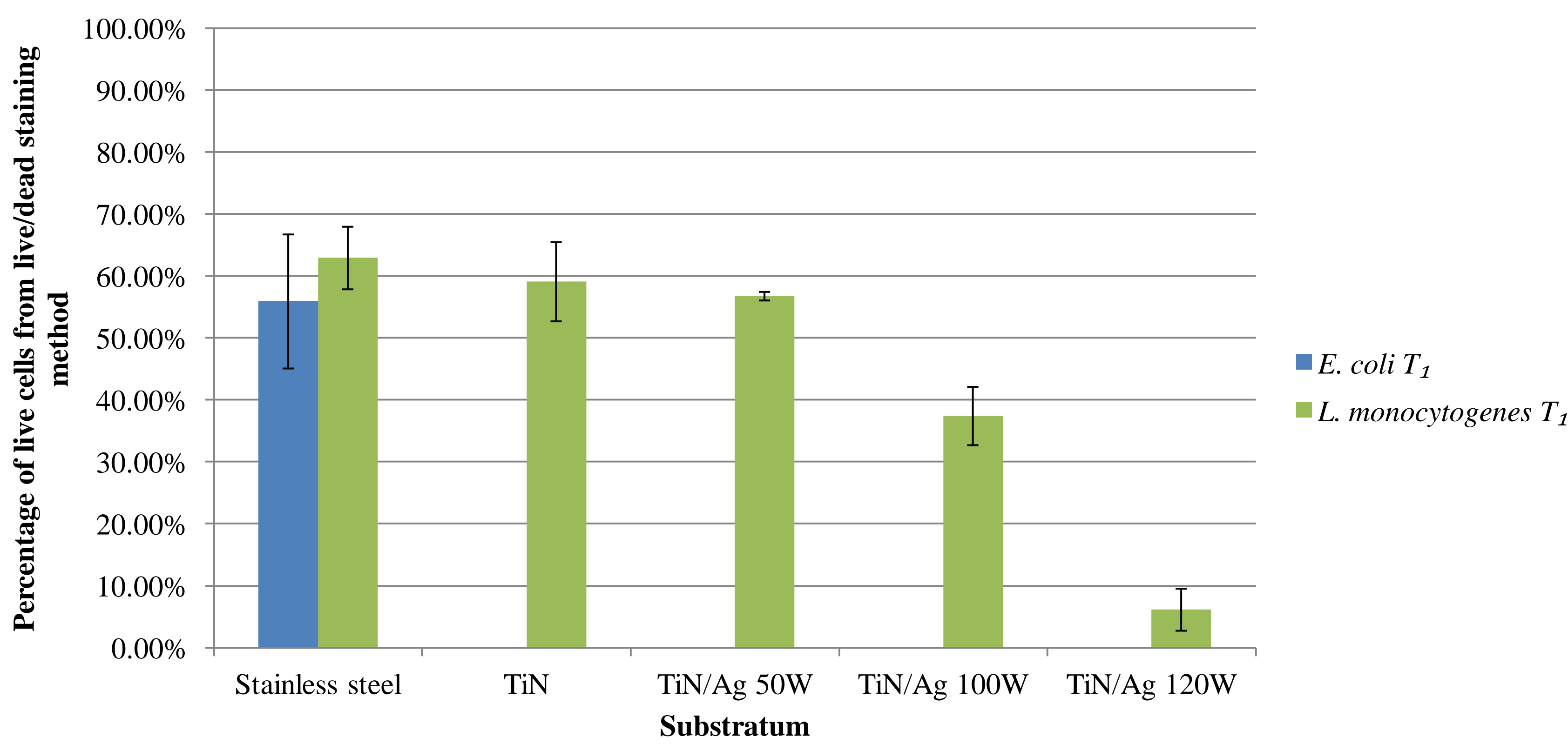


Figure 3.26. Viability of cells after for 1 hour of drying for *E. coli* and *L. monocytogenes* – 2nd use of substrata

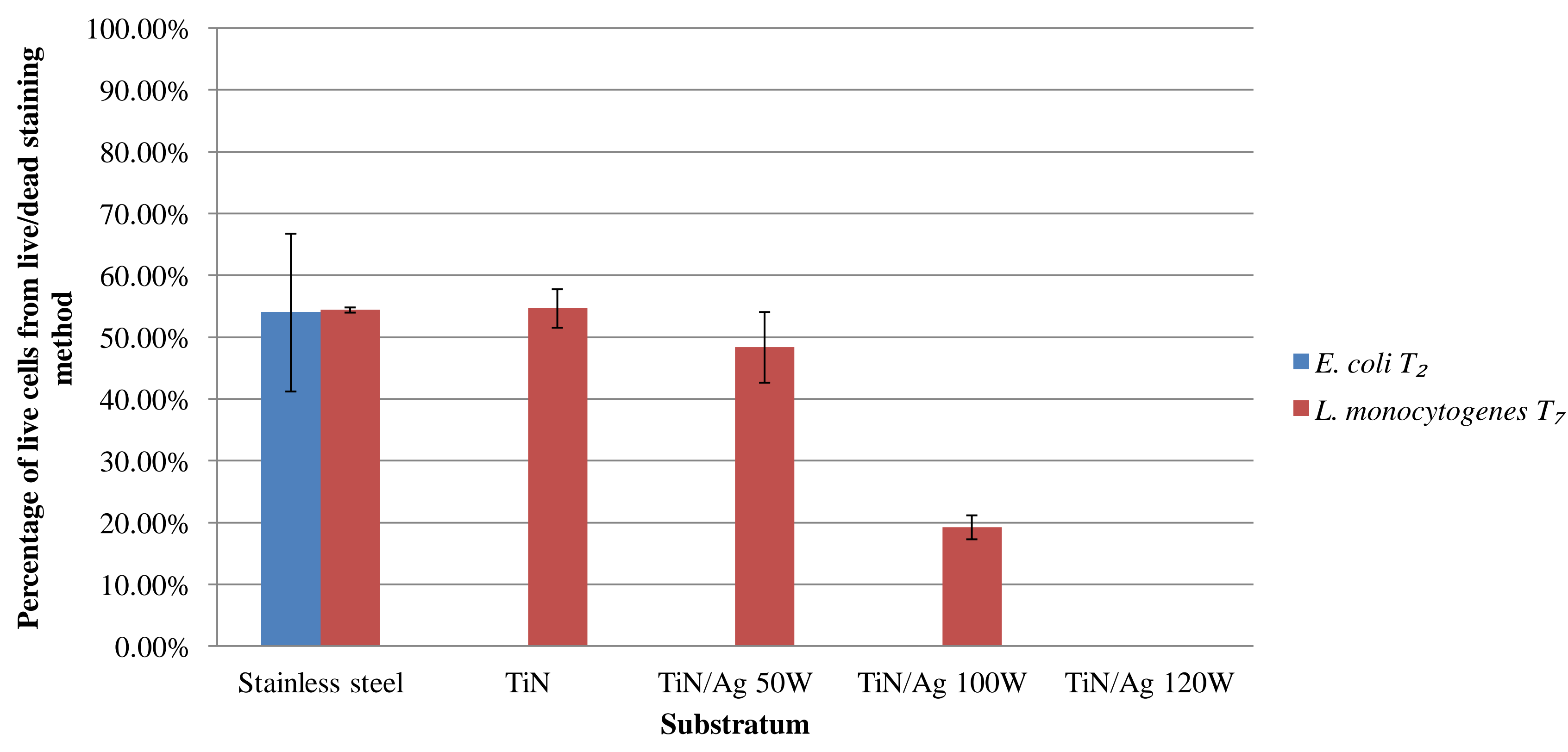


Figure 3.27. Viability of cells after for 2 hour of drying for *E. coli* and 7 hours for *L. monocytogenes* – 2nd use of substrata

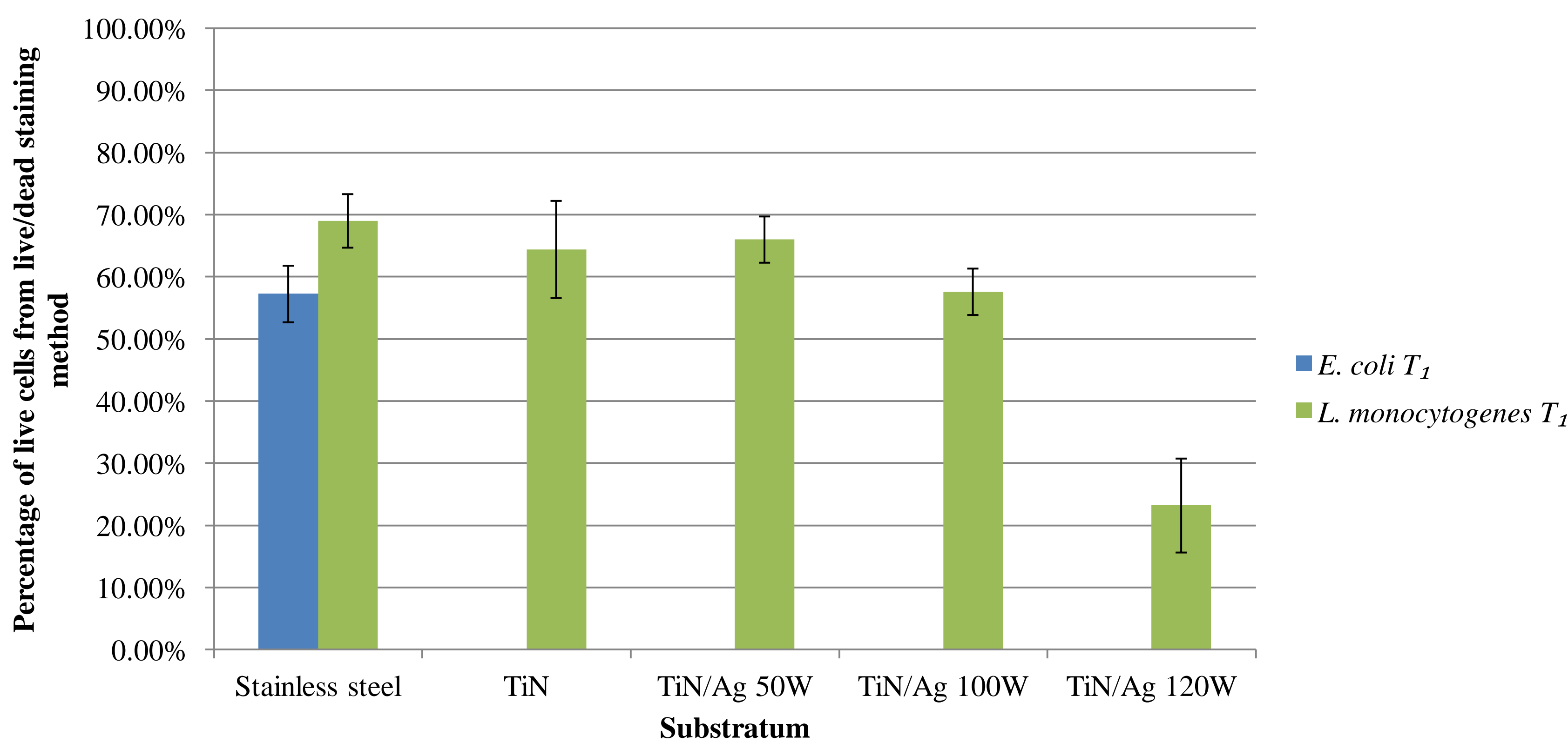


Figure 3.28. Viability of cells after for 1 hour of drying for *E. coli* and *L. monocytogenes* – 3rd use of substrata

