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An Investigation and Examination of the Levels and Types of Bacterial Contamination on the Surface of Clean Room Operators' Garments

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A thesis submitted in partial fulfilment of the requirements of the Robert Gordon University for the degree of Doctor of Philosophy

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Author's Declaration

I hereby declare that this is an original piece of work which I have undertaken and which has not previously been submitted for a higher degree or qualification of this university or another institute of learning. The research was completed at the Robert Gordon University under the supervision of Dr Andrew Lamb and Dr Noelle O' Driscoll. All results and work, other than my own, are clearly acknowledged and cited by reference.

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Abstract

The contamination of sterile pharmaceutical products is a serious event which has in the worst case scenario led to patient death. Operators are the primary source of clean room contamination, with the majority of their detritus being identified as skin squames and their related microorganisms. The ability of operator associated bacterial contamination to disseminate through specialist garments worn in the clean room environment is apparent in the literature. However, despite the fibres of such garments being identified as a suitable substrate for bacteria to adhere to and grow upon, the bacterial bioburden of the surface of clean room operators' garments is an area which severely lacks in published research. Reported here is the recovery, enumeration and comparison of the levels of bacteria on the surface of reusable antistatic carbon filament polyester clean room garments, using the direct agar contact method, following their laundering with and without terminal gamma sterilisation, immediately following their donning with operators dressing wearing either no gloves, non – sterile gloves or sterile clean room gloves, and following their wear within the clean room environment, with respect to gender. The aforementioned method, with its recovery efficiency shown to be unaffected by agar composition (NA or TSA), recovered bacteria from the surface of garments laundered with and without gamma sterilisation. Such terminal decontamination was shown to reduce the surface bacterial bioburden of the garments, especially at the chest and umbilicus regions, which were shown to harbour higher levels of bacteria than the other sites tested. The direct agar contact method, showing an increase in recovery efficiency following a 48 hour agar incubation period as opposed to a 24 hour period, also recovered bacteria from the surface of clean room garments donned by operators dressing wearing either no gloves, non – sterile gloves and sterile clean room gloves. Bacteria were transferred onto the surface of these garments via the hand borne route, with the chest and oral cavity regions being found to harbour more bacteria than the other sites tested. Overall, glove type was shown to have no effect upon the resultant bacterial bioburden of the surface of the garments, suggesting expensive clean room gloves could be substituted for their cheaper non – sterile equivalents or no gloves during the donning process without subsequently increasing the surface bacterial bioburden of the garment. The direct agar contact method also recovered bacteria from the surface of clean room garments worn by male and female operators, following

their working period within a clean room environment. Gender was found to significantly affect the surface bacterial bioburden of the garments, with the surface of those garments worn by male operators being more contaminated than the surface of those worn by their female counterparts. In addition, the donning of a clean room hood was shown to reduce the levels of bacteria at the chest and posterior cervicis regions of suits worn by both genders. Overall, the direct agar contact method was identified as a successful tool to recover, enumerate and estimate the surface bacterial bioburden of reusable antistatic polyester carbon filament clean room garments. Finally, using 16S rRNA gene sequencing, found to be more reliable and accurate at identifying unknown isolates than traditional phenotypic first - stage tests, which were subsequently found to misidentify > 85 % of the isolates tested, a self - selected representative number of isolates recovered from the surface of garments during the laundering and gender comparison studies were predominantly identified as skin commensal species of *Staphylococcus* and *Micrococcus*, as well as environmental species of *Bacillus*. The knowledge contained within this thesis, with respect to clean room operators and their specialist garments, contributes towards improving contamination control standards within clean room facilities.

Abbreviations

ANOVA – Analysis of Variance

API – Analytical Profile Strip

ARI – Aberdeen Royal Infirmary

ASTM – American Society for Testing Materials

BLAST – Basic Local Alignment Search Tool

Bp – Base Pair

BS EN - British Standard European Norm

CDC – Centers for Disease Control & Prevention

CFD – Computational Fluid Dynamics

CFU – Colony Forming Unit

DLVO – Derjaguin and Landau, Verway and Overbeek

DNA – Deoxyribonucleic Acid

dNTP - Deoxyribonucleotide Triphosphate

EDTA - Ethylenediaminetetraacetic acid

EPS – Extra Polymorphic Substances

EtOH – Ethylene Oxide

EU – European Union

EU GMP – European Union Good Manufacturing Process

FDA – Food and Drug Administration

GMP – Good Manufacturing Practice

HEPA – High Efficiency Particulate Air

HVAC – Heating Ventilation & Air Conditioning

IEST – Institute of Environmental Science and Technology

ISO – International Standards Organisation

kGy – Kilogray

MALDI - TOF MS - Matrix – Assisted Laser Desorption / Ionisation Mass Spectroscopy

MCP – Microbe Carrying Particles

MDPH – Massachusetts Department of Public Health

MgCl – Magnesium Chloride

MHRA - The Medicines and Healthcare products Regulatory Agency

MRSA – Methicillin - resistant *Staphylococcus aureus*

NA – Nutrient Agar

NASA – National Aeronautics and Space Administration

NCTC – National Collection of Type Cultures

NECC – New England Compounding Company

NHS – National Health Service

OD – Optical Density

OF – Oxidation / Fermentation

PCR – Polymerase Chain Reaction

PHE – Public Health England

RESA – Research Ethics Self - Assessment

RGU – Robert Gordon University

rRNA - Ribosomal Ribonucleic Acid

SAL – Sterility Assurance Level

SEM – Scanning Electron Microscopy

SDS – Sodium Dodecyl Sulphate

SIP – Sample Item Proportion

TBE – Tris / Borate / EDTA

TE – Tris / EDTA

TRIS – Tris (hydroxymethyl) aminomethane

TSA – Tryptone Soya Agar

UV – Ultra Violet

VDmax - Maximum Validation Dose

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Chapter 1:

A General Clean Room Overview

1.1 Introduction

A clean room is defined as -

> *"A room in which the concentration of airborne particles is controlled and which is constructed and used in a manner to minimise the introduction, generation and retention of particles inside the room, and in which relevant parameters, e.g. temperature, humidity, and pressure, are controlled as necessary"* (Thomas 2006 p. 38).

The significant role clean rooms play in the manufacture of products which are required to be particulate and microbial free is highlighted in the literature (Holbrook 2009; Kenny 2010; Quinn and Brachmann 2014). These studies emphasise the importance of clean room environments within an array of industries, with many companies relying on these conditions to manufacture their products.

1.1.1 Clean Room History

The literature recognises modern day clean rooms as the result of a number of contributions and developments from a range of disciplines, with the earliest efforts predating any mechanical effort to clean air (Holbrook 2009; Kenny 2010; Quinn and Brachmann 2014). The first simple concepts were reportedly devised by Swiss watchmakers in the early 19th Century (Holbrook 2009), who prevented dust entering the back of wrist watches by either covering them with a jar (Kenny 2010) or moving their workshops to a rural location, mitigating particles such as those originating from unpaved roads (Quinn and Brachmann 2014). Furthermore, it is reported that this era, along with the early 20th Century, saw the creation of sterile surgical operating theatres, recognising the need to control biological contamination within the hospital setting (Holbrook 2009) (Figure 1 – 1).

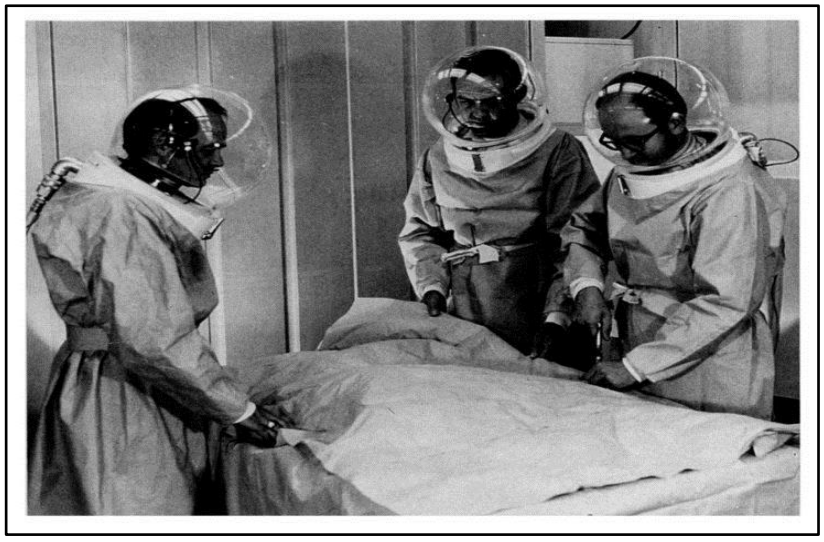


Figure 1 – 1: Surgeons working in a clean room at St. Luke's Hospital Denver in 1972. Clean conditions and specialist clothing were used to lower the risk of infections during surgery. Both the room and the clothing were based on space program experience and were developed under NASA contract by the Martin - Marietta Corporation (Taken from: Bilstein 1989) (NASA Imagery – not copyright protected).

Holbrook (2009) discusses further how clean room use developed during the First World War, where controlled areas were used to manufacture small bearings and aircraft instrumentation away from any visible contamination. In addition, Quinn and Brachmann (2014) consider the manner in which World War II saw the introduction of high efficiency particulate air (HEPA) filters, which are used to screen at least 99.97 % of airborne particles ≥ 0.3 μm entering into a room (Whyte 2010). Clean areas were subsequently used to support the production of weapons, electrical and mechanical devices including the production of the Norden bombsight and the first navigational gyroscopes, where small contaminating particles could cause these to malfunction (Holbrook 2009). Post war, clean room use shifted towards consumer goods, as well as being used by organisations including hospitals, pharmaceutical companies and the National Aeronautics and Space Administration (NASA) (Holbrook 2009).

1.1.2 Clean Room Applications

Modern day application is widespread and is documented to include advanced technologies such as the manufacture of computer integrated circuit boards (Faulkner *et al.* 1996), spacecraft assembly (Vaishampayan 2010) and Nano

system technology (Scheider 2009). This is in addition to established industries including car and plastic manufacture and food production (Schicht 2005). In fact, clean room use is immensely valuable in the pharmaceutical industry where such conditions are required to minimise or eliminate potentially fatal microbial and / or particulate contamination from parenteral and non – parenteral medications which are required to be free from microorganisms including injections, irrigation solutions, and ophthalmic and topical preparations (Dalmaso and Denoya 2015).

1.1.3 Clean Room Classifications

Clean rooms are classified according to the number of particles in the air within the room (International Standards Organisation 2015). This is based entirely upon the cumulative concentration of particles of a specified size, per cubic metre of air. The number of particles and their respective size are measured using an optical particle counter. This draws a specific quantity of air into a light scattering device which measures and records the number of particles present, ranging from 0.1 µm - 25 µm in size (NHS Scotland 2004). Clean room classification must be in accordance with the International Standards Organisation (ISO) Standard 14664 – 1: 2015 (en) document entitled "Clean Rooms and Associated Controlled Environments – Part 1" (International Standards Organisation 2015). This is part of a series of standardised documents associated with clean rooms which establishes room certification requirements in accordance with air particle concentration. ISO clean rooms are classified from ISO 1 - ISO 9 based entirely upon the maximum number of particles equal to or larger than 0.1, 0.2, 0.3, 0.5, 1.0 and 5.0 µm per cubic metre of air, as shown in Table 1 - 1. For example, a room classified as ISO 6 is permitted a maximum concentration of 35,200 particles per cubic metre of air, equal to or larger than 0.5 µm in diameter.

Table 1 - 1: The classification of International Standards Organisation (ISO) clean rooms according to the maximum number of particles per cubic metre of air. (*Table 1 — ISO Classes of air cleanliness by particle concentration* taken from ISO 14644 – 1 : 2015, Cleanrooms and associated controlled environments — Part 1: Classification of air cleanliness by particle concentration, is reproduced with the permission of the International Organization for Standardization, ISO. This standard can be obtained from any ISO member and from the website of the ISO Central Secretariat at the following address: www.iso.org. Copyright remains with ISO).

Clean Room Class	Maximum number of particles per cubic metre of air equal to and larger than the sizes below[a] (µm)					
	0.1	**0.2**	**0.3**	**0.5**	**1.0**	**5.0**
ISO 1	10[b]	[d]	[d]	[d]	[d]	[e]
ISO 2	100	24[b]	10[b]	[d]	[d]	[e]
ISO 3	1,000	237	102	35[b]	[d]	[e]
ISO 4	10,000	2,370	1,020	352	83[b]	[e]
ISO 5	100,000	23,700	10,200	3,520	832	[d, e , f]
ISO 6	1,000,000	237,000	102,000	35,200	8,320	293
ISO 7	[c]	[c]	[c]	352,000	83,200	2,930
ISO 8	[c]	[c]	[c]	3,520,000	832,000	29,300
ISO 9[g]	[c]	[c]	[c]	35,200,000	8,320,000	293,000

a – All concentrations in the table are cumulative, e.g. for ISO Class 5, the 10,200 particles shown at 0.3 um include all particles equal to and greater than this size.
b – These concentrations will lead to large air sample volumes for classification. Sequential sampling procedure may be applied.
c – Concentration limits are not applicable in this region of the table due to very high particulate concentrations.
d – Sampling and statistical limitations for particles in low concentrations make classification inappropriate.
e – Sample collection limitations for both particles in low concentrations and sizes greater than 1 um make classification at this particle size inappropriate, due to potential particle losses in the sampling system.
f – In order to specify this particle size in association with ISO Class 5, the macro particle descriptor M may be adapted and used in conjunction with at least one other particle with ISO Class 5, the macro particle descriptor M may be adapted and used in conjunction with at least one other particle size.
g – This class is only applicable for the in – operation state.

Clean rooms which manufacture sterile medicinal products within the European Union (EU) do so in accordance with "The Rules Governing Medicinal Products in the European Union Volume 4 - Guidelines for Good Manufacturing Practices for Medicinal Products for Human and Veterinary Use" (European Commission 2008). Annex 1 of these guidelines - The Manufacture of Sterile Medicinal Products, covers the manufacture of sterile medical products prepared aseptically, as well as those terminally sterilised following manufacture. Clean rooms which are used to manufacture such products are divided into four grades (A, B, C and D) and are classified entirely upon the maximum number of particles greater or equal to 0.5 µm and 5.0 µm, per cubic metre of air, when the room is both at rest and operational (European Commission 2008), as shown in Table 1 – 2. For example,

a Grade B clean room in operation is permitted a maximum concentration of 2,900 particles per cubic metre of air, equal to or larger than 5.0 µm.

Table 1 - 2: The classification of pharmaceutical manufacturing Grade A - D clean rooms according to the maximum number of particles permitted per cubic metre of air, both whilst at rest and in operation (Authorised reproduction from: European Commission 2008) (© European Union, 1995 - 2017).

	Maximum number of particles per cubic metre of air equal to and larger than the sizes below (µm)			
Clean Room Grade	**Resting**		**Operating**	
	0.5	**5.0**	**0.5**	**5.0**
A	3,520	20	3,520	20
B	3,520	29	352,000	2,900
C	352,000	2,900	3,520,000	29,000
D	3,520,000	29,000	-	-

Grade A clean conditions are required for processes in which products are at unusual risk of contamination (European Commission 2008). This can include the filling of aseptic products or the preparation of aseptically prepared products which are not subsequently filtered (MHRA 2017). The lower grades of clean rooms are used during the stages of sterile manufacturing in which products are less at risk of contamination (European Commission 2008). For example, Grade C clean room uses can include the filling of products which are to be terminally sterilised. Whereas, Grade D clean room use includes the preparation of materials which are required during the product filling process (MHRA 2017).

1.1.4 Clean Room Design

To maintain the classification and integrity of a clean room environment it is fundamental that the introduction, generation and retention of particles within the facility are considered during its design. Firstly, the facility's infrastructure must not contribute towards such particulate introduction, retention and generation; therefore this should be constructed of smooth, continuous and impervious materials (European Commission 2008). Ceilings and fitted utilities

should be sealed, and sinks forbidden where not essential (Beaney 2016). In addition, room corners should be coved and room fixtures and equipment should be kept to a minimum (European Commission 2008). To reduce the introduction of particles into the clean room environment incoming air should be filtered through HEPA filters (Whyte 2010), in addition an elevated degree of over pressure must be produced between differently classified rooms, avoiding particles entering the room from that of a lower classification (European Commission 2008). It is important that any pressure differences between rooms are monitored continuously to ensure that an over pressure of at least 10 Pascal between a classified area and an adjoining room of a lower class exists and that there is at least 15 Pascal between a classified and any unclassified area (Beaney 2016). An interlocking door warning system should be in place to ensure no two air - locked doors are opened at one time, preventing contaminated air passing directly from one space into another (MHRA 2017). A heating, ventilation and air conditioning (HVAC) system must be installed to regulate such air flows, over pressures and changes within a clean room facility using four key principals - filtration, directional air flow, air movement and dilution (Halls 1994). This system also allows the room temperature and humidity to be regulated (Sandle 2011a). Clean rooms which manufacture pharmaceutical products require at least twenty volumetric air changes per hour (NHS Scotland 2004). This should be delivered by either an effective unidirectional or non - unidirectional airflow, designed to control particulate contamination whilst delivering an efficient flow of air into the room (Whyte 2010), examples of which are show in Figure 1 – 2.