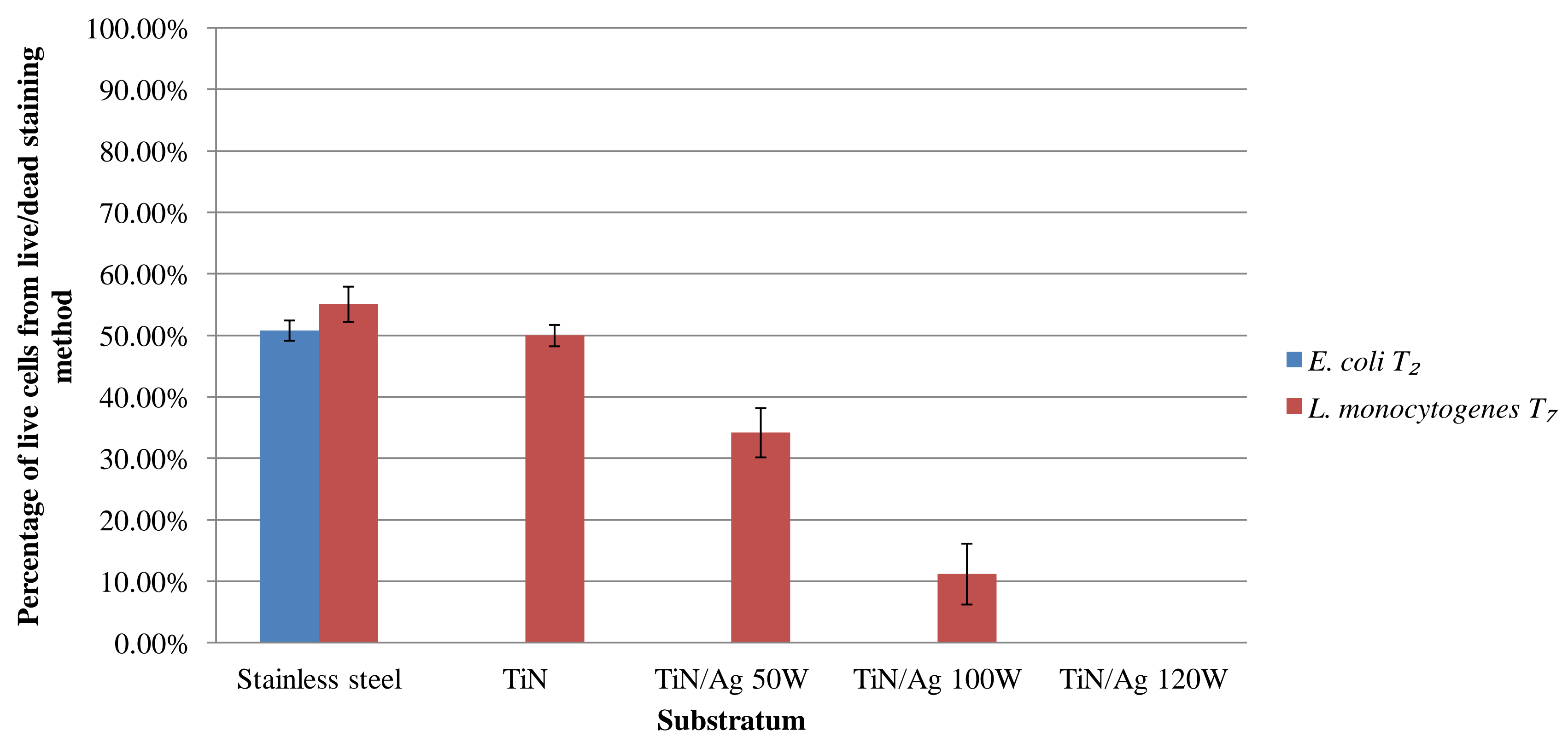


Figure 3.29. Viability of cells after for 2 hour of drying for *E. coli* and 7 hours for *L. monocytogenes* – 3rd use of substrata

3.4.6. Survival of *E. coli* in the presence of food soil on surfaces and re-use

Previous work with meat and *E. coli* was done (Whitehead *et al*, 2010) which led to the same selection of microorganism and food soil to investigate the effect of meat soil on *E. coli* survival on test surface

3.4.6.1. Swabbing assay

The initial recovery of the cells at T_0 from the inoculation was similar to those recovered without the presence of meat extract (*Section 3.4.4.1.*). The survival of *E. coli* on stainless steel was increased from 4 hours to at least 16 hours by the presence of meat soil (see *Figure 3.11*). The soil was providing a protective effect but it appears some antimicrobial silver ions diffused through the soil. Viability decreased as silver concentration increased, on silver coatings. The meat soil was source of nutrient and had a protective effect which was confirmed by the increase of survival of *E. coli* from 2 hours without meat soil to 16 hours with meat soil.

When re-using the surfaces, there was no survival on TiN/Ag 120W after 16 hours (*Figure 3.30.* to *3.32.*). There was a significant difference between survival in TiN and TiN/Ag 50W, and TiN/Ag 50W and TiN/Ag 100W ($P < 0.01$) for all of the re-uses, thus showing a significant decrease of the cell viability as silver concentration increased. There was no significant difference in the number of recovered cells between each re-use on all the surfaces. Generally, there was a decrease of survival over time with apparent trend to increase kill with increase concentration of silver in the coatings.

The presence of meat extracted proved to increase the survival of *E. coli* on all test surfaces. In food factories, regular cleaning procedures are important for the maintenance of a good hygiene (Wirtanen, 1995). Cleaning and re-using of the surfaces may modify the surface of the substrata (Akamatsu *et al*, 1991; Singh *et al*, 1997). The wear of the surfaces may affect the retention of cell and soils, thus potentially enhancing the survival of bacteria (Verran *et al*, 2010). On silver coating surfaces, the presence of soil and re-use of the surfaces did not enhance survival. Indeed, no viable cells were recovered after 16 hours at the highest concentration of silver, thus the antimicrobial effect of silver was also retained.

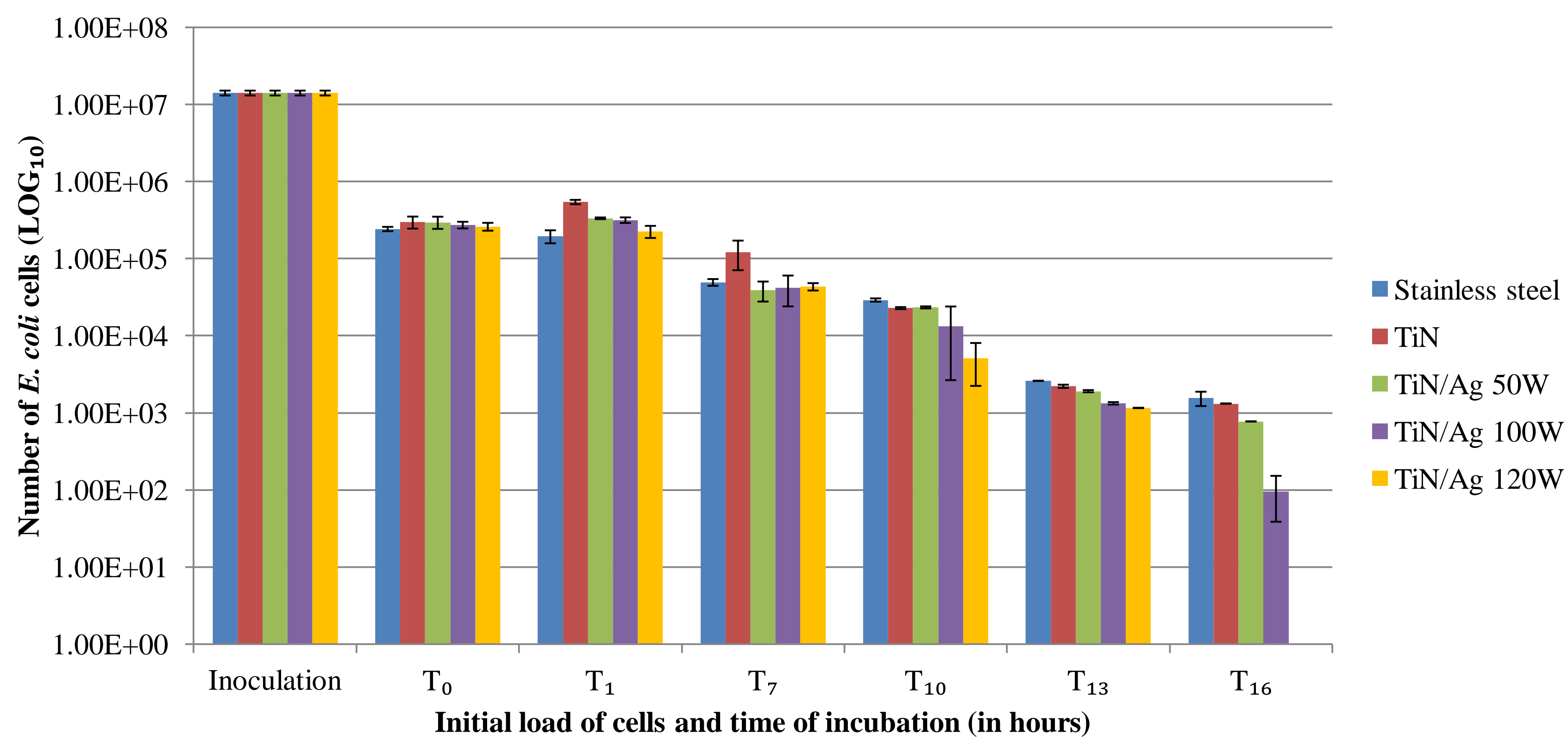


Figure 3.30. E. coli cells recovered after 0, 1, 7, 10, 13 and 16 hours of incubation with meat extract (50:50) at 20ºC, 52% ERH – 1st use of substrata

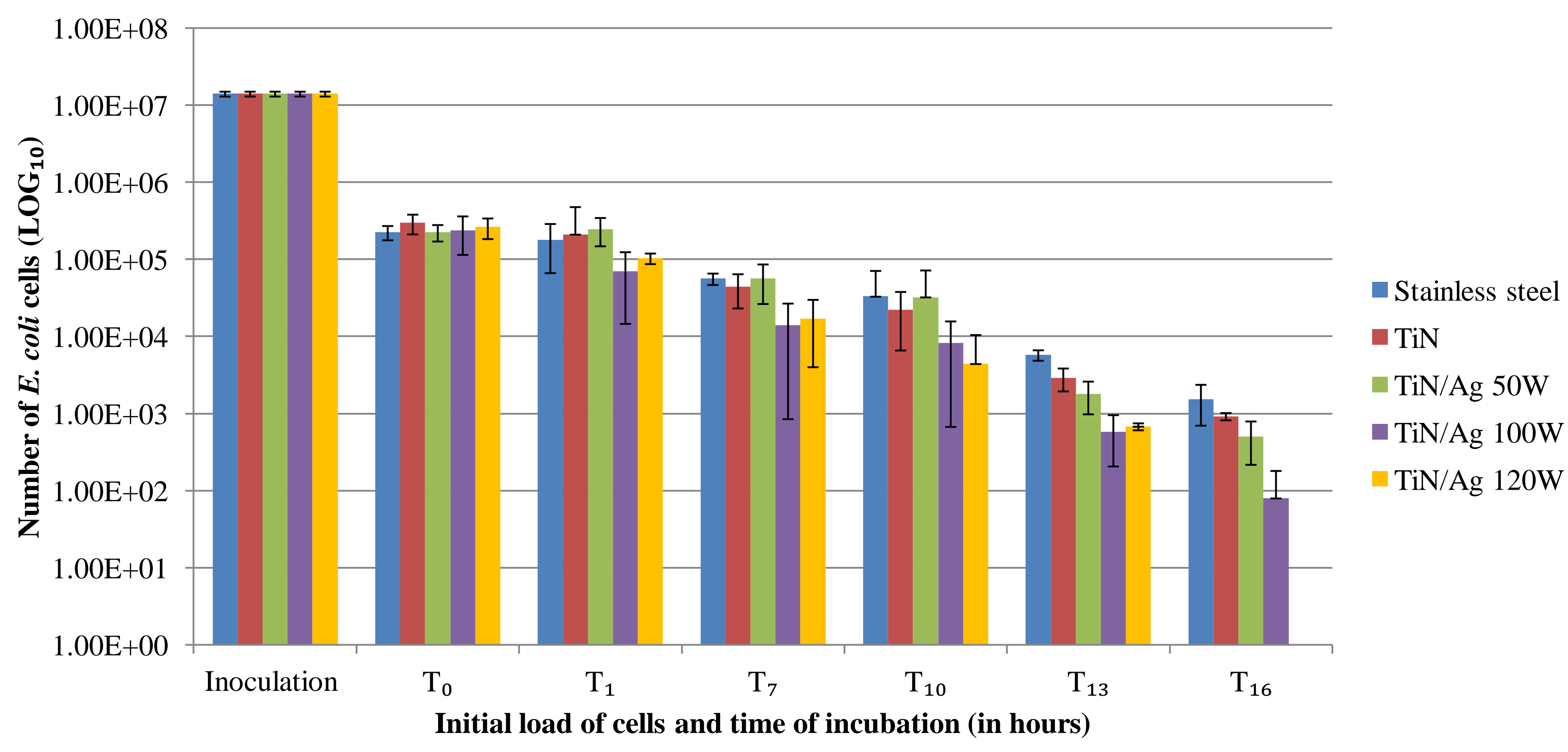


Figure 3.31. E. coli cells recovered after 0, 1, 7, 10, 13 and 16 hours of incubation with meat extract (50:50) at 20ºC, 52% ERH – 2nd use of substrata