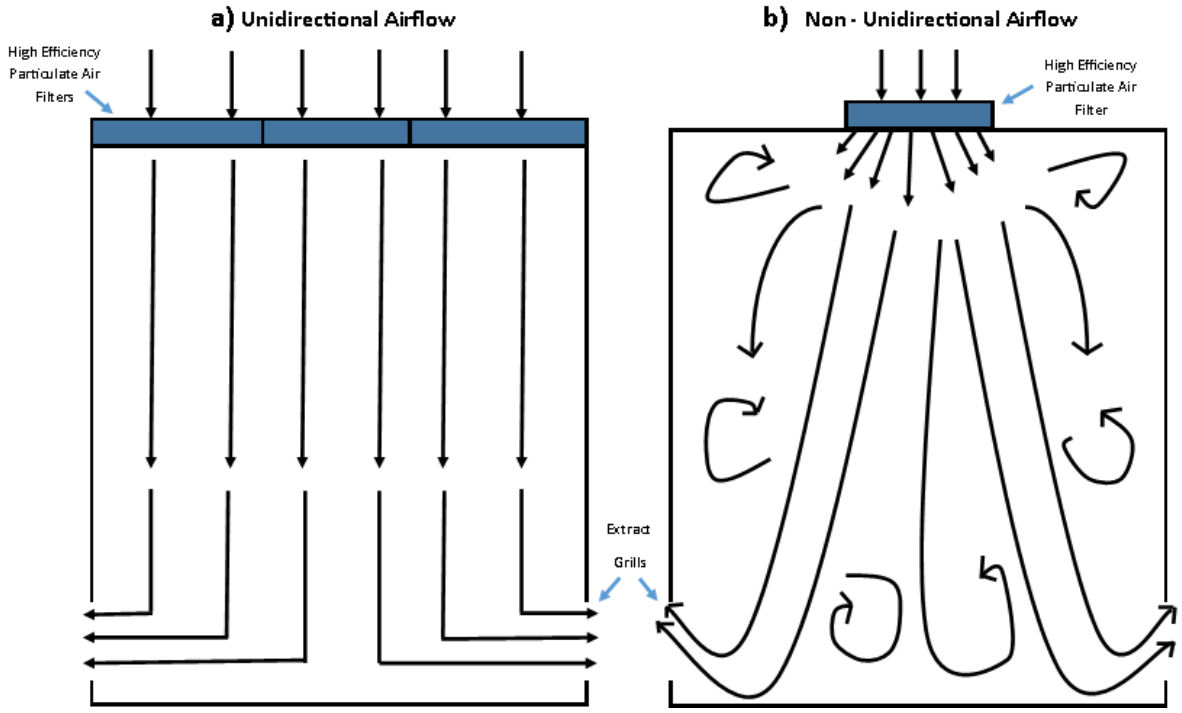


Figure 1 - 2: An example of (a) a unidirectional airflow pattern and (b) a non - unidirectional airflow pattern within a clean room. Arrows illustrate the direction of the airflow into the room, within the space and its exit at extract grills.

A unidirectional air flow pattern (Figure 1 – 2 (a)) is the movement of high speed air throughout the entire room in an unimpeded straight fashion, sweeping away any particles (Sandle 2011a). In contrast, a non - unidirectional or turbulent air flow pattern (Figure 1 – 2 (b)) is the supply of high velocity air through the room's ceiling and its exit at low level grills. The high speed of the air ensures it is in constant turbulent motion, thus avoiding contamination settling. Any remaining particles are diluted with clean filtered air (Sandle 2011a). Computational fluid dynamics (CFD), the analysis of a fluid flow by the means of a computer based equation, is reported as a potentially valuable tool in simulating airflow and the dispersion of contaminants within a room (Lee *et al.* 2002). Such a technique can be a successful tool to assist in the design of clean rooms (Lei 2001), allowing air concentration, pattern and speed to be investigated throughout such an environment (Lee *et al.* 2002). Lee *et al.* (2002) discuss that although CFD simulation results have previously been shown to be in agreement with qualitative and quantitative experimental data (Hosni *et al.* 1996; Gilham *et al.* 1997), disagreement with results has also been reported (Ellacott and Reid 1999). Despite this, such simulations have also previously

been shown to successfully determine the source and route of contaminants within a clean room environment (Noh *et al.* 2009).

1.1.5 Clean Room Contamination

Clean room contamination has been divided into four distinct categories - particulate, biological, chemical or energy (Bryant 2010) - examples of which can be seen in Figure 1 – 3. Although the primary requirement in clean rooms which manufacture sterile pharmaceutical products is to control the number of airborne particles within the room (European Commission 2008), in many technological advanced clean room applications there is also the requirement to control chemical and energy pollutants (Berndt 2011).

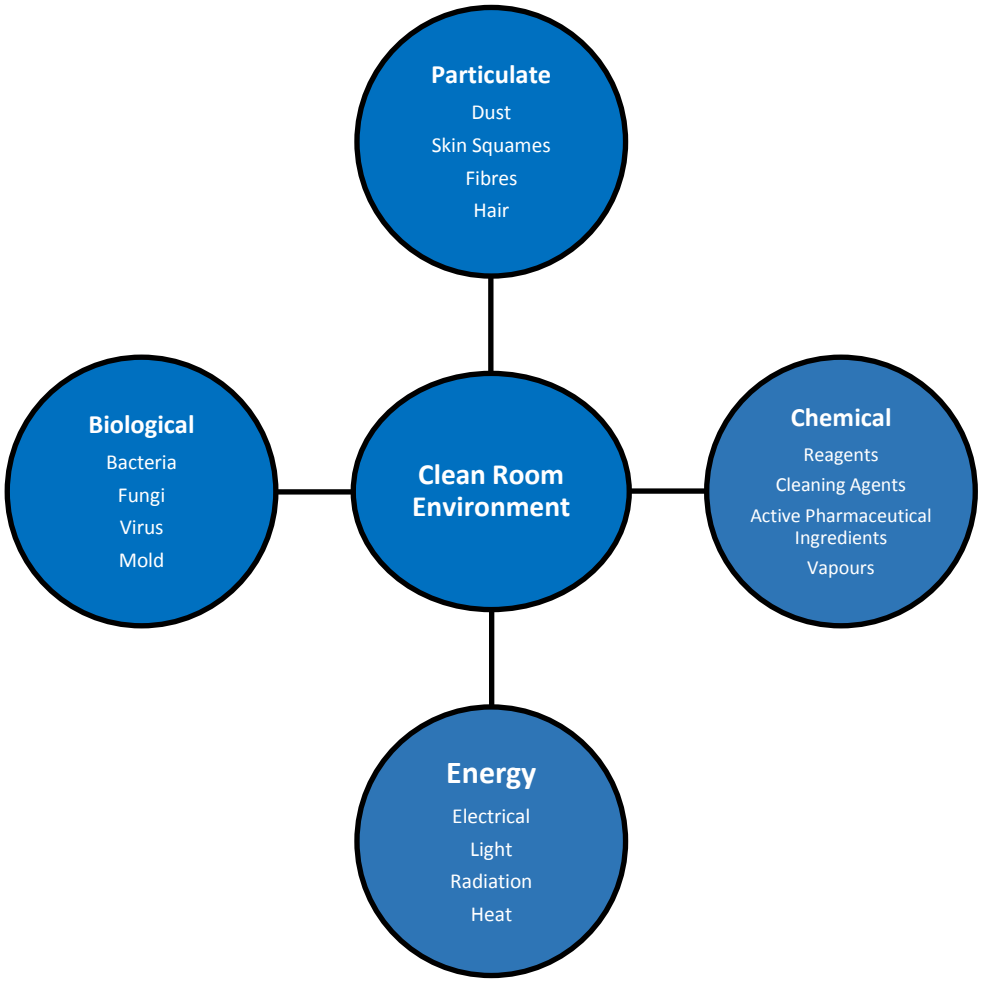


Figure 1 – 3: Some examples of the four major categories of clean room contaminants.

1.1.5.1 Particulate Contamination

Particulate contamination is described as a minute portion of assorted material which can be challenging to chemically quantify (Tran *et al.* 2006). Such contaminants can range in size from sub – micron level up to hundreds of microns, examples of such particles and their sizes can be seen in Figure 1 – 4.

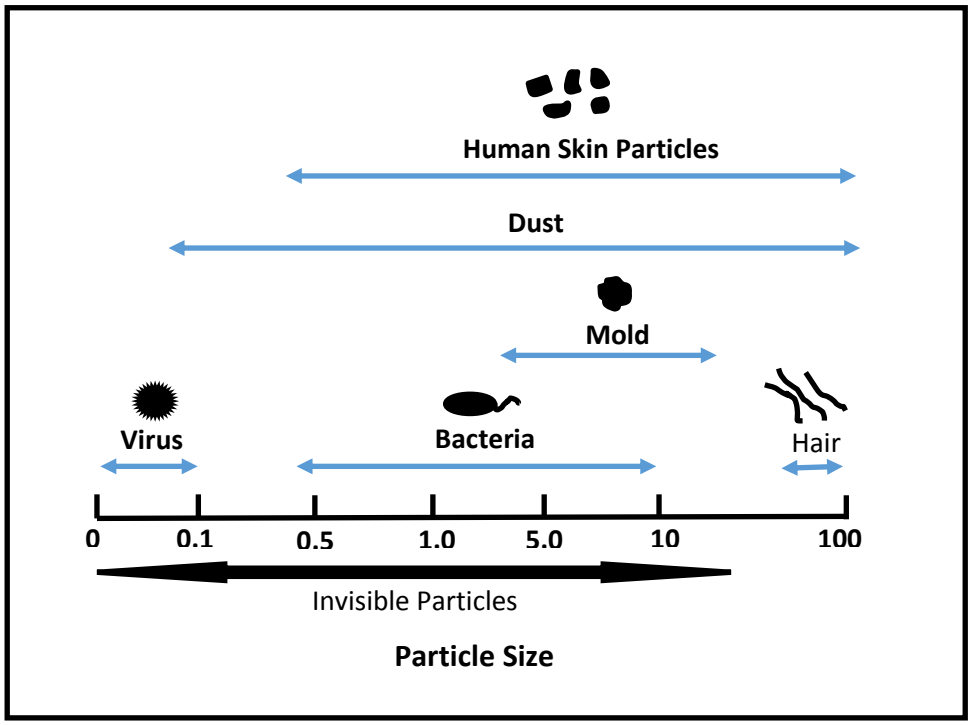


Figure 1 - 4: Typical clean room particulate and microbial contaminants by size.

Particles which can pose as a threat to a clean room environment and its products are classified as viable or non - viable (Whyte and Hejab 2007). Non – viable particles, which are free of organisms, can include dust, dirt and fibres (Kastango 2005). It has previously been reported that non – viable particles can settle into clean room products (Whyte and Hejab 2007). Despite this, historically, as reported by Whyte *et al.* (1979) and Whyte (1983) (cited in Whyte and Hejab 2007) the incidence of viable particles in pharmaceutical products was not thought to be significant, furthermore, Whyte (1987) (also cited in Whyte and Hejab 2007) found that such contamination was unlikely to be to the detriment of the end user. However, if non – viable particles are present in an intravenous medication these have the potential to block veins, capillaries and arteries if they enter into the bloodstream (Tran *et al.* 2006). In fact, such

contamination of intravenous pharmaceutical products has been reported by Tran *et al.* (2006) to have resulted in a variety of serious health conditions including pulmonary inflammation and dysfunction, allergic reactions and even death (Lehr *et al*. 2002; Gecsey and Harrison 2005). In contrast, viable particles (also known as microbe carrying particles - MCPs) are those which act as a transmission mode for microorganisms (required for microbes to move from one area to another) (Elliot 2009), carrying one or more microorganisms (Kastango 2005; Ramstorp *et al.* 2005; Broad *et al.* 2007). If viable particles are introduced into a pharmaceutical product these can have a negative effect on its sterility (Kelly 2005). In fact, microbial contamination of sterile products is a serious occurrence. Not only are such events reported to have led to expensive shut downs and increased facility expenditure (Champagne 2008), but in the worst case scenario microbial contamination of sterile pharmaceutical products has led to patient deaths (MDPH 2012; Pharmacy Practice News 2013; Public Health England 2014; Sprinks 2014; Torjesen 2014). The requirement for such products to be sterile is critical as parenteral products are directly administered into the patient's bloodstream, bypassing their innate immune system (Siew 2013). Furthermore, patients receiving such products may have weakened immunity responses, such as those receiving parenteral nutrition, where the malnutrition suffered by such patients suppresses their immune system (Karacabey and Ozdemir 2012). Therefore, overall, controlling the number of viable and non - viable particles within in the clean room environment is critical in ensuring the integrity and sterility of the product (Broad *et al.* 2007).

1.1.5.2 Microbial Contamination

Microorganisms can be classified into five different groups - algae, bacteria, protozoa, fungi and viruses (Bajpai *et al.* 2011). Although some of these microorganisms can cause disease in humans, plants and animals, many play a significant role in sustaining life on earth including oxygen production and the decomposition of organic substances to be used in the food chain, as well as being used to produce food products and pharmaceutical products such as vaccines and enzymes (Microbiology Society 2018). Although clean room air is filtered, the temperature and humidity of the environment is constantly controlled and all surfaces regularly cleaned to ensure the levels of nutrients in

the environment remain low, this will not eradicate all bacteria (La Duc *et al.* 2007), in addition, any organic matter and water sources within the environment can provide a potential source of nutrition for bacteria (Sandle 2011b). Studying the number and diversity of microorganisms within the clean room environment can help establish effective microbial control (Sandle 2011b). For example, clean room isolates have previously been identified as Gram - positive cocci, which are associated with the human skin, Gram - positive rods, which inhabit the environment and Gram - negative rods, which are found in water (Sandle 2011b; Sandle 2017). Over the years there have been a number of research studies which have focussed upon the qualitative and quantitative analysis of microbes within the clean room environment (Wu and Liu 2007, Martín *et al.* 2012; Park *et al.* 2013; Moissi – Eichinger *et al.* 2015), with the most commonly observed isolates being reported to be bacterial species of *Staphylococcus, Micrococcus*, *Corynebacterium* and *Bacillus,* as well as fungal species of *Aspergillus* and *Penicillium* (Sandle 2011b).

A study of 642 pharmaceutical products recalled between 2004 and 2011 found 79 % of these to be sterile products, of which 11 % were recalled due to microbial contamination (Jimenez and Sutton 2012). In June 2014, Public Health England (PHE) and the Medicines and Healthcare Products Regulatory Agency (MHRA) investigated the bacterial contamination of a batch of total parenteral nutrition bags, which resulted in three neonate deaths (Sprinks 2014). Twenty three cases of neonate septicaemia infection were found to be linked to a batch of total parenteral nutrition, manufactured daily on an individual patient basis by the company ITH Pharma. These were found to be infected with the environmental bacterium *Bacillus cereus* (Sprinks 2014; Torjesen 2014), the same species identified in infected babies, environmental samples from the day of manufacture and in recalled products removed from circulation. Operations at the facility were suspended and following investigation of the company's manufacturing process the MHRA concluded that the incident was isolated (Public Health England 2014). Most clean room microbial research studies focus on bacteria due to their abundance within the environment (Sandle 2011b), however, as found during the 2012 Massachusetts Department of Public Health (MDPH) and United States Food and Drug Administration (FDA) investigation into

a multi - state outbreak of fungal infections, resulting in the death of 64 individuals having received an epidural or para - spinal steroid injection (Pharmacy Practice News 2013), fungi can also be a cause for concern. Infection was found to be linked to three batches of glucocorticoid injection (17,676 doses), manufactured and distributed by the New England Compounding Company (NECC) to 23 states (MDPH 2012). The fungus *Exserholum rostratum,* was identified in recalled sealed vials (MDPH 2012; Pharmacy Practice News 2013), as well as infected patients. As a result the MDPH immediately recalled all products produced by the NECC and suspended operations at the facility (MDPH 2012). Preliminary investigations found several potential contributing factors within the manufacturing facility including soiled tacky mats, surface water, a leaking boiler, residual power and invalidated autoclaves. Furthermore, terminal product sterilization did not conform to required standards (MDPH 2012).

To observe the bacterial bioburden of a clean room facility, identify the main isolates (Akers 1997) and allowing the facility to identify the source of any contamination (Dalmaso *et al.* 2008), an environmental monitoring program should be adopted (Sheraba *et al.* 2007). This should include active and passive monitoring of the air's microbial load, as well as testing operators and room infrastructure including walls and surfaces (Dalmaso *et al.* 2008). Passive air sampling, using agar settle plates, should be undertaken to quantify the number of microorganisms depositing onto a product or surface over a period of time, whereas active air sampling, using a volumetric air sampler, should be used to actively impact air and its associated microorganisms onto the testing media (Tierney *et al.* 2010). Surface cleanliness should be monitored using contact plates (or swabs) and the number of microorganisms on operators' gloved fingers hands monitored using finger dab testing after every working session (NHS Scotland 2004). For optimal results agar plates should be incubated at either 22 ^{o}C to detect fungal growth or 37 ^{o}C to detect bacterial growth. During incubation discrete colony forming units (CFU) grow, which allow bacterial or fungal numbers to be quantified. The number of colony forming units permitted, under each sampling method, within a European Union Good Manufacturing Practice (EU GMP) operational clean room are shown in Table 1 - 3.

Table 1 - 3: Recommended limits for microbiological monitoring of grade A – D clean rooms during operation (Authorised reproduction from: European Commission 2008) (© European Union, 1995 - 2017).

Clean Room Grade	Active Air Sample (cfu / m^3)	90mm Settle Plates (cfu / 4 hours)	55mm Contact Plates (cfu / plate)	Glove print (5 fingers. Cfu / glove)
A	<1	<1	<1	<1
B	10	5	5	5
C	100	50	25	-
D	200	100	50	-

If microbial and particulate limits are exceeded and trend upwards, corrective remediation should be taken according to written operating procedures (European Commission 2008).

1.1.6 Clean Room Operators

The literature reports that operators are the primary source of clean room contamination (Whyte and Hejab 2007; Strauss *et al.* 2011; Casser 2011a, Chen *et al.* 2013; Sandle 2014). In fact, over 80 % of clean room pollutants have been identified as human in origin (Bryant 2010). These include hair, skin products, chemical and organic material, vapours from the nares and mouth, biological contamination such as bacteria, fungi and viruses (Berndt 2011), as well as the principal operator associated contaminant - skin flakes (Ramstorp *et al.* 2005). Skin flakes, also known as skin squames, are small keratin fibres and crosslinked cornified envelopes (Grice and Segre 2011). These scales or flakes of dead skin (each averaging 33 µm x 44 µm x 4 µm in size) (Sandle 2014) are constantly shed by the human body due to the continuous replacement of epidermal cells (Grice and Segre 2011). This shedding is promoted by the rubbing action of clothing against the skin's surface, with squames evading this clothing, entering into the airstream and subsequently the environment (Clark and de Calcina - Goff 2009). In fact, a previous research study by Strauss *et al.* (2011) states that for each minute a clean room operator works they will shed approximately 1.7 x 10^4 skin particles into the clean room environment, elevating the

particulate concentration of each cubic metre of air by 1742 ± 481. The literature reports that 10 % of desquamated skin particles carry approximately four microorganisms each (Sandle 2014). This is owing to the skin surface being colonised by up to one million organisms per its square centimetre (Sandle 2014) - growing predominantly on the skin cells and around the glands (Whyte and Hejab 2007), as shown in Figure 1 – 5.

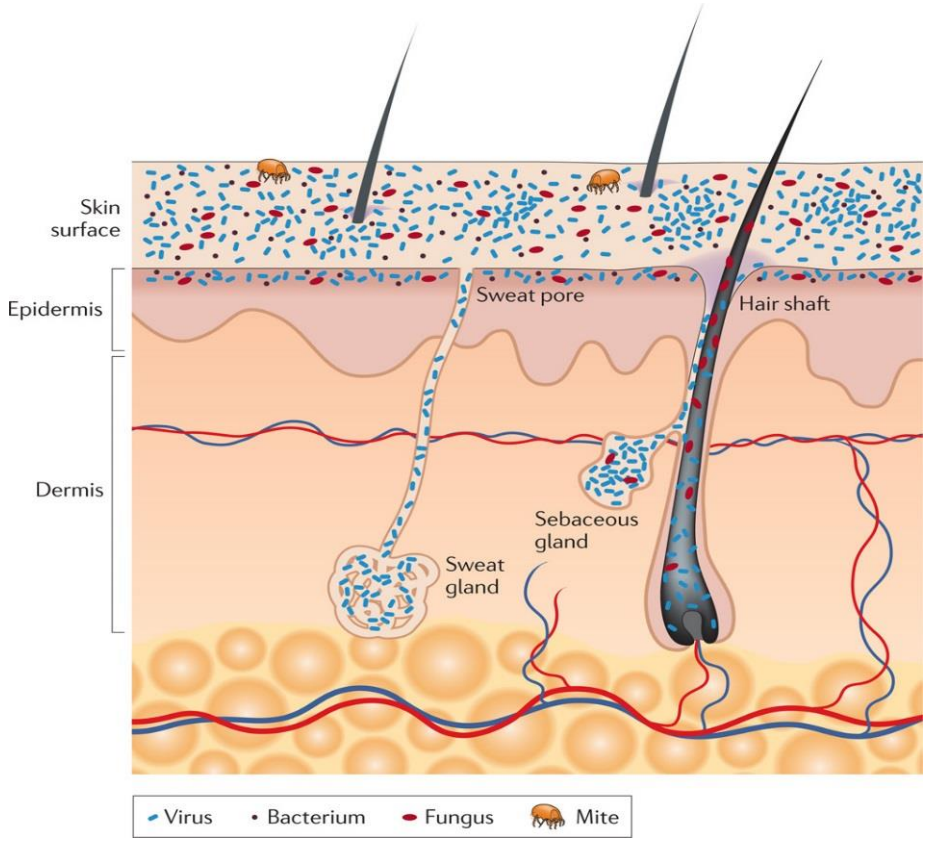


Figure 1 – 5: Schematic of skin histology viewed in cross – section with microorganisms and appendages (Reprinted with permission from Springer Customer Service Centre GmbH, Springer Nature, Nature Reviews Microbiology, The Skin Microbiome, Grice and Segre, [© Springer Nature] (2011)).

The primary bacterial skin colonizers are reported as species of *Staphylococcus, Corynebacterium, Propionibacterium, Brevibacterium* and *Micrococcus* (Grice and Segre 2011), however, the quantity and diversity of microorganisms on an individual's skin can be dependent upon a number of factors such as their genetics, lifestyle and living environment, as well as their bioburden of transient microorganisms (Zeeuwen *et al.* 2012). Furthermore, the human skin's microbial load is dependent upon body site, with moist areas and those with more

sebaceous glands and skin lipids being found to harbour more microorganisms (Reichel *et al*. 2011), as shown in Figure 1 – 6.

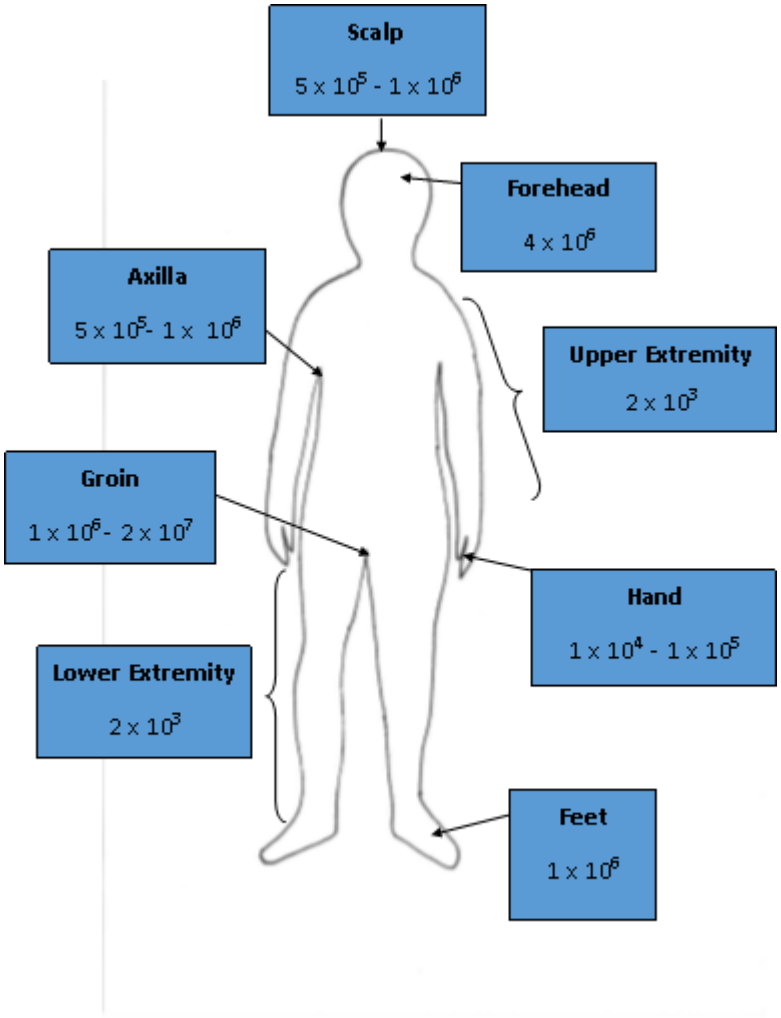


Figure 1 – 6: The approximate number of microorganisms per square centimetre of skin at various body sites (Adapted from: McGinley *et al.* 1975; Leyden *et al.* 1981; Leyden *et al.* 1987; Sandle 2014).

The variety of bacteria on the human skin is also dependent upon body site (Grice and Segre 2011; Grice *et al.* 2009). The diversity of microorganisms on the human skin at 20 different body sites, under three different microenvironments, sebaceous, moist and dry, was investigated by Grice *et al.* (2009) using 16S gene sequencing. The percentage of each bacterial group in each environment is presented in Figure 1 – 7.

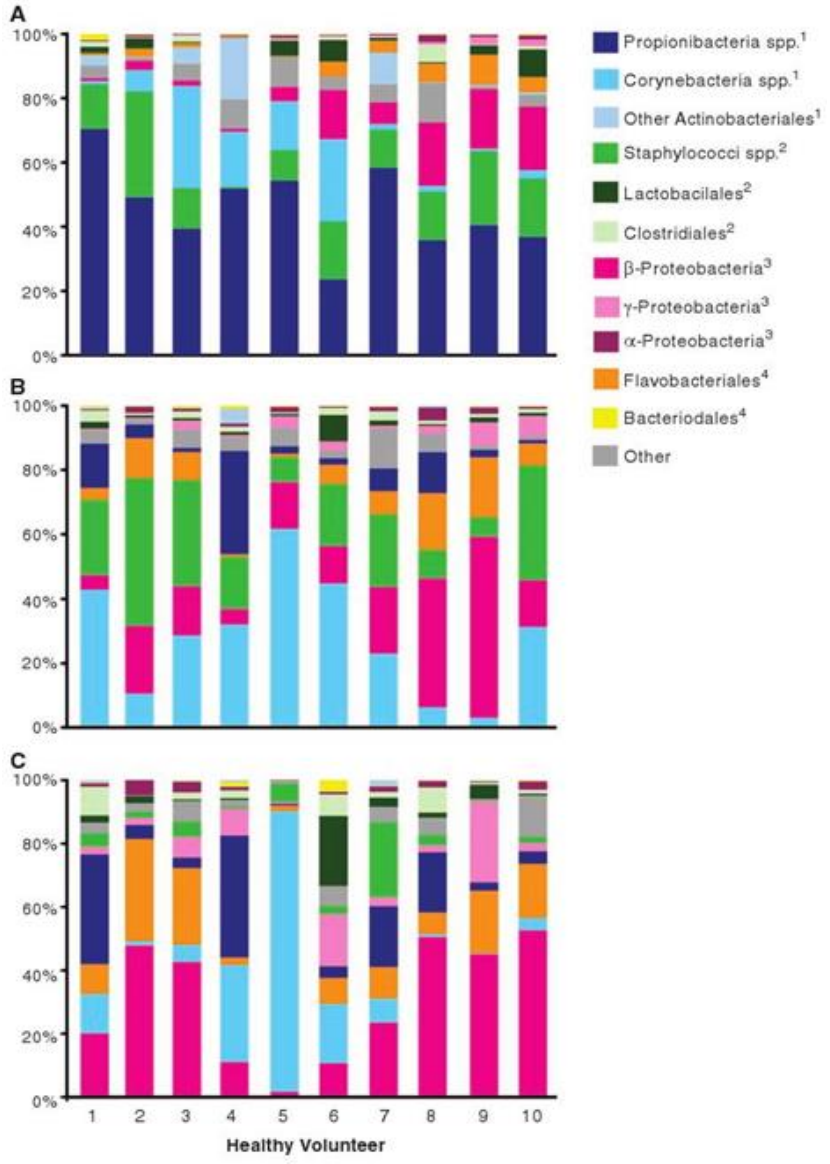


Figure 1 – 7: The 20 skin sites and associated microbiota are representative of three microenvironments: (A) sebaceous, (B) moist, and (C) dry. The relative abundance of the most abundant bacterial groups associated with each microenvironment is depicted for each healthy volunteer. Superscripts indicate phylum: 1, Actinobacteria; 2, Firmicutes; 3, Proteobacteria; 4, Bacteroidetes. (Taken from: Grice *et al.* 2009 [Topographical and temporal diversity of the human skin microbiome. *Science,* 324 (5931); 1190 - 1192]. Reprinted with permission from AAAS).

As shown in Figure 1 – 7, species of *Propionibacterium* were found to dominate the sebaceous and oily areas of the body, whereas species of *Corynebacterium* were found to be favour more moist areas (Grice *et al.* 2009). Furthermore, the diversity of bacteria at specific body sites can vary between species. An example is *Staphylococcus aureus*, which can cause disease in humans (Sattar *et al.* 2001) and which has been shown to be predominantly harboured in the nasal

cavity (Kloos and Musselwhite 1975; Grice *et al.* 2009). Whereas, Staphylococcal species *epidermidis* and *hominis* have been identified in abundance in the axilla, head, leg and arm regions (Kloos and Musselwhite 1975).

Whilst being the primary source of clean room pollutants, operators also have the greatest potential for contamination control. Over the years there has been an increase in technological advances in a bid to reduce operator associated contamination. For example, the use of isolators, used to separate the external environment from the critical working area (Siew 2013). Furthermore, in a bid to reduce contamination within the clean room environment operators must undertake regular training which covers all areas of the manufacture of sterile pharmaceutical products (European Commission 2008). One previous study by Yang and Fu (2002) found that operator movement significantly affects airflow patterns within the clean room environment, causing zones of particle recirculation to form around the operator and workbench. Therefore, the number of operators working with a room, their movement and speech all must be kept to a minimum. A high standard of personal hygiene and cleanliness of operators working in a clean room is also essential (European Commission 2008; European Commission 2013). The European Commission (2013) state that operators working in clean rooms which manufacture medicinal products for human use should follow detailed hygiene programmes including protocols which relate to their health and hygiene practices. In addition, operators must wear an arrangement of specialist clean room garments appropriate to the grade of clean room in which they will be working (European Commission 2008; European Commission 2013; Beaney 2016; MHRA 2017).

1.1.7 Specialist Clean Room Garments

The 1950's saw a shift from clean room operators wearing traditional laboratory coats to fully gowned specialist attire (Siew 2013). Nowadays, such specialist clean room garments form an active part of any clean room environmental control program (McIlvaine and Tessien 2006; Whyte and Hejab 2007; Bryant 2010; Cassar 2011; Larkin 2012; Chen *et al.* 2013). Such garments help maintain the integrity of the clean room environment by keeping the levels of particulates and associated microorganisms within the defined numbers (Cassar 2011). The European Commission (2008) address the use of specialist garments

to be worn in clean rooms which manufacture sterile medicinal products. Such guidelines appear easy to follow, however, specialist clean room garments play an important role in controlling contamination within the environment, and therefore there are many aspects associated with such attire which must be considered (Cassar 2011). The crucial factor is that these garments form a protective barrier between the operator and the environment (Sandle 2014) and have good filtration efficiency, capturing and containing particles originating from the operator (Eudy 2003). Additionally, clean room garments should possess the following characteristics (Eudy 2003; Cassar 2011; Sandle 2014):

- Produce low levels of particulate shedding
- Be loose fitting and comfortable
- Allow the body to breath
- Be comfortable to wear
- Withstand multiple laundering and sterilisation cycles
- Dissipate static
- Protect the operator

In order to achieve these objectives clean room garments are manufactured using either non – woven and woven textiles (Eudy 2014), with consideration made to the permeability, pore size, particulate retention (Ljungqvist and Reinmüller 2005) and linting properties (Eudy 2003) of the fabrics selected. Non - woven fabrics are manufactured using polyolefin fibres (Eudy 2014) which are mechanically, chemically, thermally or physically fussed or bonded together at their cross over points (Kayaoğlu 2016), as shown in Figure 1 – 8. Non – woven fabrics are commonly used to manufacture disposable clean room garments (Eudy 2014). An example of a non – woven fabric used to manufacture single - use clean room garments is Tyvek® (Whyte 2010), which is reported to exhibit excellent barrier efficiency whilst being comfortable to wear (Holbrook 2015). However, previous studies have shown that wearing garments constructed of such non – woven fabrics can reduce the permeability of air between the operator's skin and the environment, increasing the body temperature and stress of the individual wearing the garment, making it uncomfortable to wear (Hao *et al.* 2004; Laing 2008; Chen *et al.* 2013).