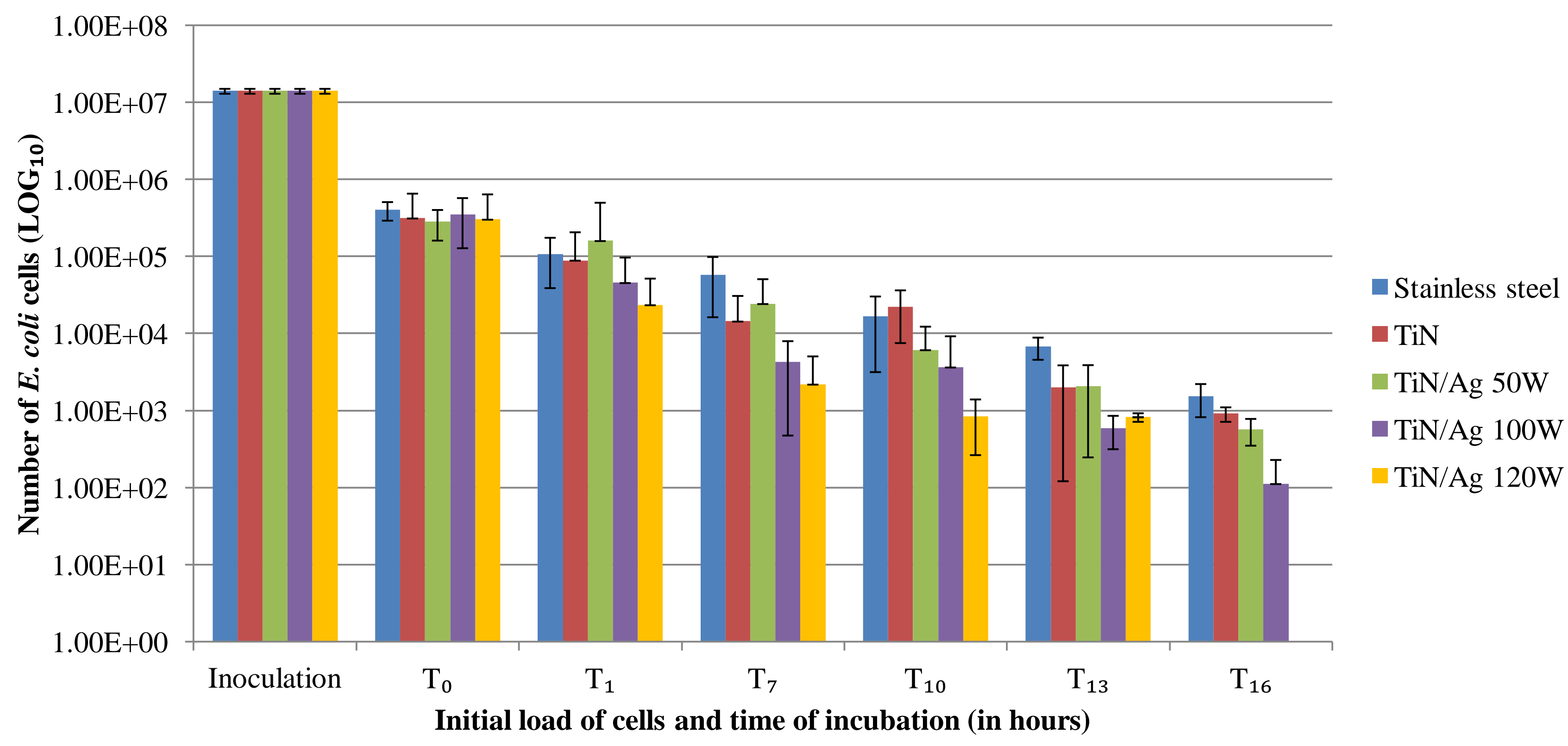


Figure 3.32. *E. coli* cells recovered after 0, 1, 7, 10, 13 and 16 hours of incubation with meat extract (50:50) at 20ºC, 52% ERH – 3rd use of substrata

3.4.6.2. Effectiveness of bacterial removal with meat extract

The presence of meat soil may enhance retention of cells on surfaces. However, as previously described (*Section 3.4.4.2.*), acridine orange was used to detect any remaining cells on all substrata after the use of *E. coli* with meat soil. No bacteria were observed under epifluorescence microscopy on all test surfaces and re-uses. Thus, swabbing was a consistent method for removing cells which was not affected by the presence of meat extract in this particular study.

3.4.6.3. Retention of *E. coli* in the presence of food soil on surfaces

The retention was very low (<0.2%) and there was no significant difference between all the substrata and their re-use in the retention of *E. coli* cells in the presence of meat extract (*Figure 3.33.*). Furthermore, there was no significant difference in the retention of *E. coli* cells with or without the presence of meat extract (*Figure 3.20.* to *3.22.*).

Further work could include a characterisation of the hydrophobicity and topography status of the surfaces after each re-use with more use, perhaps *in situ*, and with conditioning film. However, the surfaces were examined with reflected light microscopy in the following section (*Section 3.4.7.*).

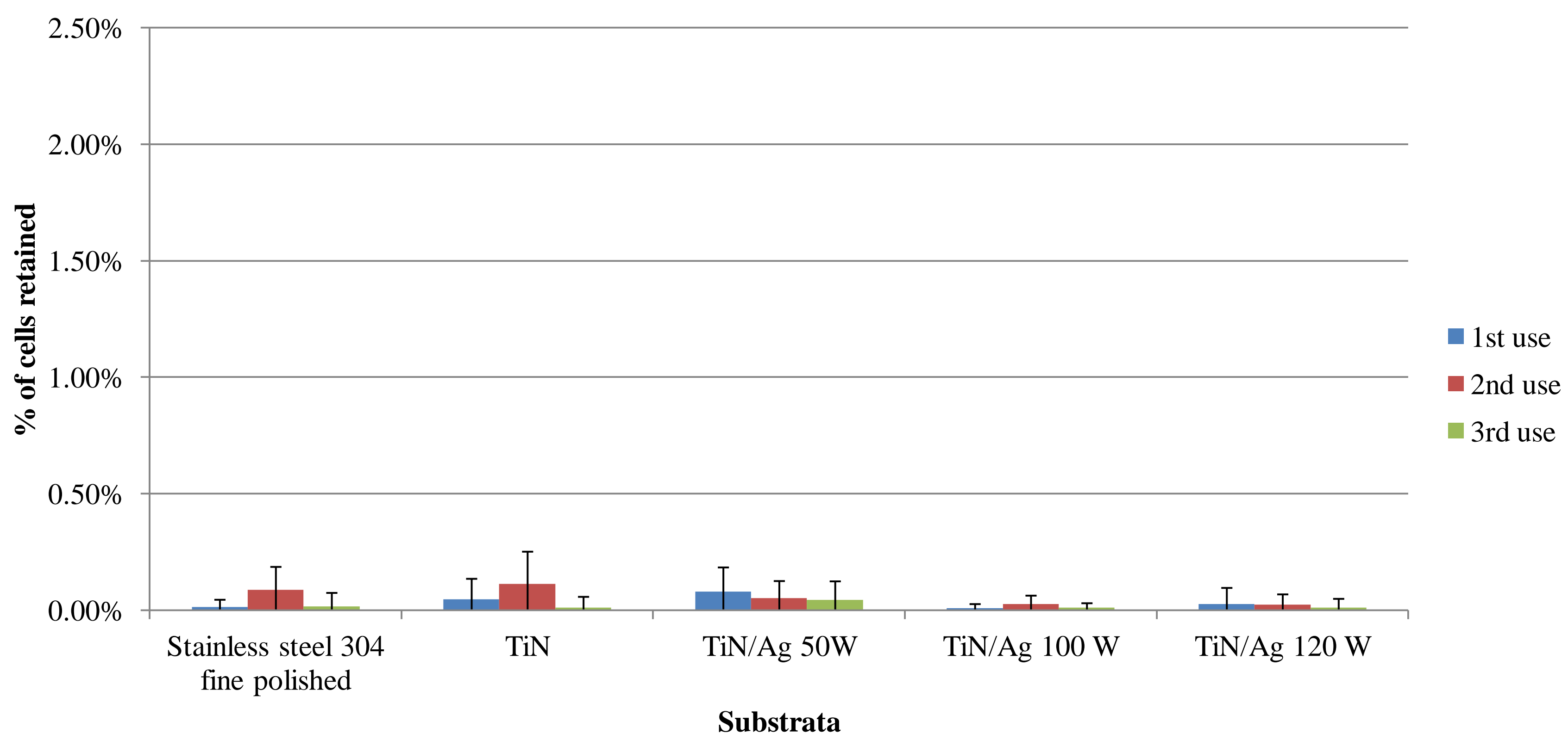


Figure 3.33. Retention of *E. coli* cells in the presence of conditioning film on substrata

3.4.7. Detection of viable and non-viable *E. coli* cells in the presence of meat extract

The aim was to determine if it would be possible to develop a method that could demonstrate the presence of undamaged and damaged *E. coli* cells within meat soil, particularly on antimicrobial surfaces. In order to understand the results, a copy of the *Table 3.3.* was added to this section.

Table 3.3. Combination of components used to investigate differential staining technique. After application of each component a drying period was employed before adding the next component as indicated in *Table 3.3.*b) enabling a range of permutations to be investigated

a)

Characters	Stains/soil/bacteria
A	*E. coli*
B	Meat extract
C	*E. coli* + Meat extract mixture (50:50)
D	FITC
E	Live/dead
F	DAPI

b)

Nº	Combinations using characters
1	A + B + D + E
2	A + B + E + D
3	C + D + E
4	C + E + D
5	B + A + D + E
6	B + A + E + D
7	A + B + E + F
8	A + B + F + E
9	C + E + F
10	C + F + E
11	B + A + E + F
12	B + A + F + E
13	B + D + A + E
14	B + F + A + E
15	C + E
16	B + A + E

A method has been successfully developed at the end of this experiment where the quantity of each component was changed from 10µL to 2µL using the most reliable and replicable combination N°14, as described at the end of these results.

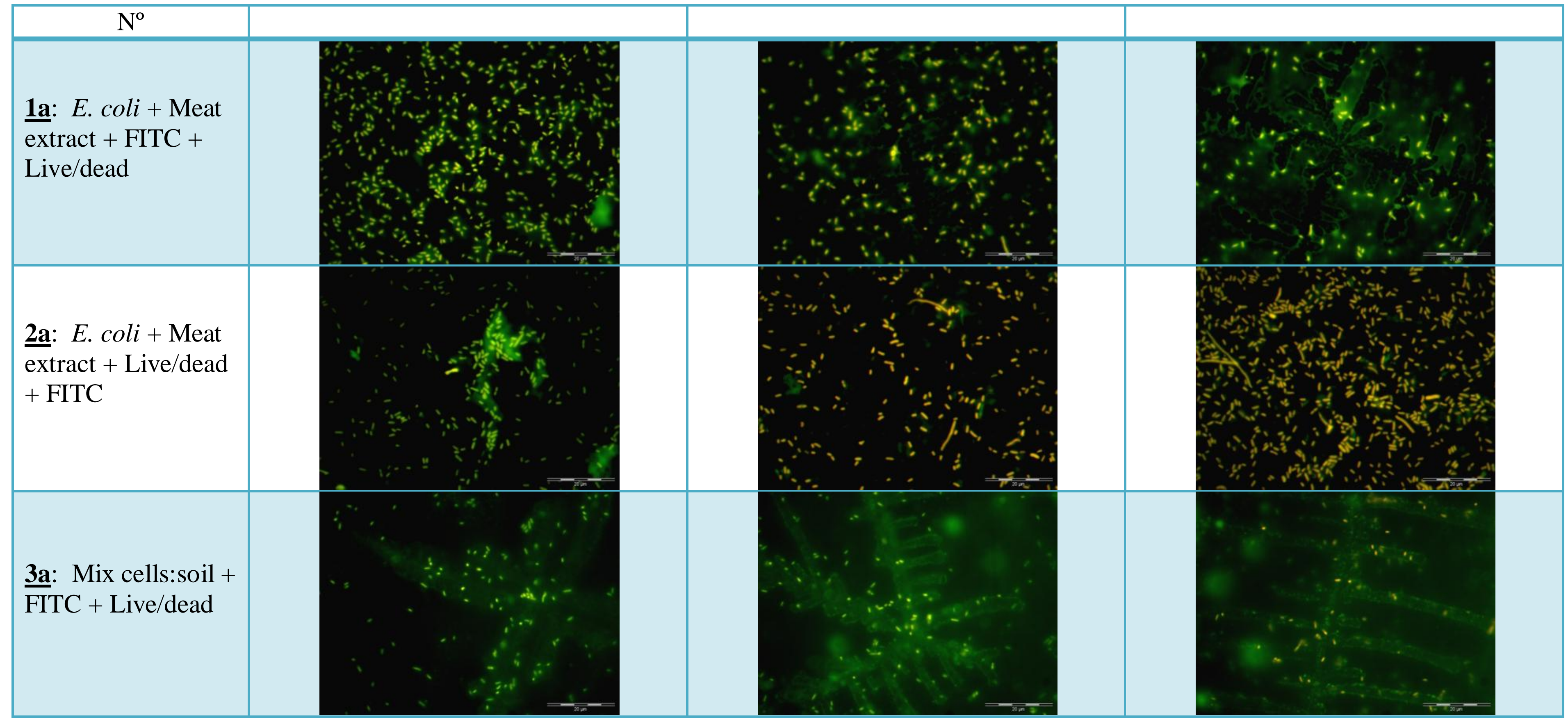
Cells were spread on the surface before the meat extract was added, or cells were mixed with meat extract. FITC and live/dead stains were applied *Figure 3.34.* and required the DAPI filter *Figure 3.35.* to visualise cells and meat extract. The use of the three different stains (FITC, SYTO 9 and Propidium iodide) may slightly alter the absorbance wavelength of each stain. Green dyes may sometimes be more visible with a blue filter when the spectra of excitation and emission are similar (www.chromatechnology.com), which explained the better visibility of meat extract with DAPI filter when using FITC. Red and green cells were briefly visible in *Figure 3.34.* 3a) & b) but all cells became yellow after 2 seconds under excitation light. This phenomenon is called photobleaching where the intensity of the excitation light reduces the lifetime of the dye rapidly by photochemical destruction (Mahmoudian *et al*, 2011). However, even in the best conditions, only meat extract and cells could be differentiated.