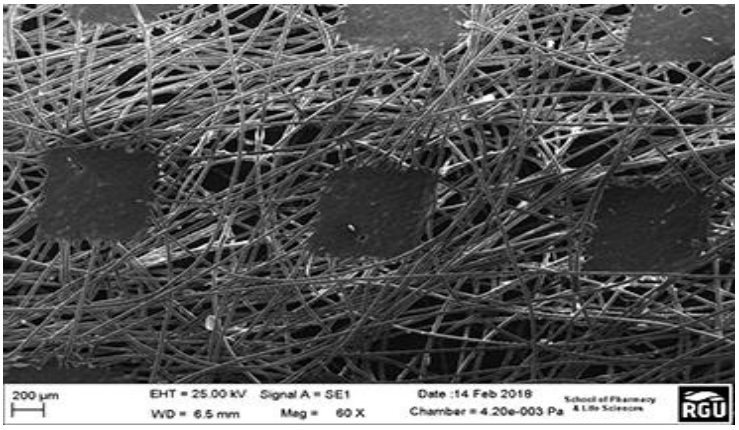


Figure 1 – 8: A scanning electron microscope (SEM) image showing the surface of a non – woven face mask, the fibres are fused together at their cross over points.

Polyester fabric is an example of a woven fabric which is commonly used to manufacture reusable clean room garments, produced by weaving continuous polyester filaments (Sandle 2014), which is made conductive by the presence of a carbon thread (Eudy 2014), as shown in Figure 1 – 9. Garments constructed of non - woven fabrics can reduce the number of operator – borne bacteria entering into the clean room environment (Whyte and Hejab 2007). However, the fibres of such fabrics can breakdown and as found by Lee *et al.* (2012) the abrasion of such carbon filament polyester fabric can also result in an increase of airborne particles.

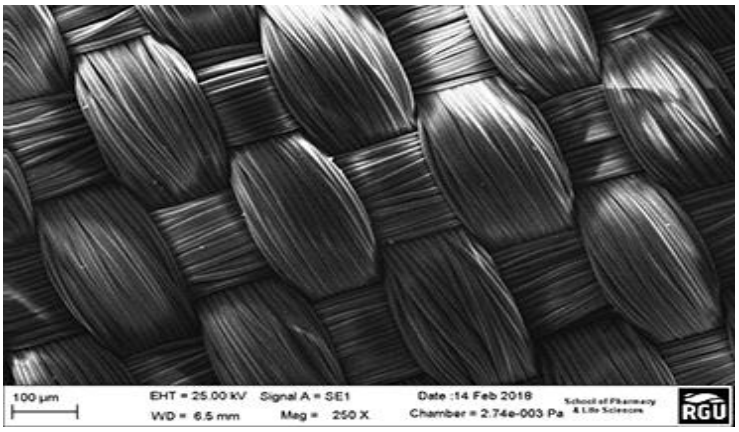


Figure 1 – 9: A Scanning electron microscope (SEM) image showing the surface of a woven antistatic carbon filament polyester clean room suit.

In clean rooms which manufacture sterile pharmaceutical products, and in order to protect the product from contamination, operators are required to wear a

specific arrangement of non – linting, sterile garments (European Commission 2008; Beaney 2016; MHRA 2017). These garments are designed so that they integrate seamlessly to one another during wear. Examples of such garments and their purpose are summarised in Table 1 – 4.

Table 1 – 4: A summary of sterile clean room garments to be worn in a pharmaceutical clean room and their purpose (Adapted From: European Commission 2008; Beaney 2016; MHRA 2017).

Type of Garment	Purpose of Garment
Hair / Facial Hair Cover	To cover and contain all hair
Sterile Gloves	To reduce the transfer of hand borne contamination and to protect the hands
Sterile Face Mask	To minimises the shedding of droplets from the oral cavity and nasal regions
Sterile Headgear	Covers the entire head and neck. Should be tucked into the neck of the protective suit
Sterile Protective Suit or Coat (single or two piece)	Covers the body, minimising particulate contamination from the skin. Should have a high neck and be tight at the wrists. Protective suit trouser legs should be tucked into over boots.
Sterile over Shoe / Boot	Covers the feet Reduces contamination being introduced into the clean room

The arrangement of clothing required to be worn within a pharmaceutical clean room manufacturing medicinal products for human use is specified by its room class and process (European Commission 2008; Beaney 2016; MHRA 2017). Operators working in a Grade A / B clean room are required to cover as much of the body as possible. To achieve this a general clean room suit, high at the neck and gathered at the wrist, should be worn. Operators should also don a protective hood, tucked into the neck of the clean room suit. In addition, a sterile face mask, footwear and sterile gloves should also be worn, with the trouser leg

of the suit tucked into footwear and sleeves tucked into gloves (Figure 1 – 10). Operators working in a Grade C or D pharmaceutical clean room are also required to don a general clean room suit (or coat – Grade D) and to cover all hair including beards, using a hair net. Shoe covers or over boots should also be worn (Beaney 2016).

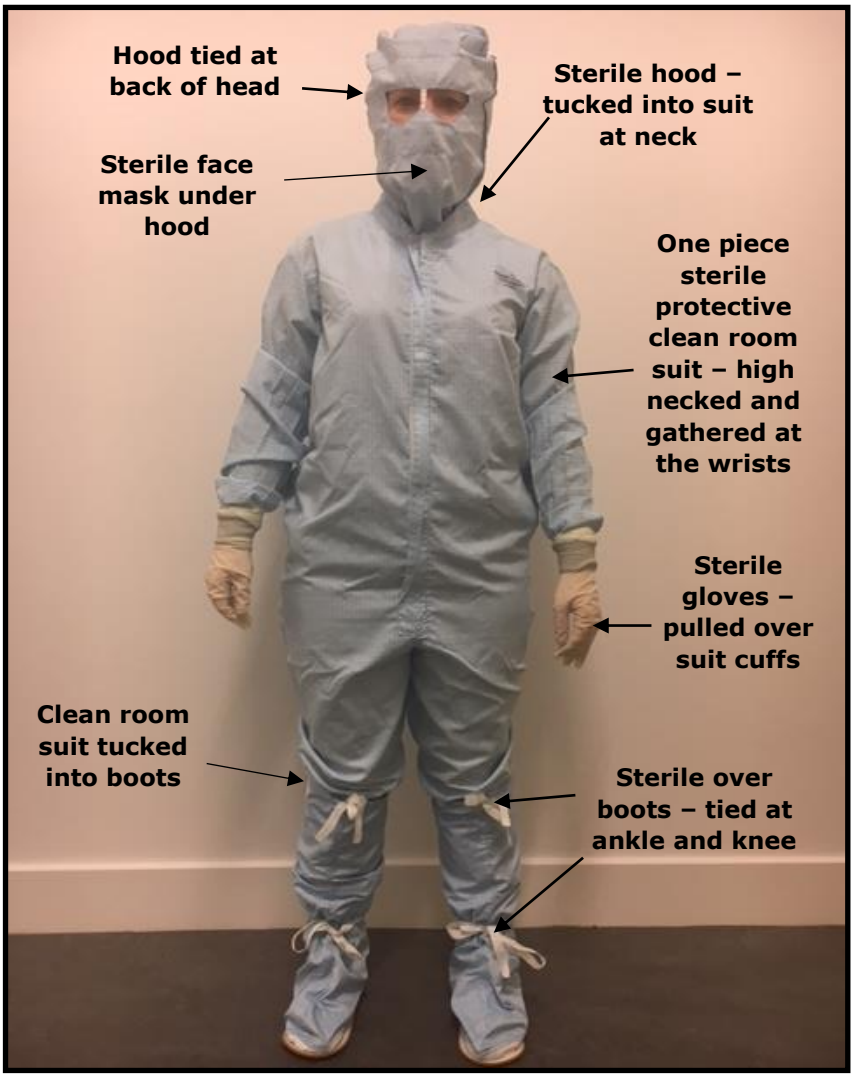


Figure 1 - 10: The typical arrangement of garments to be worn in a Grade A / B clean room (Adapted from: Beaney 2016).

Clean room garments themselves should not increase the numbers of particles and microorganisms being introduced into the environment. However, there have previously been concerns that reusable clean room garments returning from out – sourced laundering / sterilisation facilities lack in sterility (Larkin 2009), although the levels of such contamination events are not reported in the literature. Despite this, the efficiency of such laundering / sterilisation processes

is an area which lacks in research. Furthermore, following donning, the outer surface of clean room garments should remain sterile (Rhodes 2006), however, contamination of clean room garments can occur as the result of poor gowning processes, where microorganisms are transferred onto the garment by the hand borne route (Siew 2013). In fact, one case report highlighted that the contamination of a batch pharmaceutical products was in part the result of inadequate clean room gowning processes (Friedman 2005). Despite this, and reports that contaminated hands will act as a transmission mode for microorganisms (World Health Organisation 2009), there is no standardised protocol for wearing hand attire during the clean room garment donning process and no published studies which assess this process. Furthermore, to ensure the integrity of the product, during the working duration the bacterial bioburden of the surface of garments worn by operators in the clean room should remain low, preferably zero (Rhodes 2006). However, despite research showing that cleanroom garments can significantly reduce the airborne dispersion of particles from operators (Ramstorp *et al.* 2005; Whyte and Hejab 2007), such attire will not contain all sources of human particulate and biological contamination, as shown in Figure 1 – 11.

Figure 1 – 11: Diagram showing the migration of particles and their associated microorganisms through specialist clean room garments into the clean room environment (red arrows represents the movement of particles throughout the garment and their potential exit points, blue arrows represent the dissemination of particles through pores and seams in the fabric and the green arrow represents the penetration of the face mask and hood by bacteria residing in the oral and nasal cavity).

As shown in Figure 1 – 11, abrasion of the skin by garments can detach squames (Clark and de Calcina - Goff 2009), this along with clean room operator movement has been show to assist particles in travelling up and down the garment in the direction of both the head and feet (Eudy 2003), with such particles becoming part of the airstream, flowing from the garment into the environment (Clark and de Calcina - Goff 2009). Furthermore, particles including those carrying microorganisms have been shown to disseminate through

protective clean room garments (Ramstorp *et al.* 2005; Whyte and Hejab 2007), penetrating pores, seams, pinholes and defects (Laing 2008). In fact, research has shown that the surface of such clean room garments becomes contaminated with microorganisms as operators work, with the levels of such contamination increasing over time (Grangé *et al.* 2010). This is of concern as clean room garments could act as a vehicle for the transmission of microorganisms, which may have a detrimental impact upon the sterility and integrity of products manufactured within the room, and ultimately the end user's health.

1.2 Research Aims & Thesis Overview

A general review of the limited clean room literature available, as detailed above, has highlighted the importance of clean room use in the pharmaceutical industry, as well as the significance of maintaining contamination control within such facilities, especially with respect to clean room operators and their specialist garments. However, there is a severe lack of published literature which assesses this topic. Therefore, the primary aim of this study was to address this absence of research by evaluating a number of aspects associated with clean room operators and their specialist clothing and to use any findings to contribute towards improving contamination control standards within clean room facilities. The research project was split into five stages and was undertaken through addressing a series of objectives:

- To develop a surface sampling method which could subsequently be used to recover, enumerate and compare the surface bacterial bioburden of reusable antistatic carbon filament polyester clean room garments during the remainder of our study.
- To compare the bacterial bioburden of the surface of reusable antistatic carbon filament polyester clean room garments following their laundering with and without terminal gamma sterilisation.
- To compare the surface bacterial bioburden of reusable antistatic carbon filament polyester clean room suits following their donning by operators dressing wearing either no gloves, non - sterile gloves or sterile clean room gloves.

- With respect to gender, compare the bacterial bioburden of the surface of reusable antistatic carbon filament polyester clean room garments following their wear in the clean room environment.
- To seek the identification of a representative selection of bacterial isolates recovered from the surface of reusable antistatic carbon filament polyester clean room garments during the laundering and gender comparison studies by comparing the identification efficiencies of both phenotypic and genotypic identification methods.

Chapter 2:

A Comparison and Evaluation of the Quantitative Recovery of *S. aureus* NCTC 6571 from Antistatic Carbon Filament Polyester Fabric using Direct Agar Contact and Swabbing Methods of Surface Sampling

2.1 Introduction

Bacteria will attach to surfaces (Busscher and van der Mei 2012), including textiles (Bajpai *et al.* 2011), often as their preferential mode in which to grow and survive (Lindsay and Von Holy 1999). The number of bacteria present on a surface or object can be enumerated and assessed using one of a number of environmental surface sampling methods including swabbing, direct agar contact or dip slides (Salo *et al.* 2000), as well as wipes (Downey *et al.* 2012). The aim of this part of our study was to compare the recovery efficiency of the two most commonly employed aforementioned tools, contact plates and swabs, and subsequently develop a surface sampling method which could be used to successfully recover, enumerate and compare the levels of bacteria on the surface of reusable antistatic carbon filament polyester clean room garments during the remainder of this study.

2.1.1 Bacterial Adhesion to Surfaces

Bacterial adherence to a surface can be classed as either reversible, occurring in the initial stage of attachment when the bacterium is only weakly attached to the surface and lasting for only milliseconds, or irreversible, when the microorganism forms a biofilm (Sandle 2013). Biofilms are a survival mechanism by where the adhering organism protects themselves within a self - produced matrix of extrapolymetric substances (EPS) (Busscher and van der Mei 2012). The stages of biofilm formation can be seen in Figure 2 – 1.

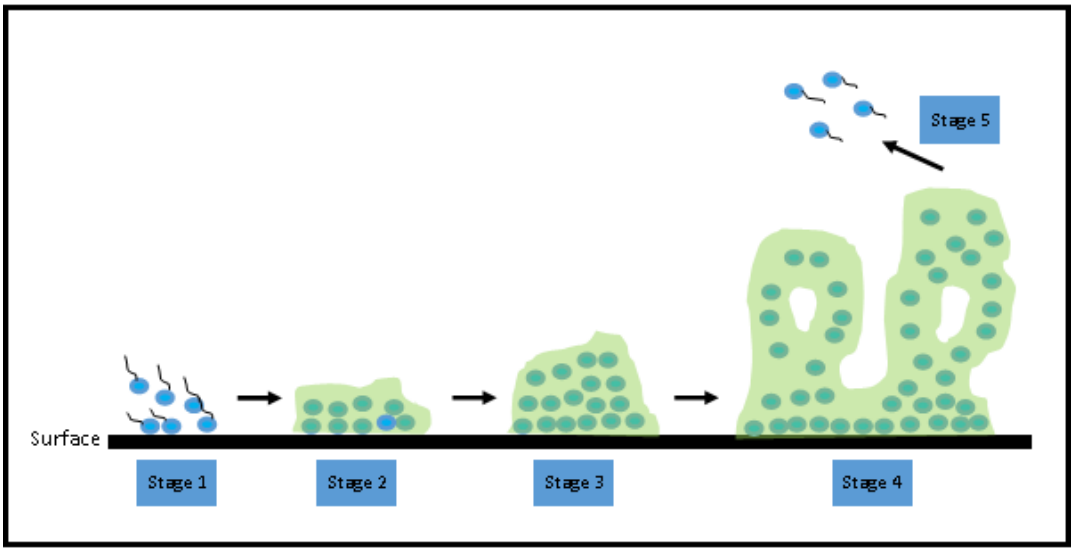


Figure 2 – 1: The five stages of biofilm formation.