Cells and meat extract were more easily distinguishable when the cells were added after the meat extract (*Figure 3.36.*)when comparing the results found with the cells deposited before meat extract. As before, using DAPI filter with the use of FITC made the meat extract more visible (*Figure 3.37.*). Cells mixed with meat extract and applied gave 'cloudy' results where most cells were not clearly visible (*Figure 3.34.*3a); *Figure 3.35.* 3b); *Figure 3.36.* 4a) and *Figure 3.37.* 4b)).

When using the DAPI stain, both cells and meat extract were clearly visible irrespective of the order of deposition (*Figure 3.38.* & *3.39.*). However, there was still no differentiation between damaged and undamaged cells.

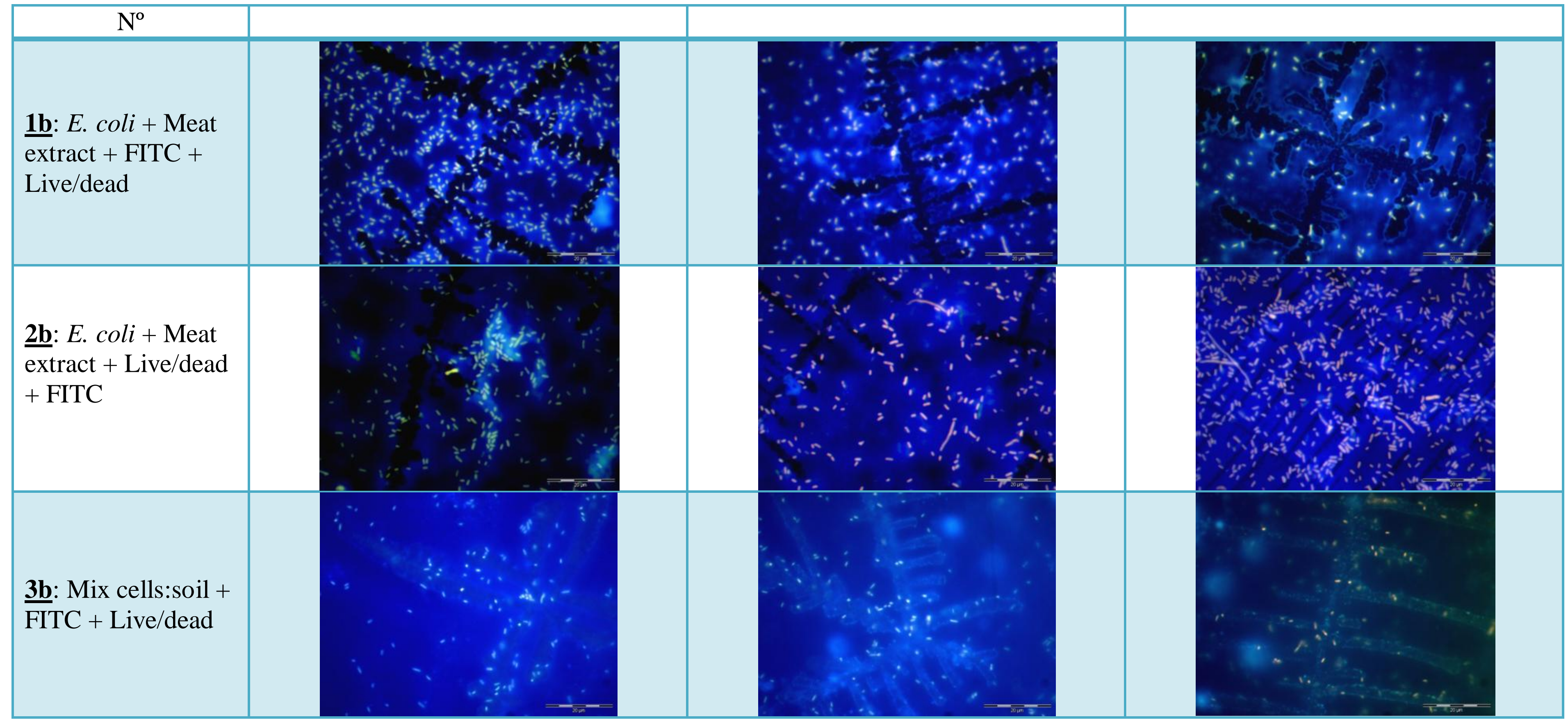
In order to improve the staining method, the FITC/DAPI stains were applied between the meat soil and cell applications. This method only improved the visibility of the meat extract which was enhanced by the use of the DAPI filter (*Figure 3.40.* & *3.41.*). Live/dead stain only was only effective with live/dead filters and DAPI (*Figure 3.42.*).

Finally, smaller quantities of each component were used (2µL). The specific combinations of components were selected from the previous results N°13 (meat extract, drying, FITC stain, drying, *E. coli*, incubation time, live/dead stain and drying) and N°14 (meat extract, drying, DAPI stain, drying, *E. coli*, incubation time T_x, live/dead stain and drying) *Figure 3.43.* Using the FITC stain (N°14), differentiation of damaged/undamaged cells and meat extract was possible only on stainless steel after 2 hours *Figure 3.43.*. However, the DAPI method was reproducible and differentiated damaged/undamaged cells and meat extract at all times on stainless steel and TiN/Ag 120W (*Figure 3.44.*). No difference between the number of damaged and undamaged cells was noted over time, in this brief study (although counts were not employed). Previous experiments (swabbing), the viability of *E. coli* did not decrease rapidly in the presence of meat, thus explaining the lack of increase in damaged cells over time (from T_0 to T_4). Future studies are needed to investigate the antimicrobial effect of silver with *E. coli* and meat extract over longer periods of time.



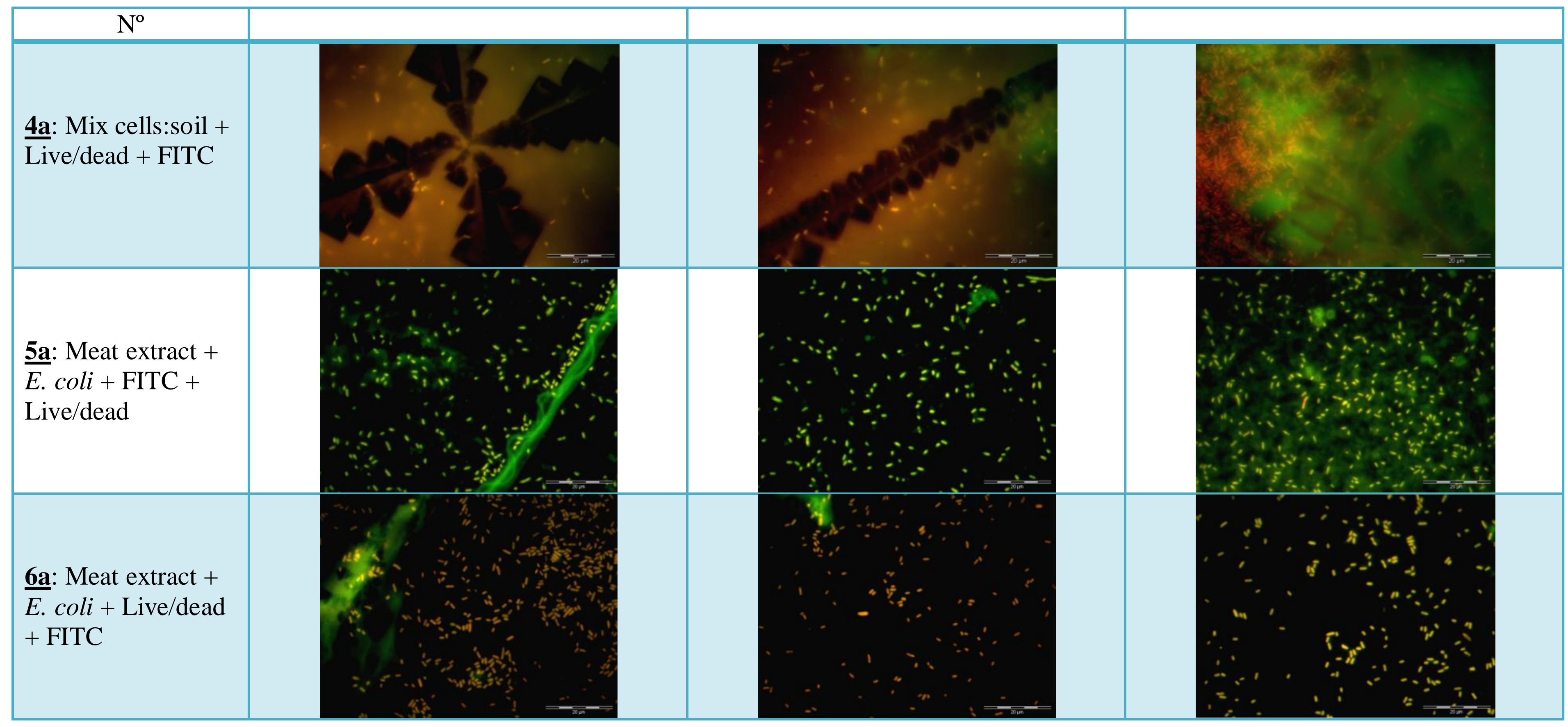
Nº			
1a: *E. coli* + Meat extract + FITC + Live/dead			
2a: *E. coli* + Meat extract + Live/dead + FITC			
3a: Mix cells:soil + FITC + Live/dead			

Figure 3.34. Differential staining using live/dead and FITC dyes with FITC and live/dead filters, not differentiating damaged and undamaged cells as well as meat in most of them



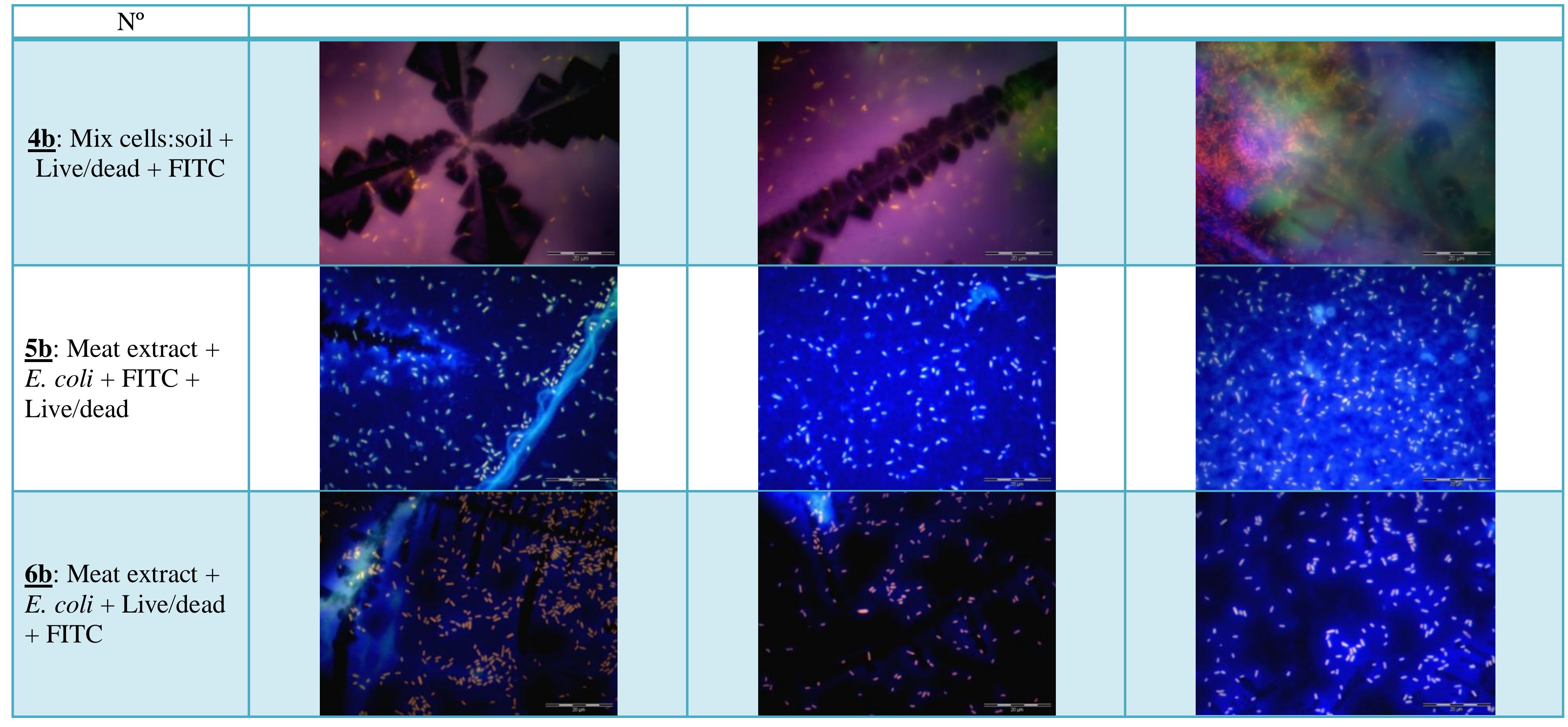
Nº			
1b: *E. coli* + Meat extract + FITC + Live/dead			
2b: *E. coli* + Meat extract + Live/dead + FITC			
3b: Mix cells:soil + FITC + Live/dead			