The first stage of biofilm formation (Figure 2 – 1) occurs as a result of attractive forces (Sandle 2013), where the organism attaches to a surface (Busscher and van der Mei 2012). Whether a bacteria will be attracted to or repelled by a surface is dependent upon the forces around the bacterial cell and the charge of the adhering surface. For example, if a negatively charge bacterium comes in the close vicinity of a positively charged surface these will readily attract one another (Montville and Schaffner 2003). This can be explained using the DLVO (Derjaguin and Landau, Verwey and Overbeek) theory, which is described as the *"net interaction (V_{TOT}) between a cell and surface as a balance between two additive factors: (V_A) resulting from van der Waals interactions (generally attractive) and repulsive interactions (V_R) from the overlap between the electrical double layer of the cell and the substratum"* (Katsikogianni and Missirilis 2004 p. 41). In Stage 2, also illustrated in Figure 2 – 1, if a bacterial cell is attracted to the surface, EPS are produced and it is at this stage in the process at which irreversible attachment occurs. Bacterial growth follows at stages 3 and 4, before the final stage can involve bacteria being released from the biofilm and potentially spreading (Sandle 2013).

Bacterial attachment to a surface is strongly influenced by a number of factors including the organism's strain (Sandle 2013) and cell characteristics (Bajpai *et al.* 2011), as well as general environmental conditions such as temperature, humidity and pH (Garrett *et al.* 2008). Attachment is also a consequence of the physical and chemical properties of the adhering surface including its shape and structure, pore size, texture and hydrophobicity (Foschino *et al.* 2003). For example bacterial cells have been shown to be more readily attracted to rougher rather than smooth surfaces, thought to be due to an increase in surface area (Scheuerman *et al.* 1998). In fact, Bellon – Fontaine *et al.* (2008) (cited in Grangè *et al.* 2010) found that bacterial adhesion to garments is effected by fabric irregularity. The composition of the adhering surface can also influence bacterial adhesion, depending upon its hydrophobicity and charge (Katsikogianni and Missirilis 2004). For example, surface polarity has been shown to be an important factor in bacterial cell adhesion and spread, with a greater degree of these being promoted by a higher degree of surface polarity (Harnett *et al.* 2007).

2.1.1.1 Bacterial Adhesion to Textiles

The composition of textiles including those used to manufacture clothing can provide a suitable substrate for bacterial adherence (Bajpai *et al.* 2011). In fact, bacteria have been shown to interact with the fibres of such textiles during a succession of stages. Firstly, bacteria will attach themselves to the fibre, next the bacteria will grow, where it may potential cause damage, before finally spreading (Hsieh and Merry 1986). Furthermore, the transfer of bacteria from a textile can occur by the fabric directly touching another surface or by the material disseminating particles which will carry microorganisms (Sattar *et al.* 2001). Neely and Maley (2000) found that although there are a number of studies which assess the ability of bacteria to survive on solid surfaces (Pettit and Lowbury 1968; Dickgiesser and Ludwig 1979; Noskin *et al.* 1995; Bonilla *et al.* 1996) research investigating the ability of microorganisms to survive on textiles is limited. Moreover, those studies which do exist assess the survival of microorganisms on fabrics including cotton, polyester and cotton / polyester blended fabrics (Hsieh and Merry 1986; Neely and Maley 2000; Schmidt – Emrich 2016), but not specifically to antistatic carbon filament polyester fabric, which is commonly used to manufacture reusable clean room garments. Despite this, these previous studies show that polyester fibres are vulnerable to microbial adherence, an example of which is shown in Figure 2 – 2.

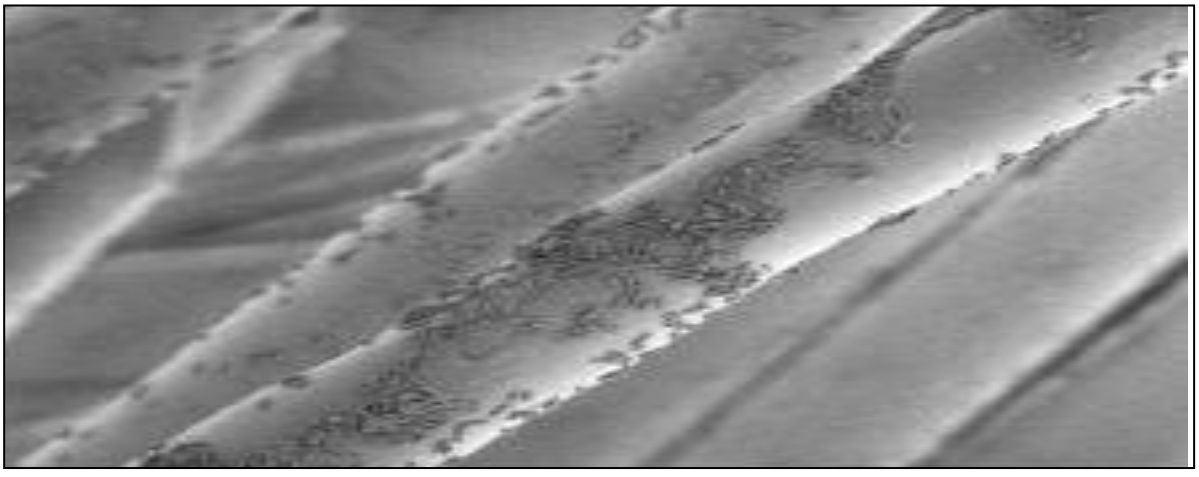


Figure 2 – 2: SEM image of *P. aeruginosa* cells on polyester fibre after 2 hours attachment (Taken from: Schmidt – Emrich *et al.* 2016, © 2016 by the authors, licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons by Attribution (CC - BY) license (http://creativecommons.org/licenses/by/4.0/).

Bajpai *et al.* (2011) studied the adherence of *E. coli* cells to three different fabric compositions - cotton, polyester and a polyester blend – greatest adherence was observed with cotton fibres, with the least number of cells adhering to the polyester fabric. However, Hsieh and Merry (1986) studying the same effect found bacterial textile adherence to be species specific with *Staphylococcus* cells adhering to polyester in greater abundance than *E. coli* cells. Although, this was also found to be affected by inoculum concentration, with greater bacterial adherence observed at higher densities. Neely and Maley (2000) found that potentially pathogenic *Staphylococcus* species were viable on polyester fabric, stored in a biosafety cabinet at 22.9 – 24.5 °C and between 30 – 49 % humidity, for between 1 and 56 days after their initial adherence. This is of concern to the clean room industry, as if Staphylococcal species such as *S. aureus* (found on the skin and in the nasopharynx of humans (Sollid *et al*. 2014) and responsible for colonising in and infecting hospital patients (Shiomori *et al.* 2002) disseminate from operators, contaminating the outer surface of clean room garments, and entering into the clean room environment or product, this could cause not cause patient disease but as previously discussed, their death. Furthermore, bacteria have also been shown to cause degradation of textile fibres (Hsieh and Merry 1986), this could lead to a reduction in the barrier efficiency of the fabric, which is an essential property of clean room garments.

2.1.2 Environmental Surface Sampling

Environmental surface sampling has become a focus of scientific research (Downey *et al.* 2012). Early surface sampling methods are reported to date as far back as 1917, however, it wasn't until the mid - 20th Century that determining the levels of bacteria on a surface using surface sampling grew, primarily in the food industry (Frawley *et al.* 2008). Surface sampling again entered the forefront of research following the 2001 anthrax bioterrorism attacks (Frawley *et al.* 2008; Downey *et al.* 2012), creating a need to adapt the commonly used but historical swabbing method of surface sampling originally developed in the early 20th century (Mannheimer and Ybanez 1917). Nowadays, surface sampling is prevalent across a wide range of industries including those associated with space exploration and medical institutions (Downey *et al.* 2012). Over the years various surface sampling research studies have been undertaken, focusing on developing a standardised surface sampling technique for the quantitative recovery, enumeration and

identification of microorganisms from surfaces. These studies assessed the recovery of biological agents from a surface using a number of different materials including swabs, contact plates, sponges and adhesive tapes (Salo *et al.* 2000; Moore and Griffith 2007; Edmonds *et al.* 2009; Downey *et al.* 2012; Galvin *et al.* 2012). However, such studies have shown that generally a lack of standardised methods and / or materials has led to inconsistent results obtained. For example, recovery of pre – inoculated bacteria from a stainless surface using cotton swabs has been shown to range from 0.47 % (Obee *et al.* 2007) to 89 % (Edmonds *et al*. 2009). In addition, bacterial recovery using macro foam swabs was shown to range from 31.7 – 49.1 % (Hodges *et al.* 2006) to 89 % (Edmonds *et al*. 2009).

The efficiency of any surface sampling technique can be assessed by contaminating a surface with a known concentration of a microorganism before recovering and quantifying this using one of the aforementioned methods. However, there are a number of factors which can affect the efficiency of a surface sampling method and which should be considered during sampling including bacterial specie and its ability to adhere to and penetrate the surface (Poletti *et al.* 1999), bacterial seeding density (Hsieh and Merry 1986; Salo *et al.* 2000; Probst *et al.* 2001; Frawley *et al.* 2008), time of agar contact (Foschino *et al.* 2003) and the moisture content of the sampling surface (Marples and Towers 1978; Craythorn *et al.* 1980; Moore and Griffith 2002a; Moore and Griffith 2002b). There are a number of research studies which have previously investigated the recovery efficiencies of various surface sampling methods and tools using this technique (Poletti *et al.* 1999; Salo *et al.* 2000; Moore and Griffith 2002a; Moore and Griffith 2002b; Foschino *et al.* 2003; Kusumaningrum *et al.* 2003; Rose *et al.* 2004; Obee *et al.* 2006; Dalmaso *et al.* 2008; Frawley *et al.* 2008; Downey *et al.* 2012), however, the majority of these previous studies cannot be compared directly to this part of our current study due to variables within the sampling materials, surfaces and methods employed. For example, most of these previous studies assess the recovery of bacteria from hard and non - porous model surfaces, which does not consider variables such as depth, composition and porosity, all of which are important when assessing a method's ability to recover bacteria from textiles. Whilst one method of recovery may recover an organism from a surface it may not recover the same quantity of an organism from another surface (Edmonds 2009).

For instance, cotton swabs were found to recover 88.7 % of liquid deposited *Bacillus atrophaeus* cells from glass whereas only 49 % of those deposited onto a vinyl tile were recovered using the same method (Edmonds *et al.* 2009). There are studies which investigate the recovery of bacteria from fabrics, however, most of these do so in the hospital setting and study surfaces such as linoleum, mattresses (Claro *et al.* 2014), bedding (Shiomori *et al.* 2002), curtains (Das *et al.* 2002), white coats (Nealy and Maley 2002; Treakle *et al.* 2009), towels, scrubs, drapes and aprons (Neely and Maley 2000). Aside from Grangè *et al.* (2010), there are a lack of studies which focus on the adherence and penetration of bacteria onto or through the surface of antistatic carbon filament polyester clean room operator's garments. Furthermore, those other studies which do exist are small in size and remain unpublished (Tayside Pharmaceuticals 2007; Tayside Pharmaceuticals 2010).

2.1.2.1 Swabbing Method of Surface Sampling

Swabbing is a method of surface sampling reported to be widely used in hygiene monitoring (Moore and Griffith 2002b) and involves rubbing a swab bud over the sampling site to collect microorganisms (Figure 2 – 3 (a)). The bud is then spread over solid nutrient medium (Figure 2 – 3 (b)) or placed into a suitable re - suspension fluid or diluent, before the level of growth achieved is semi - quantitatively evaluated (Moore and Griffith 2007) (Figure 2 – 3 (c)).

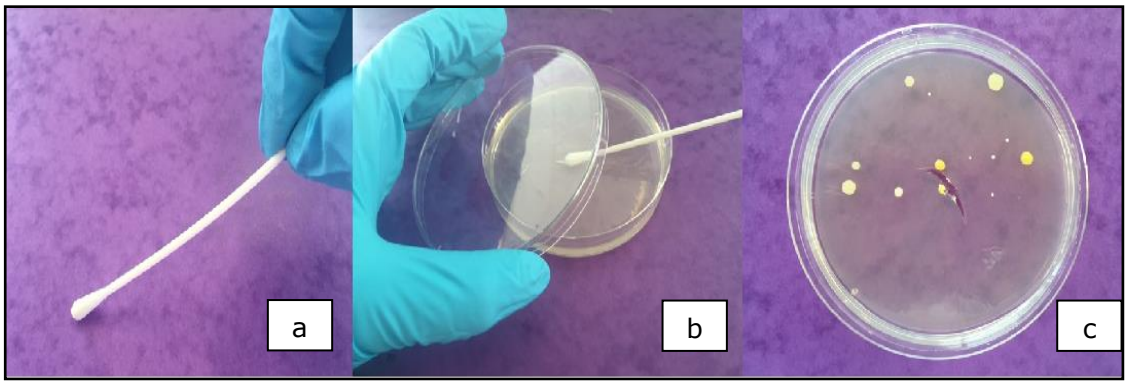


Figure 2 – 3: Images of (a) a cotton swab bud being used to recover bacteria from a surface, (b) bacteria being transferred from the swab bud onto the surface of an agar plate and (c) colonies on the agar plate following its incubation at 37 ^{o}C.

There are four types of swabs available for sampling surfaces, these are manufactured using either cotton, micro foam, rayon or polyester fibres spun into

buds at the end of a wooden or plastic handle (Edmonds 2009). Previous research has shown that the recovery efficiency of these different swab types varies significantly (Rose *et al.* 2004). The main problem is that there is no universal standardised method for swabbing. Variables can include the surface sampling size, the pattern of swabbing the surface, the pressure applied to the swab handle and whether a wetting agent is used or not (Obee *et al.* 2006). As reported in the literature, this has led to inconsistent findings. In one study by Rose *et al.* (2004) cotton, macro foam and rayon swabs were used to recover liquid deposited *Bacillus anthracis* spores from stainless steel surfaces. Recovery efficiency was found to be 27.7 %, 30.7 % and 10.0 % respectively. In another previous study, recovery efficiency was shown to differ depending upon swab bud moisture content, with a wet swab bud recovering 3.22 % of pre - inoculated bacteria from a wet surface compared to 0.29 % recovery from the same surface using a dry swab bud (Moore and Griffith 2002b). This clearly exemplifies the severity of the problem.

2.1.2.2 Direct Agar Contact Method of Surface Sampling

The direct agar contact method is another commonly used technique to monitor surface contamination levels (Hall and Hartnett 1964; Pinto *et al.* 2009; Galvin *et al.* 2012), especially in a pharmaceutical clean room where it is an important feature of the environmental surface monitoring programme (Pinto *et al.* 2009). Historically, there were various methods of applying agar to a surface including syringes, sausage skins, paper strips, muslin (Frawley *et al.* 2008), aluminium foil and milk bottle caps (Hall and Hartnett 1964). However, these were laterally replaced by direct agar contact plates (Hall and Hartnett 1964; Frawley *et al.* 2008). Contact plates, normally 55 mm Petri dishes, are filled with sufficient nutrient agar such that the solidified medium forms a convex surface (Figure 2 – 4 (a)). During sampling the surface of the agar is pressed at constant pressure onto a flat surface for a fixed period of time (Figure 2 – 4 (b)). This contact time varies considerably, ranging from periods as short as a few seconds to a number of hours (Foschino *et al.* 2003). The plates are subsequently incubated and the resultant colonies then counted and expressed in colony forming units (cfu) per plate (Figure 2 – 4 (c)) (Hall and Hartnett 1964).

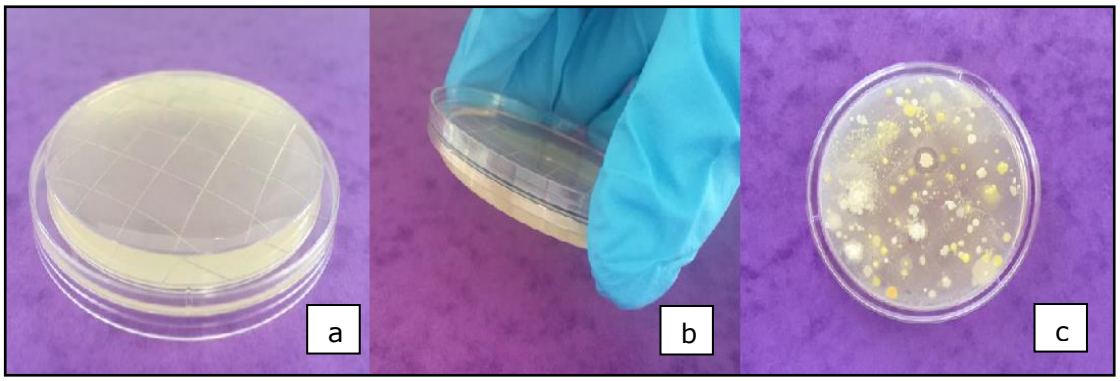


Figure 2 – 4: Images of (a) a 55 mm contact plate filled with nutrient agar to form a convex surface, (b) the surface of an agar contact plate being pressed against the test surface for a fixed period of time at constant pressure and (c) colonies on an agar contact plate following its incubation at 37 ^{o}C.