Figure 3.35. Differential staining using live/dead and FITC dyes with DAPI and live/dead filters, not differentiating damaged and undamaged cells, meat extract was more apparent with DAPI filter



Nº			
4a: Mix cells:soil + Live/dead + FITC			
5a: Meat extract + *E. coli* + FITC + Live/dead			
6a: Meat extract + *E. coli* + Live/dead + FITC			

Figure 3.36. Differential staining using live/dead and FITC dyes with FITC and live/dead filters, not differentiating damaged and undamaged cells but differentiating cells and meat extract



N°			
4b: Mix cells:soil + Live/dead + FITC			
5b: Meat extract + *E. coli* + FITC + Live/dead			
6b: Meat extract + *E. coli* + Live/dead + FITC			

Figure 3.37. Differential staining using live/dead and FITC dyes with DAPI and live/dead filters, not differentiating damaged and undamaged cells but differentiating cells and meat extract, where meat extract was more distinguishable than with FITC filter

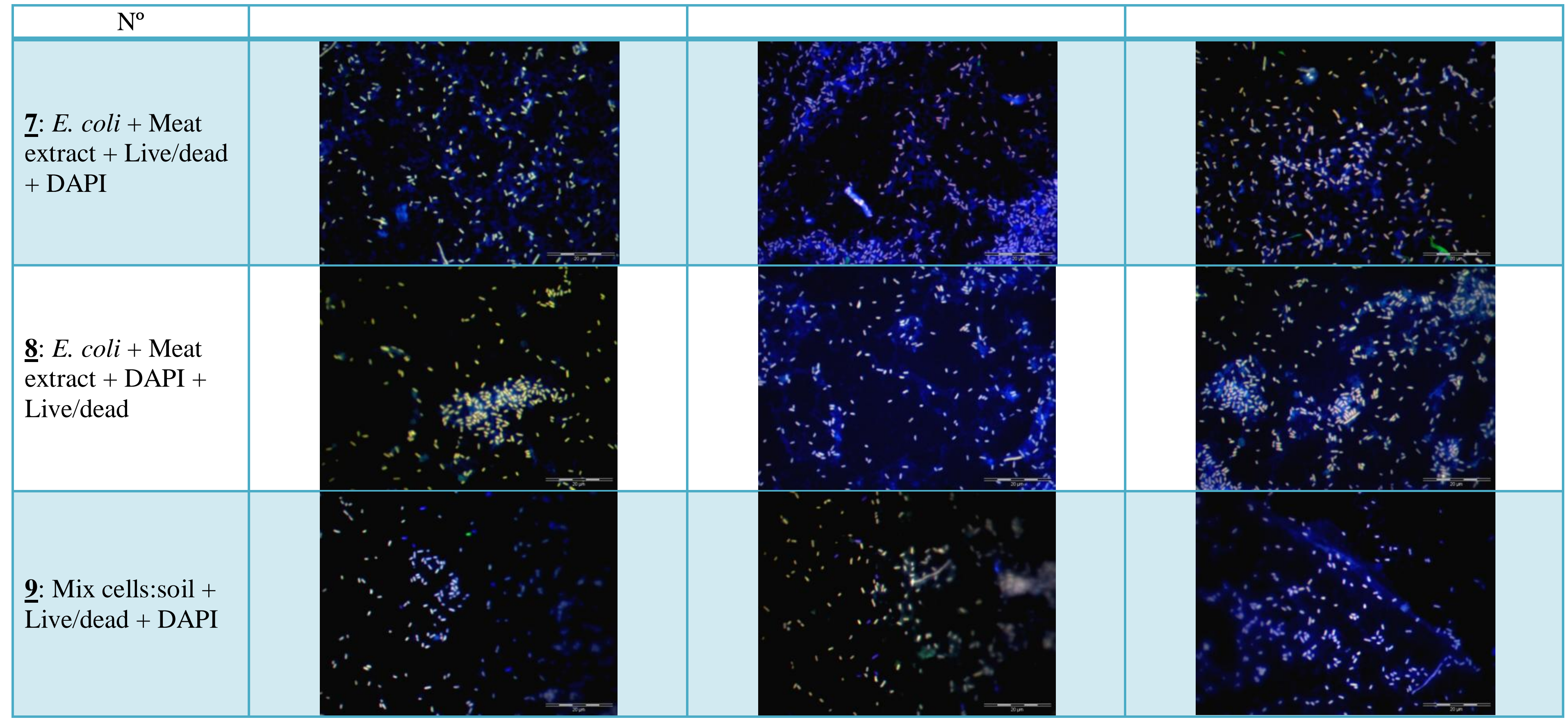
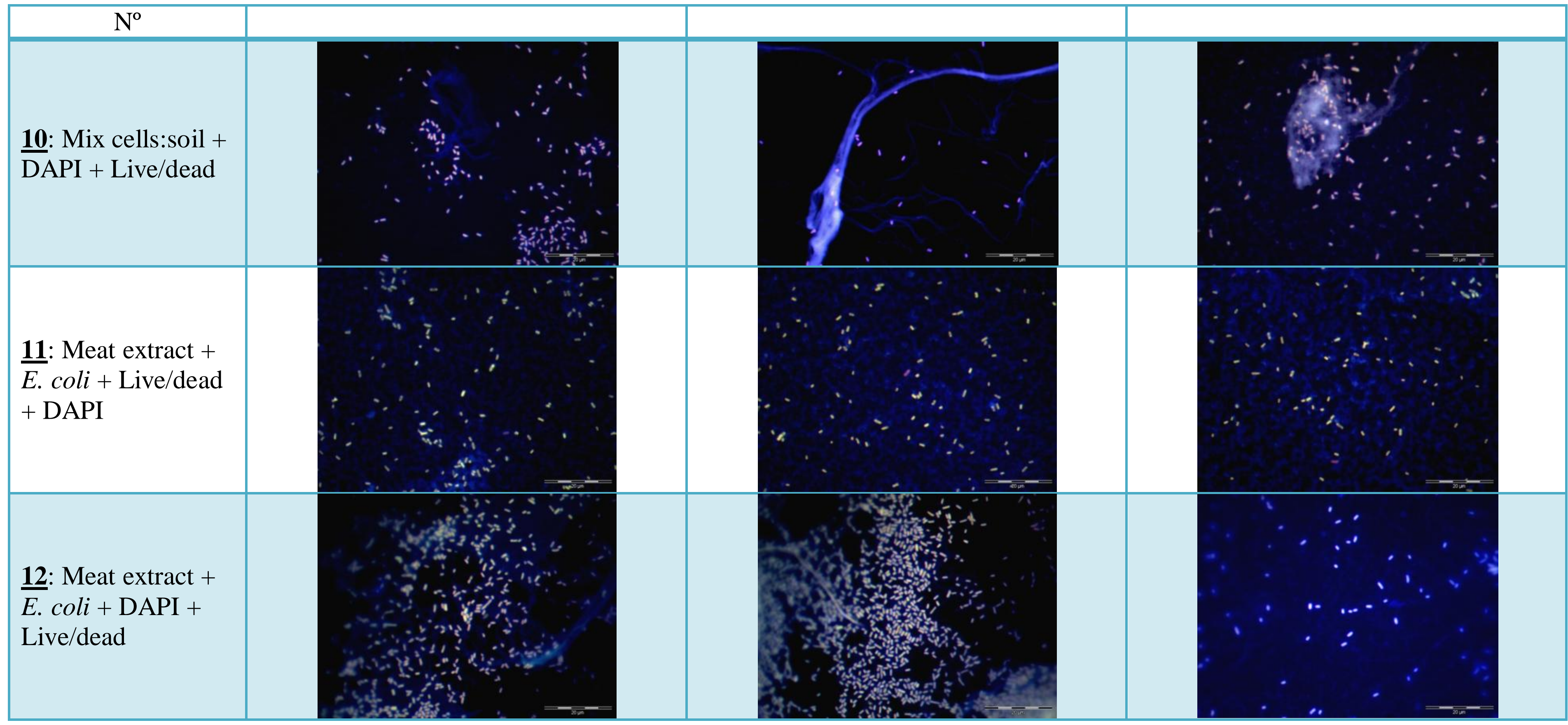


Figure 3.38. Differential staining using live/dead and DAPI dyes with DAPI and live/dead filters, not differentiating damaged and undamaged cells but differentiating cells and meat extract



Nº			
<u>10</u>: Mix cells:soil + DAPI + Live/dead			
<u>11</u>: Meat extract + *E. coli* + Live/dead + DAPI			
<u>12</u>: Meat extract + *E. coli* + DAPI + Live/dead			

Figure 3.39. Differential staining using live/dead and DAPI dyes with DAPI and live/dead filters, not differentiating damaged and undamaged cells but differentiating cells and meat extract

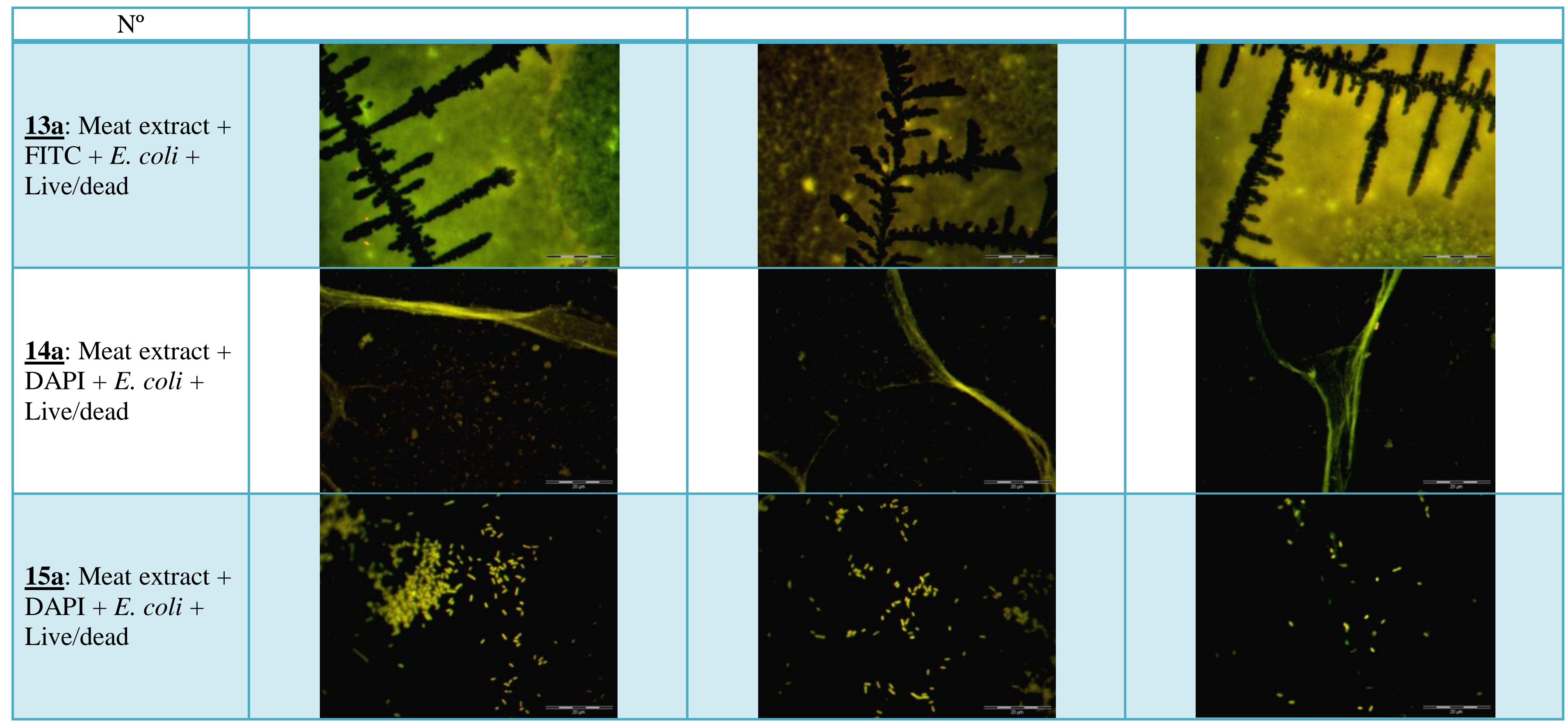
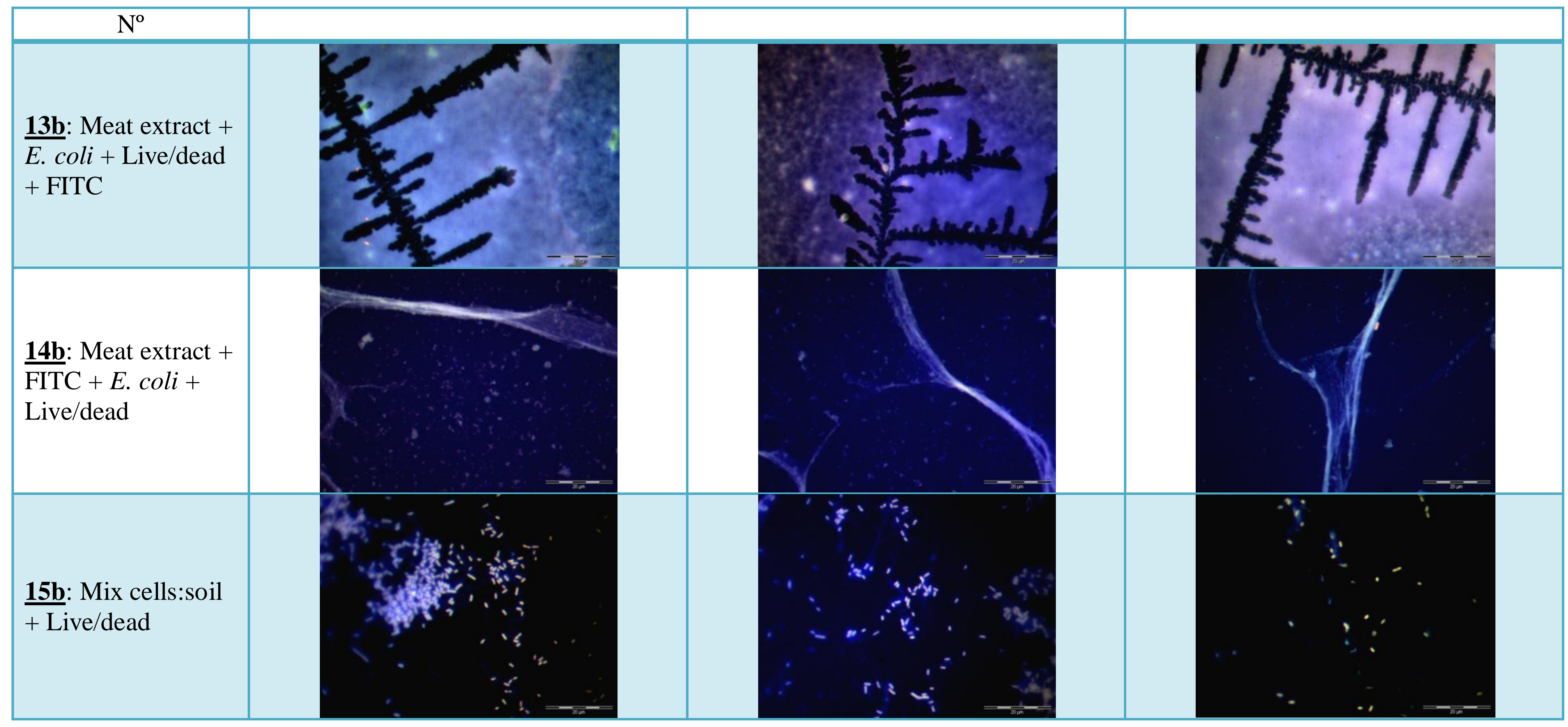


Figure 3.40. Differential staining using live/dead and FITC dyes with FITC and live/dead filters, not differentiating damaged and undamaged cells or cells from meat extract



Nº			
13b: Meat extract + *E. coli* + Live/dead + FITC			
14b: Meat extract + FITC + *E. coli* + Live/dead			
15b: Mix cells:soil + Live/dead			

Figure 3.41. Differential staining using live/dead and FITC dyes with DAPI and live/dead filters, not differentiating damaged and undamaged cells but differentiating cells and meat extract in 15b

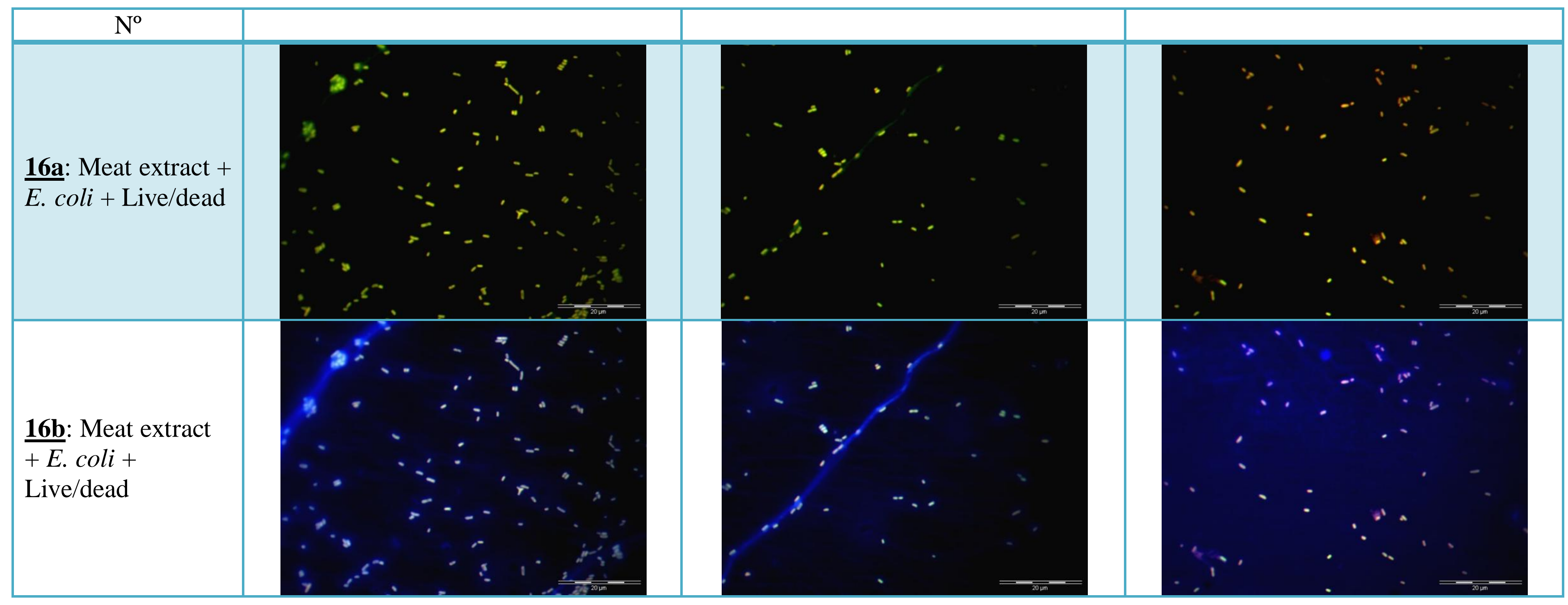
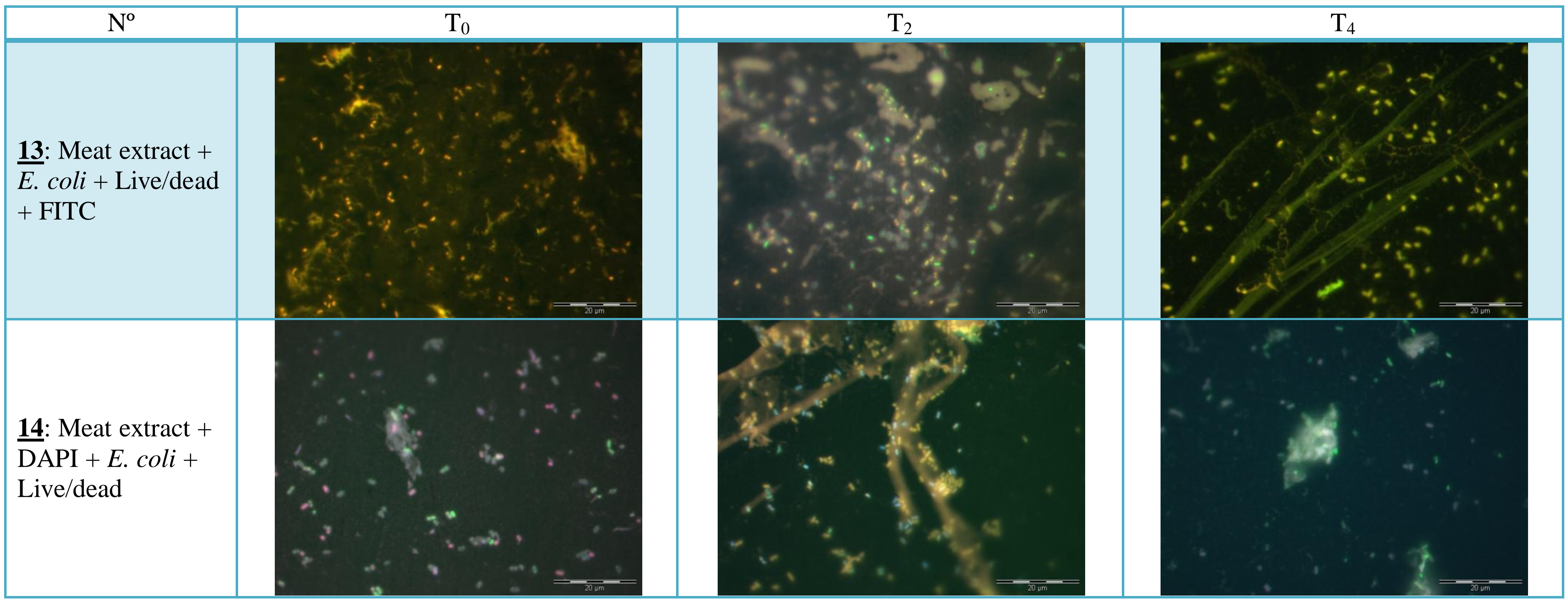
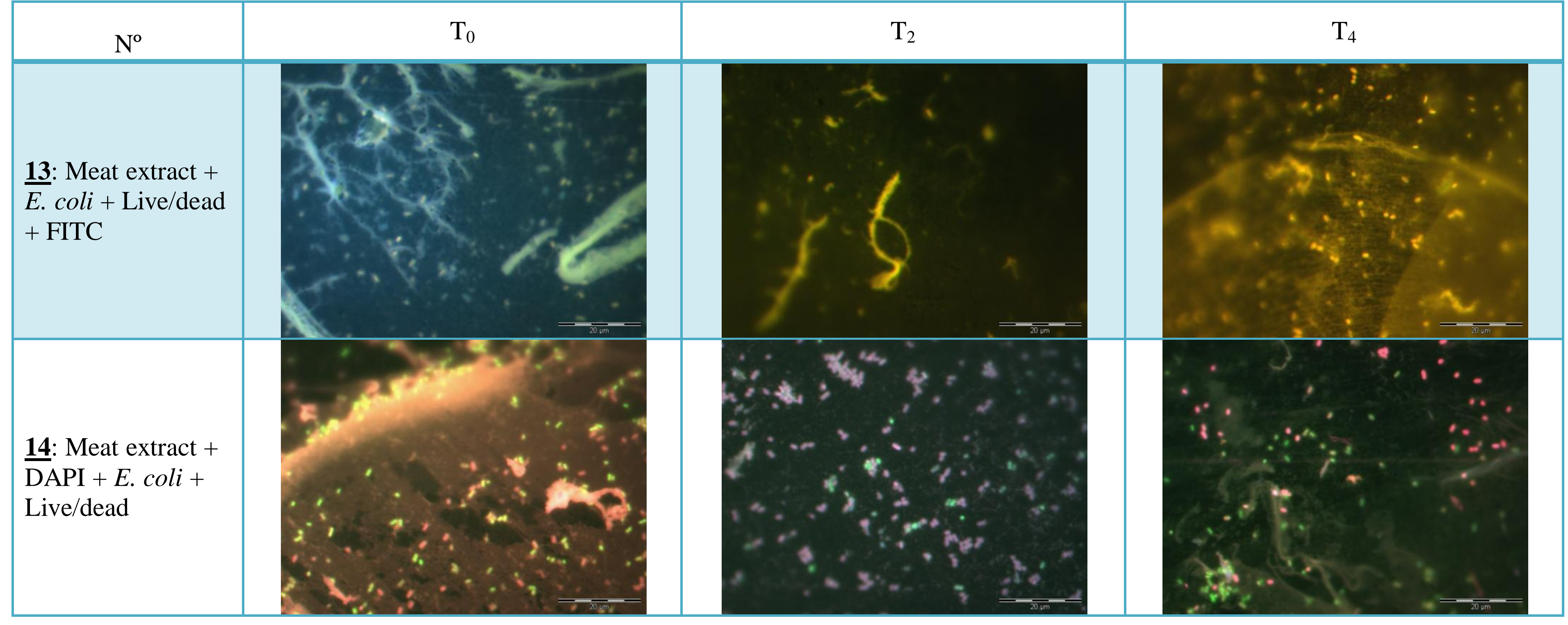


Figure 3.42. Differential staining using live/dead and live/dead filters only in 16a), live/dead and DAPI filters in 16b) showing a better differentiation of cells and meat extract with the use of DAPI filter



Nº	T_0	T_2	T_4
13: Meat extract + *E. coli* + Live/dead + FITC			
14: Meat extract + DAPI + *E. coli* + Live/dead			

Figure 3.43. Differential staining using live/dead and FITC dyes with live/dead and DAPI filters, on stainless steel after 0, 2 and 4 hours of incubation at room temperature (20ºC) before staining



Nº	T_0	T_2	T_4
<u>13</u>: Meat extract + *E. coli* + Live/dead + FITC			
<u>14</u>: Meat extract + DAPI + *E. coli* + Live/dead			

Figure 3.44. Differential staining using live/dead and FITC dyes with live/dead and DAPI filters, on TiN/Ag 120W over 0, 2 and 4 hours

3.4.8. Wear of surfaces

Images of the surfaces, using reflected light microscopy, were taken to compare the visual wear of the substrata. On stainless steel, pits and deeper scratches and debris were apparent on the surface (*Figure 3.45.*). The TiN coated surface showed similar abrasions with a peeling of the coating in some places which is more obvious in "2nd time used" (*Figure 3.46.*). The surface of TiN/Ag 50W did not show any visual difference of wear before and after use (*Figure 3.47.*). TiN/Ag 100W showed numerous scratches appearing after use, but the coating did not peel (*Figure 3.48.*). There was no visual difference before and after uses of the TiN/Ag 120W surfaces or any peeling of the coating itself (*Figure 3.49.*).