There are a number of advantages to a direct contact method of testing over other sampling materials including ease of use (Obee *et al.* 2006) and a quick sampling time (Galvin *et al.* 2012). Moreover, contact plates have been shown to be more effective at recovering bacteria from surfaces than other surface sampling techniques (Tebbutt 1991; Salo *et al.* 2000, Foschino *et al.* 2003; Obee *et al.* 2007). In 2006, Obee *et al.,* assessing the recovery efficiency of Methicillin - resistant *Staphylococcus aureus* (MRSA) from stainless steel coupons using 3 different sampling methods, found contact plates to be the most effective method of recovery, with 72.5 % of cells being recovered compared to 51.3 % recovery using dip slides and less than 1 % recovery using cotton swabs. Salo *et al.* (2000) also found that contact plates recovered 55.5 % of bacterial inoculated at low concentrations onto stainless steel, compared to a 39.8 % recovery with dip slide sampling and 45.2 % recovery with swabbing. Furthermore, Foschino *et al.* (2003) found that contact plates recovered 80 % of *E. coli* cells deposited onto a stainless steel surface when compared to a mere 1 % recovery with swabbing. However, some limitations with contact plate surface sampling exist. In particular the problem encountered when sampling uneven and unusually shaped surfaces, in which case swabbing is considered the best method of recovery (Niskanen and Pohja 1977). Surface sampling using contact plates is also limited to the surface area of the agar - 25 cm^2 (Hall and Hartnett 1964). However, in comparison to swabbing, surface bacterial recovery using a direct contact method is far less complex. Furthermore, the materials associated with this method are easy to source, prepare, handle, use and in addition are cost effective (Hall and Hartnett 1964). The biggest advantage of contact plates over swabbing is that these

eliminate the step of bacterial transfer onto agar, associated with the swabbing method (Salo *et al.* 2000), avoiding the underestimation of bacterial numbers associated with adhesion of bacterial cells to the swab head. Another major benefit of direct agar contact plates is the commercial availability of the plates, which enables their use in premises which lack laboratory facilities for their preparation (Salo *et al.* 2000). However, it is important to note that a study into the effectiveness of three brands of media in commercially filled contact plates by Pinto *et al.* (2009) found that bacterial recovery ranged from 23 % to 56 %, dependent upon agar brand. This suggests that the brand of commercial contact plate employed can vary recovery efficiency significantly. Interestingly, lowest percentage recovery was observed in contact plates filled with Oxoid media agar brand. Although this part of our study did not use commercially prepared plates, Oxoid is the brand of agar used and this may have contributed towards the efficiency rates observed. Furthermore, fluctuating factors in agar such as wetness, water evaporation, incubation time and temperature (Pinto *et al.* 2009), as well as agar contact time (Foschino *et al.* 2003), have all been shown to have an effect on bacterial recovery efficiency using contact plates.

2.2 Aim & Objectives

The aim of this aspect of the project was to develop a surface sampling method which could be used to recover, enumerate and compare the levels of bacteria on the surface of reusable antistatic carbon filament polyester clean room garments during the remainder of our study. The specific objectives were:

- To compare the efficiency of two commonly employed surface sampling methods, direct agar contact and swabbing, at recovering bacteria from antistatic carbon filament polyester fabric.
- To assess the effect swab bud moisture content plays upon the percentage of bacteria recovered from antistatic carbon filament polyester fabric using swabbing.
- To assess the retention of bacteria on swab buds following the agar transfer step of swabbing.

- To assess the effect of bacterial seeding density, agar contact time and fabric drying time upon the percentage of bacteria recovered from antistatic carbon filament polyester fabric using direct agar contact method.
- To use a continuous series of contact plates to examine the presence of bacteria on antistatic carbon filament polyester fabric following sampling with a single contact plate.

2.3 Materials & Methodology

In order to achieve the specified objectives the following materials and methods were used.

2.3.1 Preparation of 0.9 % Sodium Chloride Buffer

Sterile sodium chloride (Fisher Scientific Ltd, Loughborough, UK) buffer (0.9 %) was prepared and decanted into 10 mL and 9 mL volumes in McCartney bottles which were sterilised by autoclaving (Astell Scientific Ltd, Sidcup, UK) at 121 ^{o}C for 15 minutes.

2.3.2 Preparation of Nutrient Broth

Sterile nutrient broth (Oxoid Ltd, Basingstoke, UK) was prepared and decanted in 100 mL, 10 mL and 9 mL volumes in medical and McCartney bottles which were sterilised by autoclaving at 121 ^{o}C for 15 minutes.

2.3.3 Preparation of Nutrient Agar Plates

Agar plates were prepared by pouring 23 – 27 mL of molten sterile nutrient agar (Oxoid Ltd, Basingstoke, UK) into the base of a 90 mm diameter triple vented Petri dish (Fisher Scientific Ltd, Loughborough, UK). These were left to set in the laminar airflow cabinet (Hepaire Products Corporation, Kanata, Canada) and then stored at 4 ^{0}C in the fridge (Liebherr Group, Kirchdorf, Germany) until their use. Agar plates were removed from the fridge and left at room temperature for two hours prior to use to eliminate condensation on the surface of the agar.

2.3.4 Preparation of Contact Plates

Contact plates were prepared by pipetting 13 mL of molten sterile nutrient agar into the base of a 55 mm contact plate (Fisher Scientific Ltd, Loughborough, UK) to form a convex surface. As per Section 2.3.3, contact plates were left to set and

subsequently stored 4 °C, before being removed from the fridge 2 hours prior to use.

2.3.5 Preparation of Bacterial Stock Culture

This study was undertaken using skin commensal bacteria *S. aureus*, considered a pathogen which can colonise and cause infection in immunocompromised patients (Neely and Maley 2000; Harris *et al.* 2002). In order to prepare a stock suspension of *S. aureus* NCTC 6571 (Public Health England, Salisbury, UK) a single CryoProtect bead (Fisher Scientific Ltd, Loughborough, UK) containing the microorganism was removed from the – 80 °C freezer (New Brunswick Scientific, New Jersey, USA). This was placed into 10 mL of sterile nutrient broth (Oxoid Ltd, Basingstoke, UK) and incubated (Weiss Technik Ltd, Leistershire, UK) for 24 hours at 37 °C. Following incubation a loopful of this bacterial culture was streaked onto a nutrient agar plate and incubated at 37 °C for 24 hours to produce a master plate. A working plate was prepared by selecting one colony from the master plate and streaking this onto a nutrient agar plate. This was incubated at 37 °C for 24 hours and then stored at 4 °C along with the master plate. A fresh working plate was prepared weekly.

2.3.6 Preparation of Overnight Culture

To prepare an overnight suspension of *S. aureus* NCTC 6571, a conical flask containing 100 mL of sterile nutrient broth was aseptically inoculated with a single isolated colony of *S. aureus* NCTC 6571 from the working plate. The flask was incubated at 37 °C for 24 hours in a Max Q4000 orbital incubator (ThermoFisher Scientific, Massachusetts, USA) set at 150 rpm.

2.3.7 Preparation of Bacterial Calibration Curve

In order to develop an assay which could effectively recover known densities of bacteria from antistatic carbon filament polyester fabric a bacterial calibration curve of bacterial cell density against optical density at 650 nm (OD_{650}) was prepared. One millilitre volumes of fresh overnight culture were aseptically added to four 1.5 mL micro centrifuge tubes (Fisher Ltd, Loughborough, UK). These were centrifuged at 15,339 x g for 5 minutes using the Technico Maxi centrifuge (Fisher Ltd, Loughborough, UK). The supernatant was carefully removed and discarded,

with each pellet re - suspended in 1 mL of sterile 0.9 % sodium chloride buffer and the tubes re - centrifuged at 15,339 x g for a further 5 minutes. Again the supernatant was carefully removed and the pellets re - suspended in 0.25 mL of sterile 0.9 % sodium chloride buffer. The contents of the four micro centrifuge tubes were combined into one tube and this placed on ice. From the 1 mL of washed bacterial suspension, suitable dilutions were prepared using sterile 0.9 % sodium chloride buffer to produce four different OD_{650} values, ranging from 0.1 to 0.2, the absorbance of which were read on the Helios Epsilon spectrophotometer (Fisher Scientific Ltd, Loughborough, UK).

To determine the number of colony forming units (cfu) per millilitre of each suspension these were serially diluted 10 - fold down to 1 x 10^{-7} cfu / mL using sterile 0.9 % sodium chloride buffer. For each 10 - fold dilution series, five 20 µL volumes of the appropriate dilution was pipetted onto the surface of an over dried nutrient agar plate, with duplicate plates prepared. The plates were left to dry for 30 minutes, and then incubated at 37 ^{o}C for 24 hours. After this time the numbers of colony forming units in the lowest countable dilution were recorded and the mean colony count determined. Viable count in the original diluted suspension was then calculated. The viable counts (cfu / mL) from the four suspensions were plotted against their respective optical density values at 650 nm using Microsoft Excel 2010 (Microsoft Corporation, Washington, USA) as shown in Figure 2 – 5.

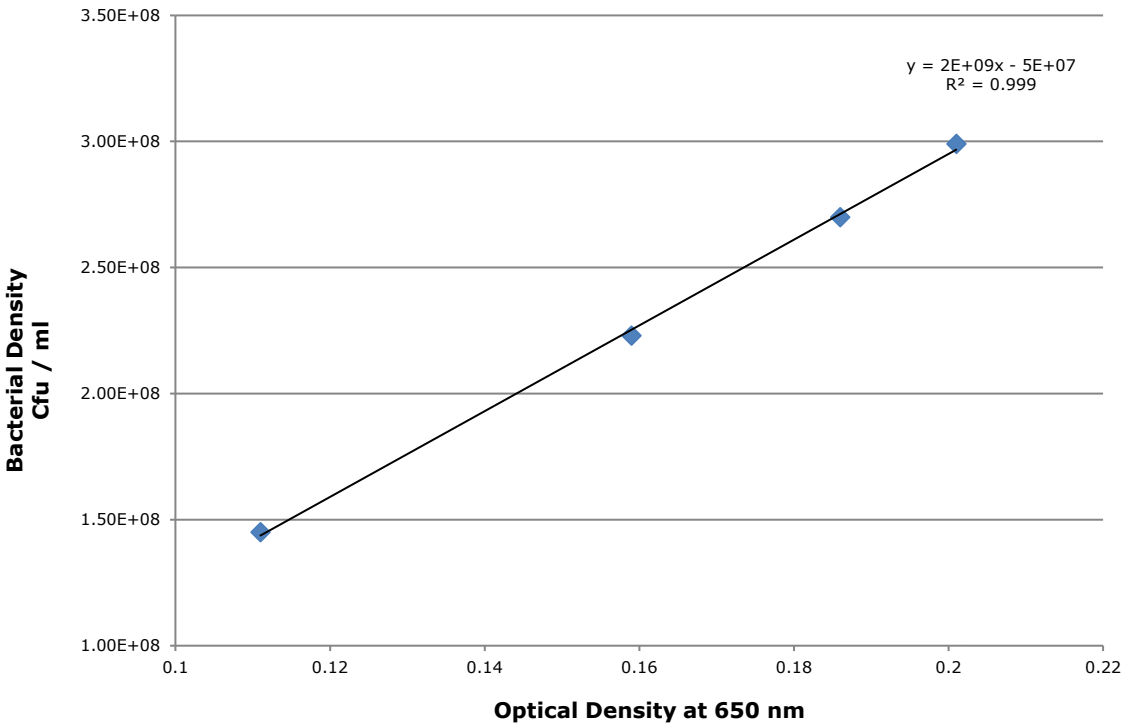


Figure 2 – 5: A Bacterial calibration curve of *S. aureus* NCTC 6571 at an optical density of 650 nm.

The equation of this curve (y = 2E+09x – 5E+07) was subsequently used to determine the concentration of *S. aureus* NCTC 6571 in overnight cultures, which were then diluted to prepare bacterial suspensions of known densities. The calibration curve was produced by necessity from a single experiment with an overnight culture involving multiple replicates (n = 3) for each data point. The calibration curve was validated by similar curves being prepared on different overnight culture (n = 3).

2.3.8 Preparation of Antistatic Carbon Filament Polyester Fabric Squares

In order to pre - inoculate sterile antistatic carbon filament polyester fabric with known densities of *S. aureus* NCTC 6571, antistatic carbon filament polyester fabric squares of 5 cm x 5 cm were cut from brand new clean room garments of the same composition (Chemsplash, Manchester, UK (formerly CCA Products)). Fabric was Chemstat® 909, extruded polyester interwoven with a conductive raised grid that is an extruded copolymer of polyester and carbon (air permeability - 0.5 mL cm^{-2} Sec^{-1}, pore size - 40 µm, static decay - 0.5 seconds) (N. O' Driscoll.,

2013, Personal Communication). These fabric squares were packaged into autoclave bags (Fisher Scientific Ltd, Loughborough, UK) and sterilised by autoclaving at 121 °C for 15 minutes.

2.3.9 Bacterial Inoculation of Fabric Squares

To pre - inoculate sterile antistatic carbon filament polyester fabric squares with *S. aureus* NCTC 6571 a fresh overnight culture was prepared and serially diluted to produce suspensions containing *S. aureus* NCTC 6571 of known densities. A 5 cm x 5 cm piece of sterile antistatic carbon filament polyester fabric was aseptically transferred into a 90 mm sterile Petri dish. This was then inoculated on the topside with 100 µL (5 x 20 µL) of bacterial suspension in a grid arrangement (Figure 2 – 6) and the fabric then dried for 60 minutes at 37 °C (to replicate dry clean room garments). The inoculated fabric was dried at 37 °C to ensure that the fabric was dry within a 60 minute time frame, due to time constraints during the study. However, if time permits the inoculated fabric could be dried over a longer period of time at ambient temperature.

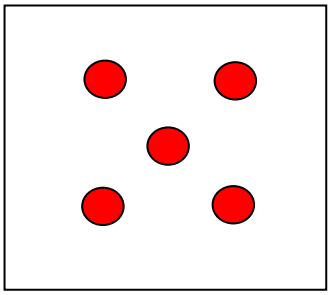


Figure 2 – 6: The inoculation arrangement of 100 µL *S. aureus* NCTC 6571 on a 5 cm x 5 cm square of sterile antistatic carbon filament polyester fabric. Each circle represents a 20 µL volume of *S. aureus* NCTC 6571 suspension of a known density.

The direct agar contact method, as described in section 2.3.10, and the swabbing method of bacterial recovery, as described in Section 2.3.11, were undertaken on the topside of the fabric square and the underside of a separate fabric square (pre – inculcated with *S. aureus* NCTC 6571 on the topside). During all assays a negative control of 100 µL (5 x 20 µL) sterile 0.9 % saline was inoculated onto separate fabric squares and tested in the same manner. Following testing all agar and contact plates were inverted and incubated at 37 °C for 24 hours (as undertaken by Tebbutt 1991; Foschino *et al.* 2003; Obee *et al.* 2006; Pinto *et al.* 2008). Following incubation the number of colony forming units per plate were

counted and the percentage of *S. aureus* NCTC 6571 recovered calculated. All assays were performed in triplicate.

2.3.10 Direct Agar Contact Method of Bacterial Recovery

The recovery of *S. aureus* NCTC 6571 from antistatic carbon filament polyester fabric squares using a single agar contact plate was assessed. An overnight culture of *S. aureus* NCTC 6571 was prepared, as described in section 2.3.6, and diluted to produce a suspension containing 1 x 10^4 cfu / mL. This suspension was pre – inoculated onto the topside of sterile antistatic carbon filament polyester fabric squares and dried as described in section 2.3.9. The lid was lifted from the contact plate and the convex agar surface was impressed onto the surface of the fabric square for five seconds at constant pressure. The underside of a separate piece of material (inoculated on the topside, in exactly the same manner as the first piece of fabric) was tested using a separate contact plate, turning the fabric in the Petri dish using sterile forceps prior to sampling.

2.3.11 Swabbing Method of Bacterial Recovery

The recovery of *S. aureus* NCTC 6571 from antistatic carbon filament polyester fabric squares using swabbing was also assessed. An overnight culture of *S. aureus* NCTC 6571 was prepared, as described in section 2.3.6, and diluted to produce a suspension containing 1 x 10^4 cfu / mL. This suspension was pre – inoculated onto the topside of antistatic carbon filament polyester fabric squares and dried as described in section 2.3.9. Using sterile forceps the pre - inoculated fabric square was transferred onto the surface of a sterile nutrient agar plate. The head of a sterile cotton swab bud (Böttger, Bodenmais, Germany) was moistened aseptically using sterile water. The moist swab head was then pressed against the surface of the pre - inoculated fabric, at constant pressure, and moved for 14 non - overlapping continuous strokes horizontally, the swab head was turned by 180 degrees and the material swabbed vertically for another fourteen non - overlapping strokes (Figure 2 – 7). The underside of a separate piece of material (inoculated on the topside, in exactly the same manner as the first piece of fabric) was tested using a separate swab, turning the fabric during its transfer to the agar's surface using sterile forceps prior to sampling. In order to assess whether swab bud moisture content effects bacterial recovery the above swabbing method was

undertaken on the topside and underside of separate fabrics squares using a swab with a dry bud.