TiN/Ag 120W showed fewer scratches or any other damages than the other substrata tested. Differences in substrata properties noted in *Chapter 2* did not affect the behaviour of the cells on the surface, indicating that topography (i.e. smooth, hygienic surfaces) is a key property. The damaged caused to the coatings gives some concern, indicating more work needed on the effect of extended use, adhesion of coatings to stainless steel, hardness and reproducibility before *in situ* assays.

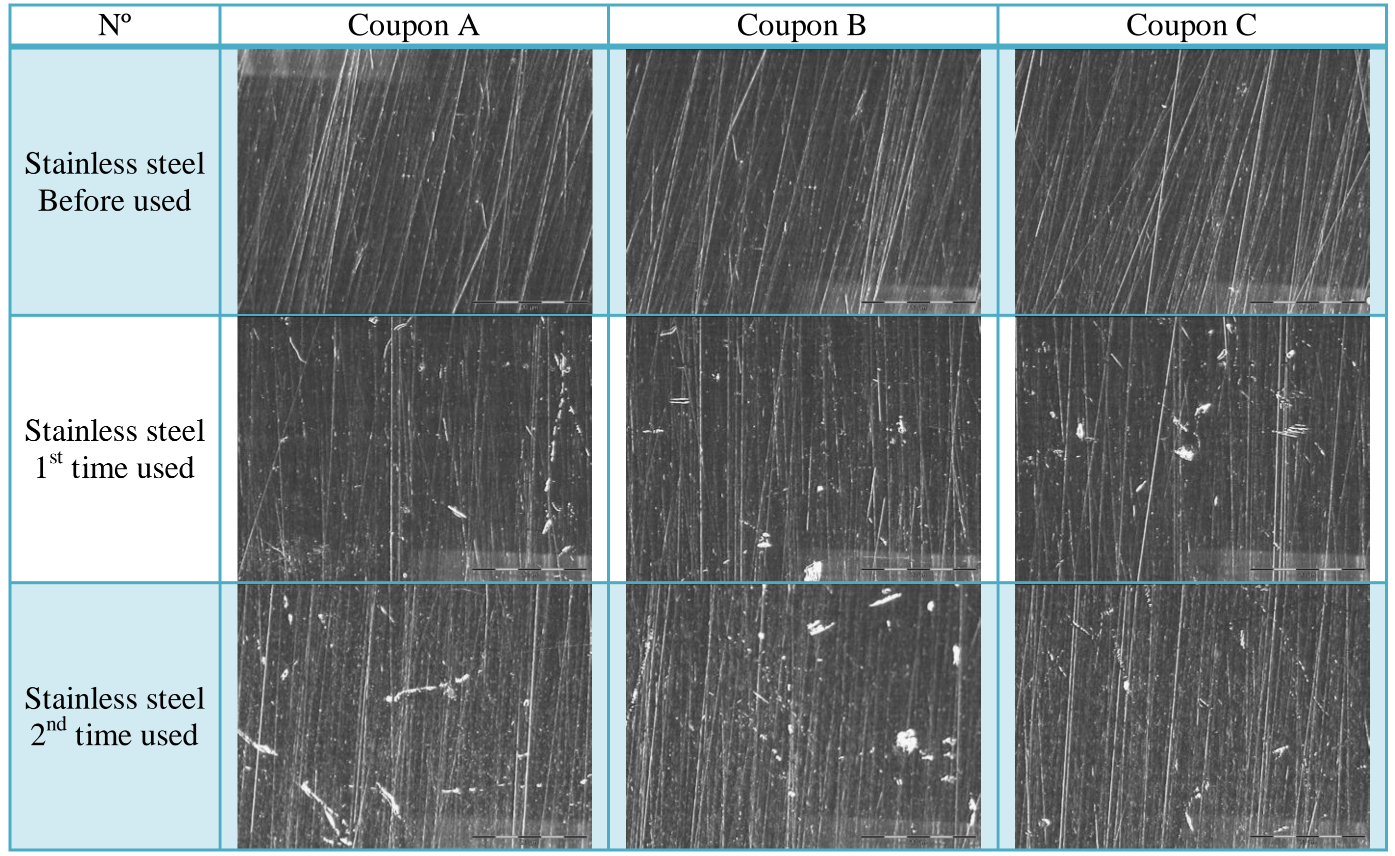


Figure 3.45. Wear and tear of stainless steel 304 fine polish before and after uses by reflected light microscopy

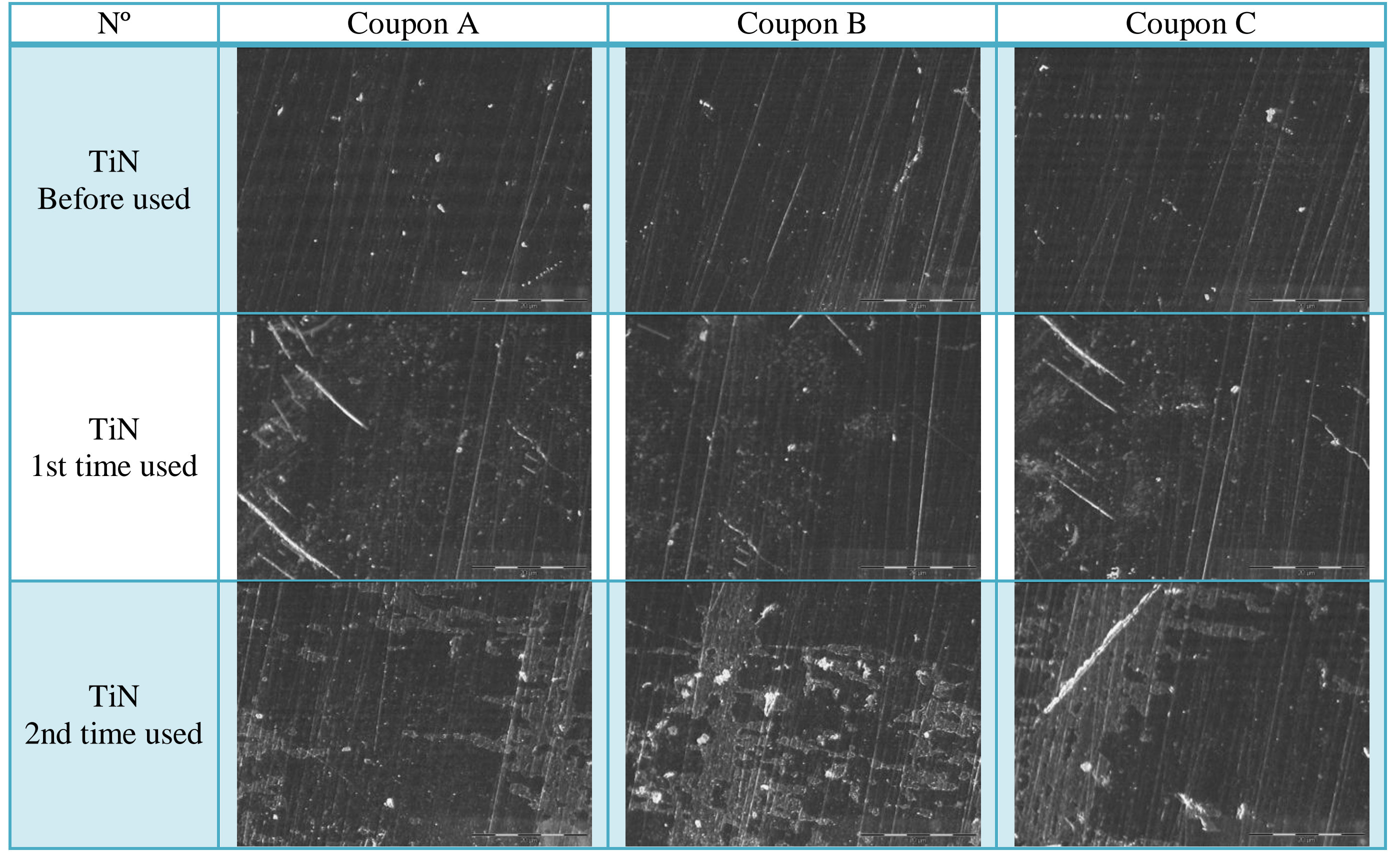


Figure 3.46. Wear and tear of TiN coated stainless steel before and after uses by reflected light microscopy

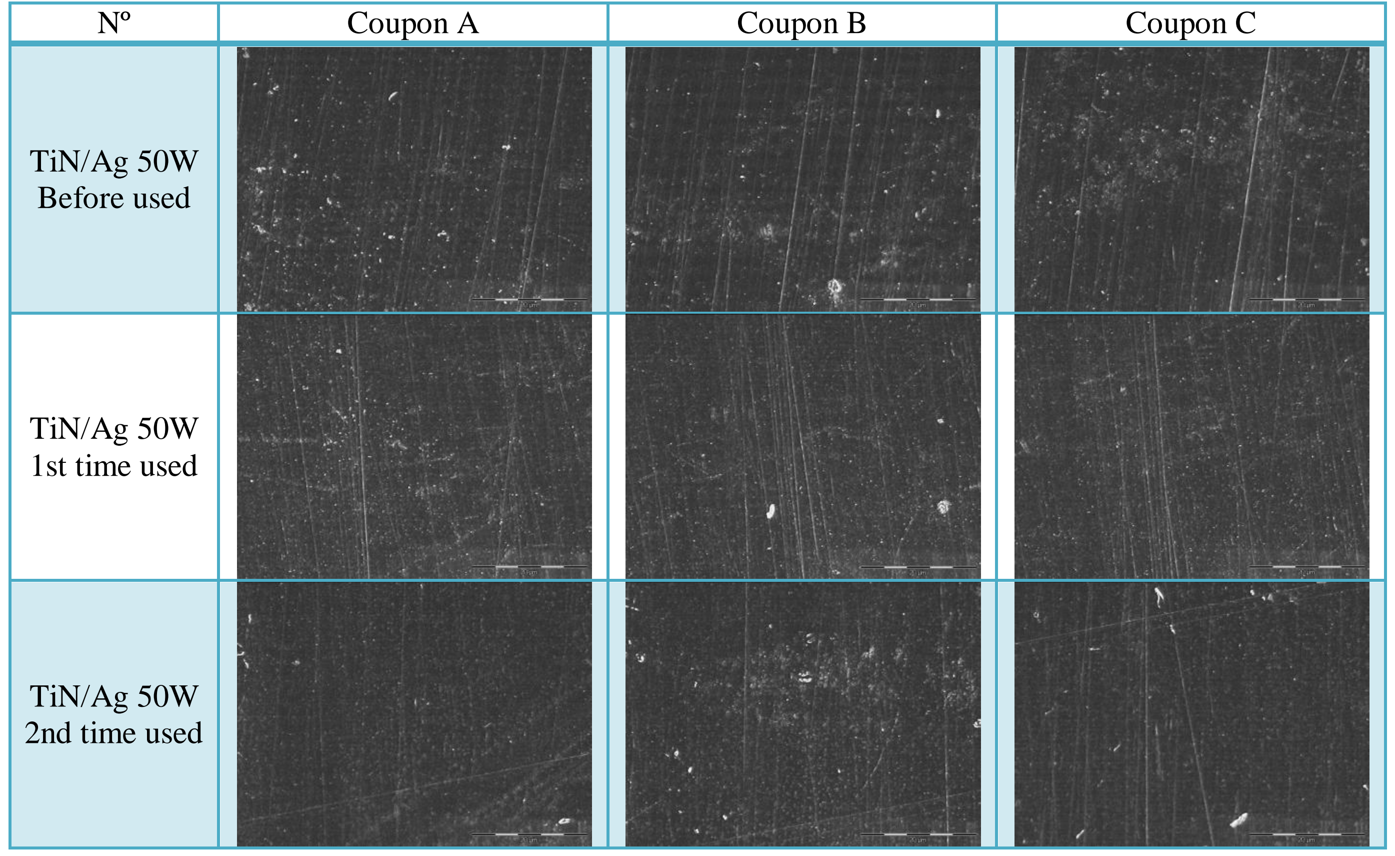


Figure 3.47. Wear and tear of TiN/Ag 50W coated stainless steel before and after uses by reflected light microscopy

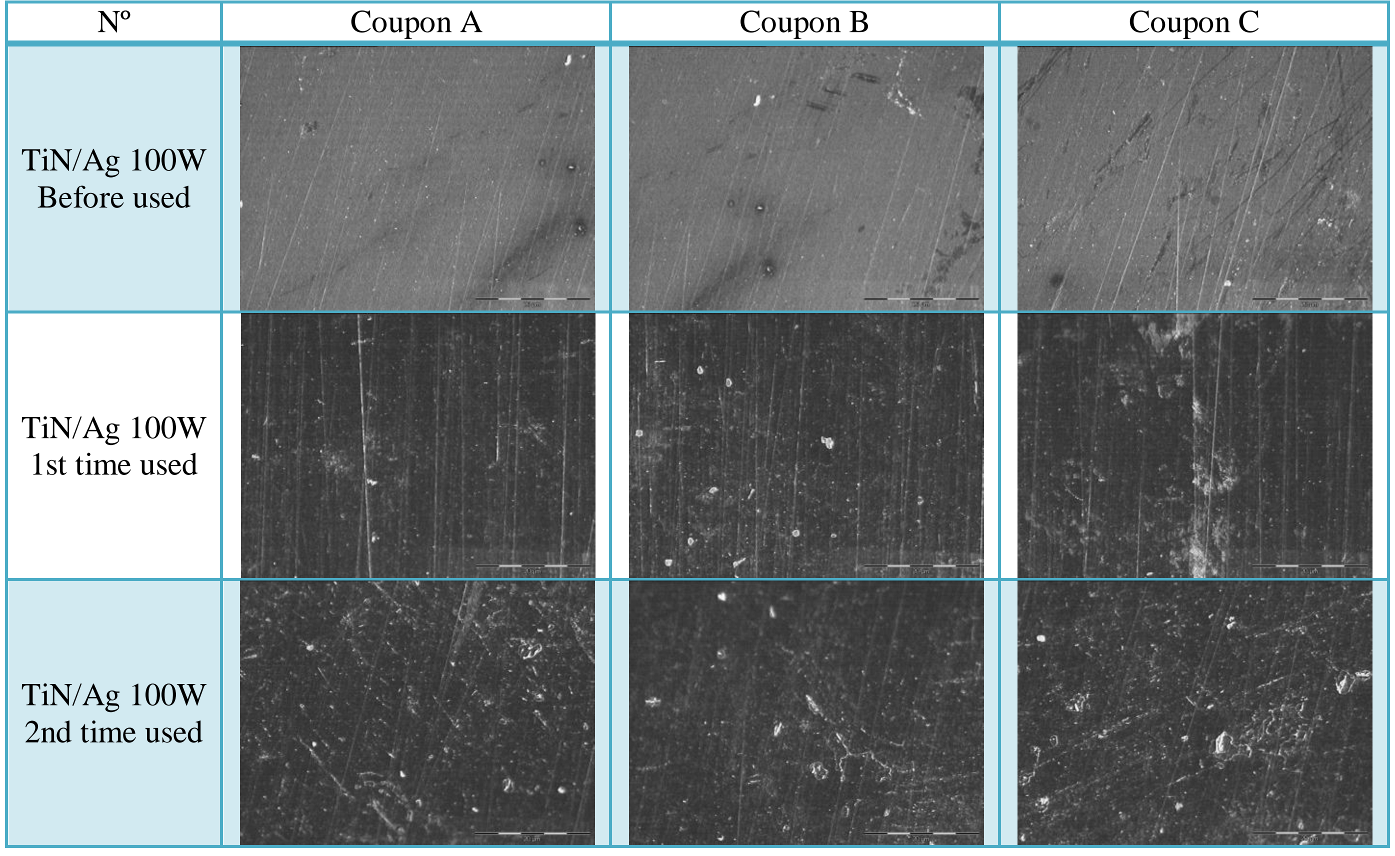


Figure 3.48. Wear and tear of TiN/Ag 100W coated stainless steel before and after uses by reflected light microscopy

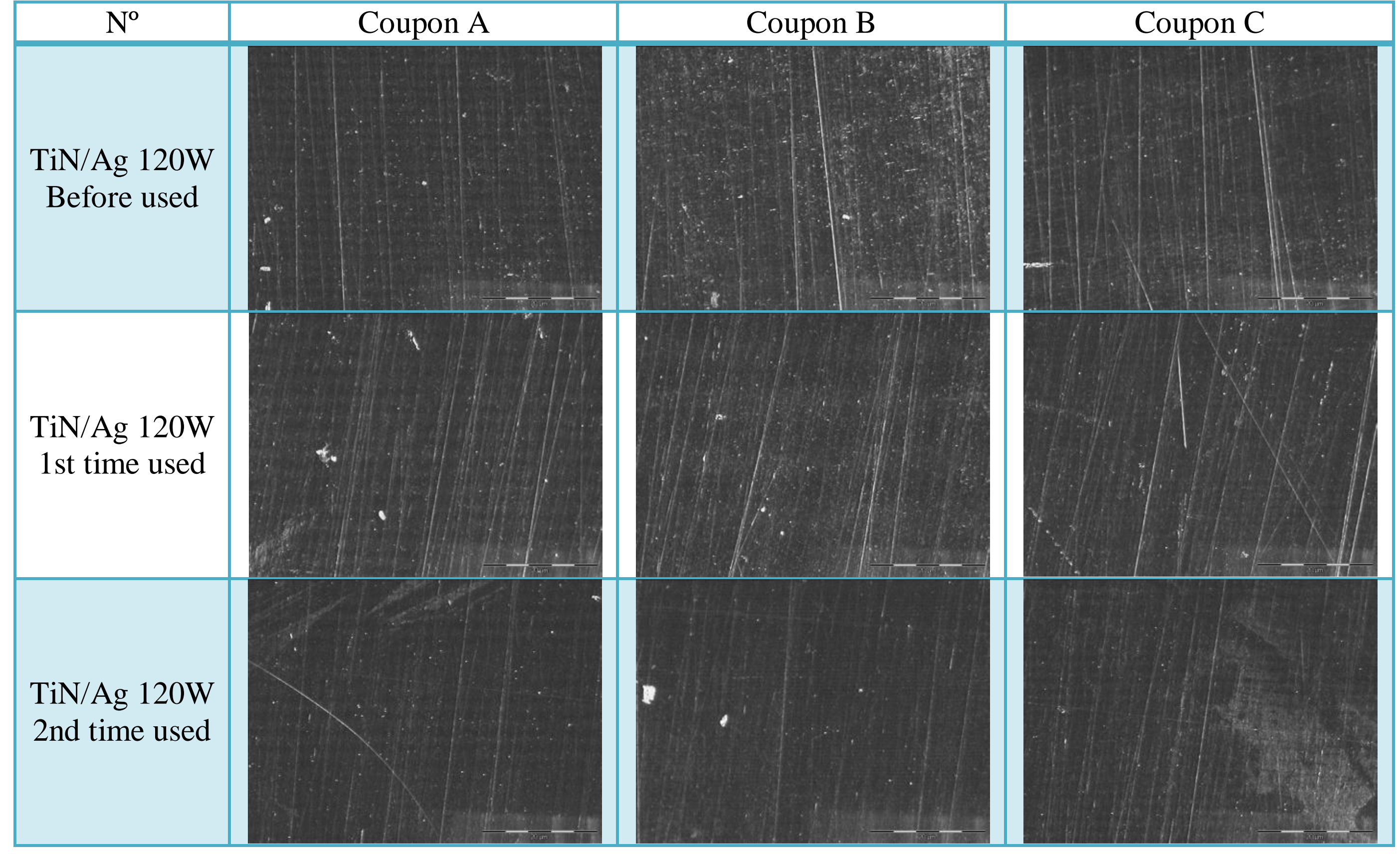


Figure 3.49. Wear and tear of TiN/Ag 120W coated stainless steel before and after uses by reflected light microscopy

3.5. Conclusion

A method has been developed to enable assessment of the survival of *L. monocytogenes* and *E. coli* on surfaces under different humidities. In general, high humidity and optimum growth temperature increased survival. The swabbing technique for removal of cells was consistent and reliable for the substrata used, even in the presence of conditioning film. In general, Gram-positive *L. monocytogenes* survived better that Gram-negative *E. coli* on stainless steel and antibacterial silver coatings. High humidity (86% ERH) did not improve the antimicrobial effect and silver released from surfaces. However, a reduction in cell viability was observed on surfaces when comparing coated surfaces to stainless steel. For *L. monocytogenes* and *E. coli*, increasing the concentration of silver decreased the viability of the cells. *L. monocytogenes* cells were retained in higher number than *E. coli* cells but the overall number of cells retained was low due to the smoothness of the substrata used. Retention was comparable on stainless steel and coatings. The re-use of the surfaces did not affect the number of cells recovered or the cell retention of *E. coli* and *L. monocytogenes* on all substrata. The antimicrobial effect of silver was maintained, although there was evidence of damage to the coatings. Finally, a differential staining method has been successfully developed to detect live/dead *E. coli* cells in meat extract on stainless steel and TiN/Ag 120W.

The substrata used in this study have not previously been used against organisms of interest to the food industry, and some potential has been revealed with suggestions made for future work.

Chapter 4

Summary

4.1.Summary

The purpose of this study was to identify the factors affecting survival of *E. coli* and *L. monocytogenes* on stainless steel and TiN-TiN/Ag nanocomposite coatings.

Stainless steel 304 fine polish was used as a control and base for the coatings because it is widely used in the food industry (www.outokumpu.com). TiN thin film coating on stainless steel was also used as control because other coatings were alloyed with different concentrations of silver to enable its antimicrobial properties to be assessed (Dowling *et al*, 2003; Wang *et al*, 2006; Dong *et al*, 2008; Martínez-Castañón *et al*, 2008; Rai *et al*, 2009; Sharma *et al*, 2009). TiN is widely used for its hardness and resistance on cutting tools (Zhang and Zhu, 1993; Watmon and Ijeh, 2010).

Throughout this study, all surfaces proved to be smooth, thus hygienic, presenting small surface features (*Chapter 2*) which retained few cells (*Chapter 3*). Re-us of the substrata would probably affect their topography, with new surface features such as scratches becoming apparent, thus increasing the potential for cell retention (Whitehead and Verran, 2006).However, our results showed that the behaviour of *E. coli* and *L. monocytogenes* cells remained similar after three uses of the substrata, indicating that the coatings were relatively robust, with potential for use in the food industry.

Ideally, characterisation of the topography and hydrophobicity of the substrata would have been done before and after each re-use if given more time. This would have allowed a better understanding of the changes taking place on the surfaces when re-used.

E. coli and *L. monocytogenes* were inhibited on TiN/Ag 50W, 100W and 120W. Increase of humidity increased the survival of microorganisms but not the antimicrobial effect of silver containing coatings as had been hypothesised (Juan *et al*, 2010). Survival decreased at the optimum temperature of the microorganisms when compared to survival at room temperature (Mai and Conner, 2007

The presence of food soil on the surface may affect the retention of cells (Zoltai *et al*, 1981; Donlan and Costerton, 2002). Meat extract did not affect the viability of cells on the surface and few cells were retained on surfaces. Nevertheless, an increase in silver concentration in the coatings showed significant reduction in the survival of *E. coli* and *L. monocytogenes*. Although the survival of *E. coli* and *L. monocytogenes* increased in the presence of meat extract, the antimicrobial properties of silver remained active, although reduced/retarded. Lloret *et al* (2012) also showed that silver ions from cellulose/silver nanocomposites were effective in inhibiting microbial growth in contact with meat or fruit exudates.

In general, Gram positive *L. monocytogenes* survived better than Gram negative *E. coli* through all the conditions tested in this study. Their differences in cell structure were possibly the reason responsible for the difference in survival (Iida and Koike, 1974). *L. monocytogenes* has a thicker peptidoglycan layer than *E. coli* which makes it more resistant to the environment (Edwards and Stevens, 1963).

Epifluorescence microscopy is a well established method used in screening cell:substratum attachment and retention in the presence and absence of soil (Holah and Thorpe, 1990; Hood and Zottola, 1997; Wirtanen *et al*, 1996; Whitehead *et al*, 2005b; Verran *et al*, 2008). A novel method was developed to enable to differentiation of live/dead *E. coli* cells and meat extract. Whitehead *et al* (2009) demonstrated that

depending on the combination and type of food soil and microorganism, different stain formulation were needed. In some cases, the substratum may interfere with the stain (www.invitrogen.com), thus preventing it from performing. Thus the concentration of food soil and cells affected the capability of the dyes to differentiate live/dead cells. Differential staining of live/dead cells in presence of food soil requires development specific to each soil:cell combination (Verran and Whitehead, 2006). However, once perfected, this method can be used to determine the effectiveness of cleaning and disinfecting regimes on the retention of cells and organic soil, and also to assess the antimicrobial properties of treatments on surfaces.

4.2. Conclusion

TiN/Ag surfaces may have potential as hygienic coatings in the food industry. Silver is not leached from the surfaces, and they retained antimicrobial properties with the re-use. Future work in terms of surface characterisation of topography, surface wear and *in situ* testing is required.

Chapter 5

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