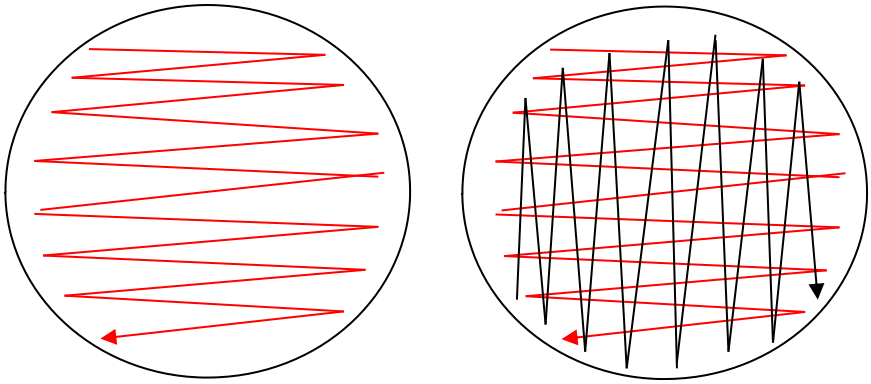


Figure 2 – 7: Pattern of swabbing undertaken on 5 cm x 5 cm antistatic carbon filament polyester fabric squares pre – inoculated on the topside with 100 µL (5 x 20 µL) of *S. aureus* NCTC 6571 at a density of 1 x 10^4 cfu / mL. The same pattern was repeated during the agar transfer step of the swabbing procedure.

In order to assess whether *S. aureus* NCTC 6571 was still present on the swab bud following the agar transfer stage of swabbing, using sterile scissors, the bud of the swab was cut from the shaft and immersed in a sterile universal containing 10 mL of nutrient broth. This was shaken vigorously for 60 seconds in a bid to detach any *S. aureus* NCTC 6571 remaining on the bud. This solution was diluted 10 – fold down to 1 x 10^{-3} by successively removing 1 mL of the media and adding this to 9 mL of fresh nutrient broth in a universal bottle. All bottles were incubated for 24 hours at 37 ^{o}C. Visual observation of growth was made in each of the bottles following incubation.

2.3.12 Assessment of Bacterial Seeding Density Using Direct Agar Contact

In order to assess the effect of bacterial seeding density upon the percentage recovery of *S. aureus* NCTC 6571 from the surface of antistatic carbon filament polyester fabric squares, the direct agar contact method was undertaken on fabric squares pre - inoculated on the topside with *S. aureus* NCTC 6571 at a density of 1 x 10^2, 1 x 10^3, 1 x 10^4 cfu / mL (all considered low bacterial seeding densities) and 1 x 10^5, 1 x 10^6, 1 x 10^7 and 1 x 10^8 cfu / mL (all considered high bacterial

seeding densities). An overnight culture of *S. aureus* NCTC 6571 was prepared, as described in section 2.3.6, and diluted to produce a series of suspensions containing 1 x 10^8 cfu / mL down to 1 x 10^2 cfu / mL. These suspension were pre – inoculated onto the topside of separate antistatic carbon filament polyester fabric squares and the fabric dried as described in section 2.3.9. The direct agar contact method, as described in Section 2.3.10, was undertaken on both the topside and the underside of a separate fabric squares.

2.3.13 Assessment of Agar Contact Time using Direct Agar Contact

In order to assess agar contact time against percentage recovery efficiency, the direct agar contact assay was undertaken on antistatic carbon filament polyester fabric squares at either 5 second, 30 second or 60 second intervals. An overnight culture of *S. aureus* NCTC 6571 was prepared, as described in section 2.3.6, and diluted to produce a suspension containing 1 x 10^4 cfu / mL. This suspension was pre – inoculated onto the topside of antistatic carbon filament polyester fabric squares and dried as described in section 2.3.9. The direct agar contact method, as described in Section 2.3.10, was undertaken on both the topside and the underside of separate fabric squares for either 5 second, 30 second or 60 second intervals.

2.3.14 Assessment of Continuous Direct Agar Contact

To assess the presence of *S. aureus* NCTC 6571 on the surface of squares pre – inoculated with the bacterium, and following their sampling with a single contact plate, a continuous direct contact method, using ten separate contact plates, was undertaken. An overnight culture of *S. aureus* NCTC 6571 was prepared, as described in Section 2.3.6, and diluted to produce a suspension containing 1 x 10^4 cfu / mL. This suspension was pre – inoculated onto the topside of antistatic carbon filament polyester fabric squares and dried as described in section 2.3.9. The direct contact method as described in 2.3.10 was undertaken on the topside of a fabric square. This method was repeated on the same fabric square with a further nine contact plates, continuously, directly one after the other on the same piece of fabric at five second intervals. The underside of a second piece of fabric (pre - inoculated on the topside in exactly the same way as the first piece of fabric) was tested with ten separate contact plates in the same manner as above.

2.3.15 Assessment of Fabric Drying Time

To assess the effect of fabric drying time upon recovery efficiency the direct contact method was undertaken on pre – inoculated fabric squares dried for 30 minutes (to replicate moist clean room garments). An overnight culture of *S. aureus* NCTC 6571 was prepared, as described in section 2.3.6, and diluted to produce a suspension containing 1 x 10^4 cfu / mL. This suspension was pre – inoculated onto the topside of antistatic carbon filament polyester fabric squares, as described in section 2.3.9 and dried for 30 minutes at 37 ^oC. The direct agar contact method, as described in Section 2.3.10, was undertaken on both the topside and the underside of separate fabric squares.

2.3.16 Statistical Analysis of Results

Results were analysed using GraphPad Prism 4.0 (GraphPad Software Inc., La Jolla, CA). Two – Way Analysis of Variance (ANOVA) at a 95 % confidence level was used to statistically analyse the difference between the variables.

2.4 Results

In a bid to develop a surface sampling assay which could subsequently be used to recover, enumerate and compare the levels of bacteria on the surface of reusable clean room garments during the remainder of this study the recovery efficiency of both a direct contact and swabbing methods (moist and dry swab bud) of surface sampling were compared. To achieve this each method was undertaken separately on 5 cm x 5 cm antistatic carbon filament polyester fabric squares, pre – inoculated on the topside with 100 µL (5 x 20 µL) of *S. aureus* NCTC 6571 at a density of 1 x 10^4 cfu / mL (1000 cfu / fabric square) and dried for 60 minutes at 37 ^oC (to replicate dry clean room garments). The percentage recovery efficiency of each methods at recovering *S. aureus* NCTC 6571 from the topside and underside of separate fabric squares is compared in Figure 2 – 8.

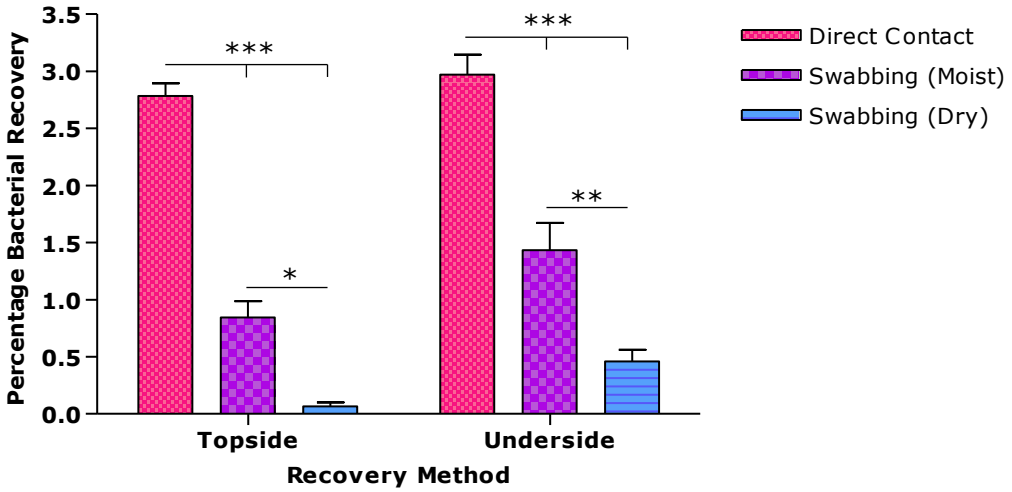


Figure 2 – 8: A comparison of recovery method, direct agar contact and swabbing (moist and dry swab buds), against the percentage of bacteria recovered from the topside and underside of separate 5 cm x 5 cm antistatic carbon filament polyester fabric squares pre – inoculated on the topside with 100 µL of *S. aureus* NCTC 6571 at a density of 1 x 10^4 cfu / mL (1000 cfu / fabric square) (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$).

As shown in Figure 2 - 8, poor recovery efficiencies were observed, with each of the surface sampling methods employed recovering less than 3 % of the pre – inoculated bacteria. However, the direct agar contact method was shown to recover a significantly higher percentage of the bacteria from both the top and underside of separate fabric squares compared to each of the swabbing methods undertaken (*** $P < 0.001$). The direct agar contact method was found to recover 2.78 % of *S. aureus* NCTC 6571 from the topside of pre – inoculated fabric squares compared to only 0.84 % bacterial recovery from the same side using a swab with a moist bud and 0.07 % using a swab with a dry bud. In addition, the direct agar contact method was found to recover 2.97 % of *S. aureus* NCTC 6571 from the underside of separate fabric squares, in comparison to only 1.43 % recovery using a swab with a moist bud and 0.46 % using a swab with a dry bud. Despite the low recovery efficiencies achieved with swabbing, as also shown in Figure 2 – 8, a moist swab head was found to recovered significantly more bacteria than its dry counterpart from both the topside of the fabric square (* $p < 0.05$) and underside (** $p < 0.01$). Additionally, although not shown to be statistically significant, in each case a higher percentage of bacteria were recovered from the underside of the fabric squares compared to that recovered from the topside of the fabric. The recovery of bacteria from the underside of the fabric squares, pre – inoculated with

bacteria on the top side, proves that *S. aureus* NCTC 6571 will permeate antistatic carbon filament polyester fabric.

Due to reports that swab buds will retain microorganisms during the agar transfer step of swabbing (Rose *et al.* 2004; Dalmaso *et al.* 2008), under quantifying the number of bacteria on a surface, the percentage of bacteria retained on swab buds following testing was therefore assessed. To achieve this, following the agar transfer step of swabbing, each swab bud was immersed in a sterile universal containing 10 mL of nutrient broth and this serially diluted down to 1 x 10^{-3}. These were then incubated for 24 hours at 37 °C. Following incubation visual inspection for growth was undertaken and the results presented in Table 2 – 1.

Table 2 – 1: Examination of the retention of *S. aureus* NCTC 6571 on moist and dry swab buds following the agar transfer step of swabbing, undertaken on the topside and underside of separate 5 cm x 5 cm antistatic carbon filament polyester fabric squares, pre – inoculated on the topside with 100 µl of *S. aureus* NCTC 6571 (1000 cfu / fabric square) ((+) - represents growth, (-) – represents no growth).

		Growth following 24 hours incubation at 37 °C			
Swab Bud	**Orientation of Fabric**	Bottle Containing Swab bud	Dilution Factor		
			1 x 10^{-1}	1 x 10^{-2}	1 x 10^{-3}
Moist	Topside (n = 9)	+	+	-	-
	Underside (n = 9)	+	+	-	-
Dry	Topside (n = 9)	+	-	-	-
	Underside (n = 9)	+	-	-	-

As shown in Table 2 – 1, growth was visually observed in all bottles which contained the swab buds, following their incubation. This suggests that bacteria were still present on the swab buds following the agar transfer stage of swabbing. The presence of growth in bottles containing the moist swab bud and their suspensions diluted to 1 x 10^{-1}, but no observation of growth made in their suspensions diluted to 1 x 10^{-2}, suggests that between ~ 10 – 90 colonies of *S. aureus* NCTC 6571 were still present on the moist swab buds following the agar transfer step of swabbing. However, the presence of bacteria in the bottles

containing the dry swab bud but the absence of growth in their suspensions diluted to 1 x 10^{-1} suggests that less than 10 bacteria were present on the swab bud following the agar transfer stage. Due to the lower recovery efficiency observed using the swabbing method of bacterial recovery (Figure 2 – 8), as well as the ability of swab buds to retain microorganisms during the agar transfer stage of the process (Table 2 - 1), the direct agar contact method was subsequently investigated during the remainder of this part of the study.

The low recovery efficiencies observed in this study using the direct agar contact method suggest that bacteria were still present on the surface of antistatic carbon filament polyester fabric squares following their sampling. In order to assess this theory, the direct agar contact method, using a continuous series of 10 contact plates, was undertaken on the topside and underside of separate fabric squares. The percentage recovery of each contact plate (1 – 10), and the cumulative percentage recovery from both the topside and underside of separate fabric squares is shown in Table 2 – 2.

Table 2 – 2: The percentage of *S. aureus* NCTC 6571 recovered from the topside and underside of separate 5 cm x 5 cm antistatic carbon filament polyester fabric squares, pre – inoculated on the topside with 100 µl of *S. aureus* NCTC 6571 (1000 cfu / fabric square), using the direct agar contact method. Average percentage recovery is shown for each contact plate, at each fabric orientation, as well as the cumulative percentage of bacteria recovered using a succession of 10 contact plates (n = 9).

	Topside of Fabric Square		Underside of Fabric Square	
Contact Plate	Percentage of *S. aureus* NCTC 6571 recovered (%)	Cumulative Percentage of *S. aureus* NCTC 6571 recovered (%)	Percentage of *S. aureus* NCTC 6571 recovered (%)	Cumulative Percentage of *S. aureus* NCTC 6571 recovered (%)
1	2.8	2.8	3.0	3.0
2	3.5	6.3	2.3	5.3
3	3.5	9.8	2.0	7.3
4	4.2	14.0	2.8	10.1
5	3.1	17.1	2.8	12.9
6	4.0	21.1	3.9	16.8
7	4.1	25.2	3.8	20.6
8	4.2	29.4	2.6	23.2
9	4.9	34.3	3.3	26.5
10	6.1	40.4	4.0	30.5

The overall cumulative percentage of bacteria recovered from the topside and underside of separate fabric squares (Table 2 – 2), pre – inoculated on the topside with 100 µL of *S. aureus* NCTC 6571 at a density of 1 x 10^4 cfu / mL (1000 cfu / fabric square), is graphed in Figure 2 – 9.

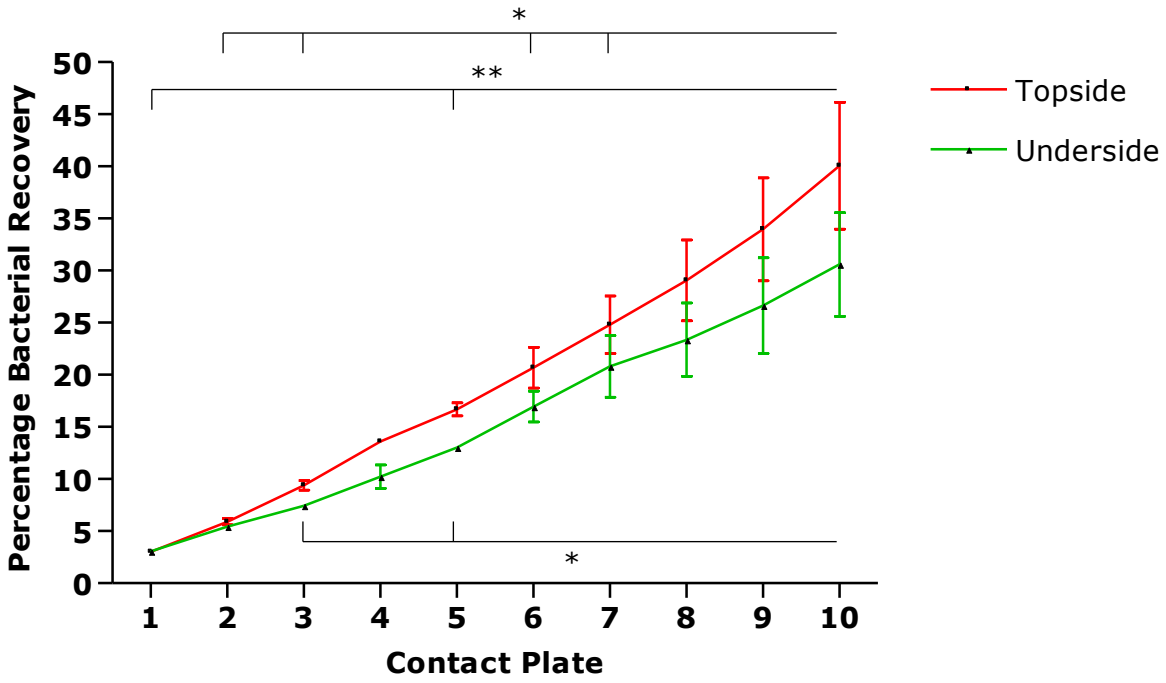


Figure 2 - 9: The cumulative percentage of *S. aureus* NCTC 6571 recovered from the topside and underside of 5cm x 5 cm antistatic carbon filament polyester fabric squares, pre - inoculated on the topside with 100 µL of the organism at a density of 1 x 10^4 cfu / mL (1000 cfu / fabric square), using a succession of 10 contact plates (** $p < 0.01$; * $p < 0.05$).

The results of this assay confirms that *S. aureus* NCTC 6571 was still present on the fabric squares following their testing with the initial contact plate. Furthermore, the 10th contact plate, used to test the topside of the fabric square, was shown to recover a higher percentage of bacteria than its predecessors. This was shown to be significantly more than the 1st, 5th (** $p < 0.01$), 2nd, 3rd, 6th and 7th (* $p < 0.05$) contact plates. Additionally, the 10th contact plate, used to test the underside of a separate fabric square, was also shown to recover a higher percentage of bacteria than the previous 9 plates, statistically significant between the 3rd plates and the 5th plates (* $p < 0.01$). As shown, despite a higher percentage of bacteria being recovered from the underside of the fabric compared to the topside of the

fabric using the initial contact plate, overall recovery of bacteria was greater from the topside of the fabric using the subsequent 9 contact plates.

In a bid to improve the recovery efficiency of the direct agar contact method a number of variable factors associated with this technique were assessed including bacterial seeding density, time of agar contact and fabric drying time. Previous research studies have shown that bacterial density on a surface can affect a surface sampling method's recovery efficiency (Salo *et al.* 2000; Probst *et al.* 2011). In order to investigate this theory with respect to the retrieval of bacteria from antistatic carbon filament polyester fabric the direct agar contact method was undertaken upon such fabric squares pre - inoculated on the topside with 100 µL of *S. aureus* NCTC 6571 at a density of 1×10^2 cfu / mL (10 cfu / fabric square), 1×10^3 cfu / mL (100 cfu / fabric square), 1×10^4 cfu / mL (1000 cfu / fabric square) (all considered low bacterial seeding densities), as well as those inoculated in the same manner with the same bacterium at a density of 1×10^5 cfu / mL (10,000 cfu / fabric square), 1×10^6 cfu / mL (100,000 cfu / fabric square), 1×10^7 cfu / mL (1,000,000 / fabric square) and 1×10^8 cfu / mL (10,000,000 cfu / fabric square) (all considered high bacterial seeding densities). An image showing colony forming units on contact plates, used to test the topside of fabric squares pre - inoculated at each of these densities, and incubated for 24 hours at 37 ^{o}C is shown in Figure 2 – 10. Similar levels of growth were observed on plates used to test the underside of separate fabric squares inoculated at the same densities (image not shown).

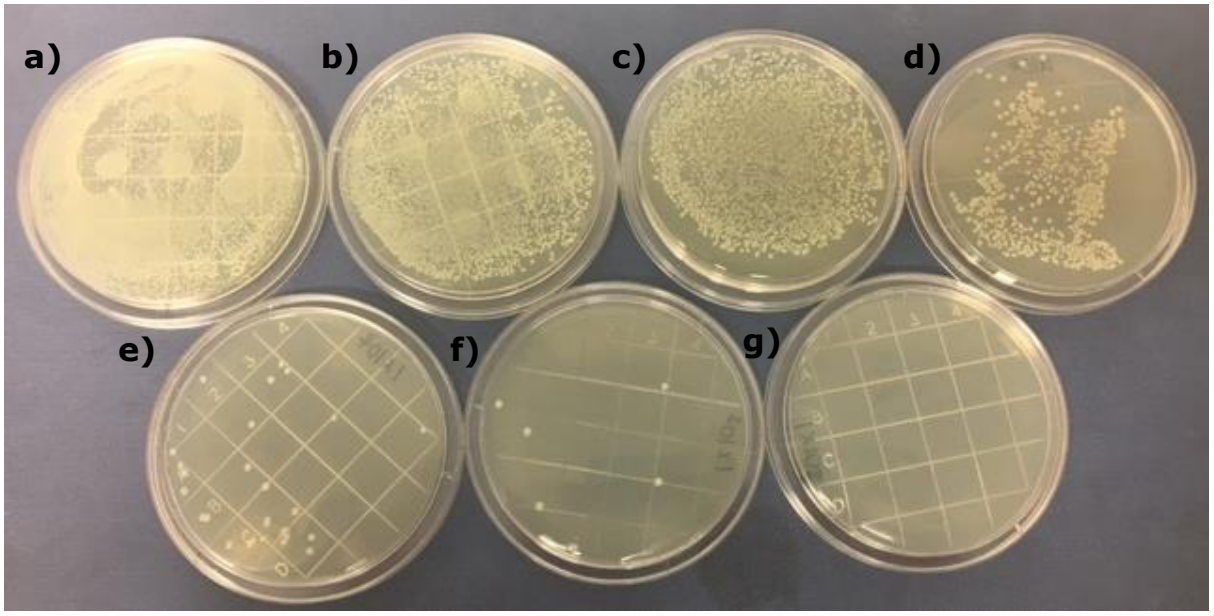


Figure 2 – 10: *S. aureus* NCTC 6571 colony forming units on the surface of contact plates following their incubation at 37 °C for 24 hours. Contact plates were used to recover the bacteria from the topside of antistatic carbon filament polyester fabric squares pre – inoculated on the topside with 100 µL of *S. aureus* NCTC 6571 at various seeding densities a) 1 x 10^8 cfu / mL (10,000,000 cfu / fabric square), b) 1 x 10^7 cfu / mL (1,000,000 cfu / fabric square), c) 1 x 10^6 cfu / mL (100,000 cfu / fabric square), d) 1 x 10^5 cfu / mL (10,000 cfu / fabric square), e) 1 x 10^4 cfu / mL (1000 cfu / fabric square), f) 1 x 10^3 cfu / mL (100 cfu / fabric square), g) 1 x 10^2 cfu / mL (10 cfu / fabric square) and dried for 60 minutes at 37 °C.

A shown in Figure 2 – 10, following their incubation, contact plates used to test fabric squares pre - inoculated with 100 µL of *S .aureus* NCTC 6571 at a density of 1 x 10^6 cfu / mL (c), 1 x 10^7 cfu / mL (b) and 1 x 10^8 cfu / mL (a) were considered too dense to count. Furthermore, those contact plates used to test fabric inoculated with *S. aureus* NCTC 6571 at a density of 1 x 10^2 cfu / mL (g) did not display any colonies following their incubation. Contact plates used to test fabric squares pre - inoculated with 100 µL of *S. aureus* NCTC 6571 at a density of 1 x 10^3 cfu / mL (f), 1 x 10^4 cfu / mL (e) and 1 x 10^5 cfu / mL (d) all displayed countable growth following their incubation. Therefore, the percentage bacterial recovery from both the topside and underside of fabric squares, pre – inoculated on the topside with *S. aureus* NCTC 6571 at a density of 1 x 10^3, 1 x 10^4 and 1 x 10^5 cfu / ml, using the direct agar contact method, are compared in Figure 2 – 11.

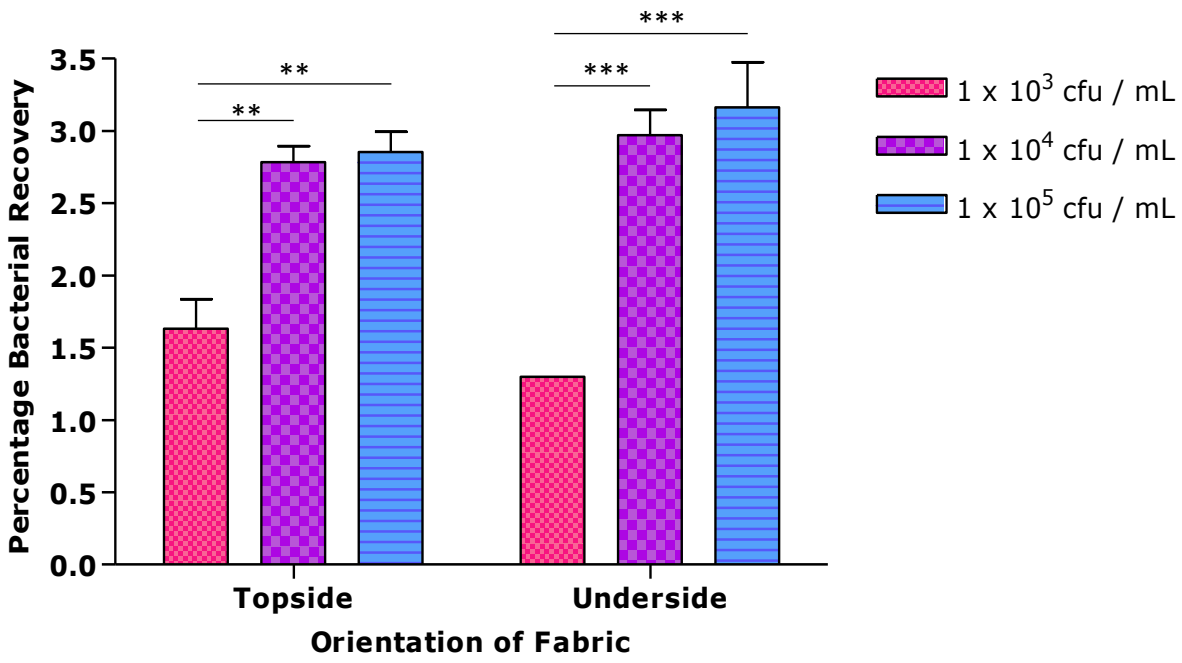


Figure 2 – 11: A comparison of bacterial seeding density against the percentage of *S. aureus* NCTC 6571 recovered from the topside and underside of separate 5 cm x 5 cm antistatic carbon filament polyester fabric squares, pre - inoculated on the topside with 100 µL of the bacteria at either 1 x 10^3 cfu / mL (100 cfu / fabric square), 1 x 10^4 cfu / mL (1000 cfu / fabric square) or 1 x 10^5 cfu / mL (10,000 cfu / fabric square), using the direct agar contact method (*** $p < 0.001$; ** $p < 0.01$).

As shown in Figure 2 – 11, a rise in bacterial seeding density saw an increase the percentage of *S. aureus* NCTC 6571 recovered from the fabric using the direct agar contact method, thus suggesting a correlation between bacterial seeding density and percentage bacterial recovery. Contact plates used to test the topside of fabric pre – inoculated with 100 µL of *S. aureus* NCTC 6571 at a density of 1 x 10^3 cfu / mL recovered a significantly lower percentage of the bacteria (1.63 %) than those used to test the fabric squares of the same orientation, pre – inoculated with the same culture at higher densities of 1 x 10^4 cfu / mL (2.78 %) and 1 x 10^5 cfu / mL (2.85 %) (** $p < 0.05$). This observation was also made with contact plates used to test the underside of separate fabric squares, pre – inoculated with 100 µL of *S. aureus* NCTC 6571 on the topside. A significantly lower percentage of bacteria were recovered from the underside of fabric squares pre – inoculated on the topside with *S. aureus* NCTC 6571 at a density of 1 x 10^3 cfu / mL (1.30 %) compared to the percentage of the same bacterium recovered from the underside of fabric squares pre – inoculated on the topside with higher bacterial densities of 1 x 10^4 cfu / mL (2.97 %) and 1 x 10^5 cfu / mL (3.16 %) (*** $p < 0.001$). The

overall low percentage recovery observed with contact plates used to test fabric pre - inoculated with *S. aureus* NCTC 6571 at a density of 1 x 10^3 cfu / ml may be due to there being no growth observed on a number of the contact plates following their incubation – reducing their overall recovery efficiency. Despite an increase in the percentage recovery of *S. aureus* NCTC 6571 being observed on contact plates used to test the top and underside of separate fabric squares, pre – inoculated with *S. aureus* NCTC 6571 at a density of 1 x 10^5 cfu / mL, compared to the percentage of the same bacteria being observed on contact plates used to test fabric squares, pre - inoculated with the same volume of bacteria at a density of 1 x 10^4 cfu / mL, this was not found to be statistically significant. Furthermore, following the incubation of plates used to test fabric pre - inoculated with *S. aureus* NCTC 6571 at a density of 1 x 10^5 cfu / mL, quantifying the number of colonies per plate proved difficult, due to the considerable number of colonies present on each plate (> 300), as well as colonies merging, which made it difficult to differentiate between single and groups of colonies (Figure 2 – 10 (d)). Overall, this resulted in the assay at this density becoming very time consuming. Therefore, it was decided that for the remainder of this part of the study the direct agar contact method would be undertaken on fabric pre – inoculated with 100 µl of *S. aureus* NCTC 6571 at a density of 1 x 10^4 cfu / mL (1000 cfu / fabric square).

Aside from bacterial seeding density, the effect of agar contact time against bacterial recovery efficiency during the direct agar contact method has previously been investigated (Foschino *et al.* 2003). Therefore, in order to assess this factor with respect to *S. aureus* NCTC 6571 recovery from antistatic carbon filament polyester fabric and to determine whether a 5 second agar constant time (initially employed in this study) was a sufficient contact time to achieve optimum bacterial recovery from clean room garments constructed of such fabric, an assessment of agar contact time was undertaken. The percentage of bacteria recovered from the top and underside of separate fabric squares, pre - inoculated on the topside with 100 µL of *S. aureus* NCTC 6571 at a density of 1 x 10^4 cfu / mL, and recovered using the direct agar contact method employed for either 5, 30 or 60 seconds, is shown in Figure 2 – 12.

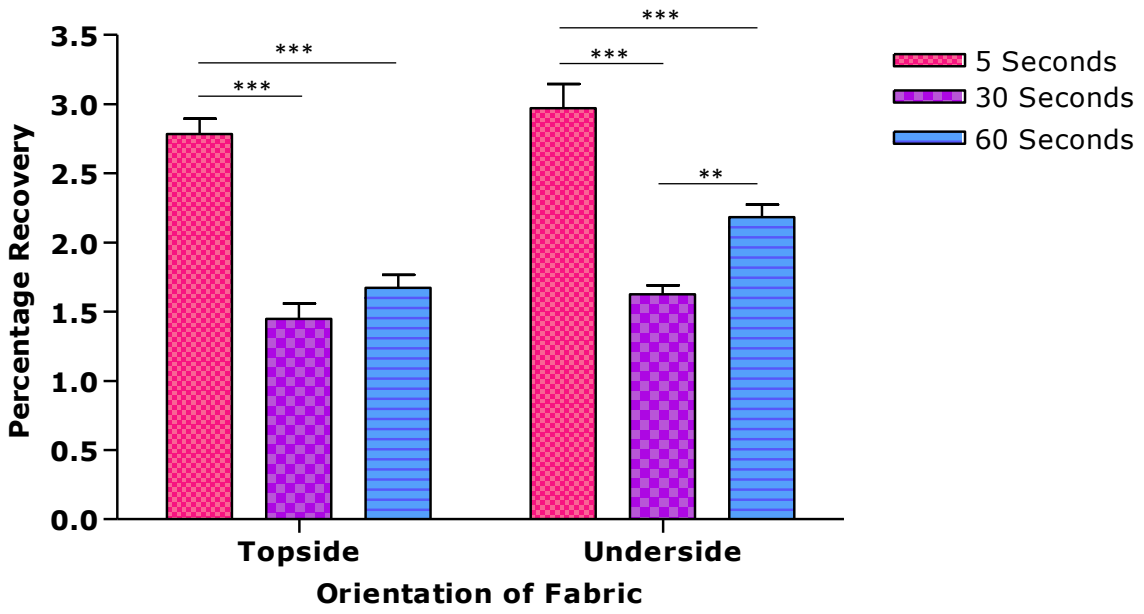


Figure 2 – 12: A comparison of agar contact time (5, 30 or 60 seconds) against the percentage of *S. aureus* NCTC 6571 recovered from both the topside and underside of separate 5 cm x 5 cm antistatic carbon filament polyester fabric squares, pre – inoculated on the topside with 100 µL of *S. aureus* NCTC 6571 at a density of 1 x 10^4 cfu / mL (1000 cfu / fabric square) (** $p > 0.01$; *** $p > 0.001$).

As shown in Figure 2 – 12, a five second agar contact time was shown to recover significantly more *S. aureus* NCTC 6571 from the topside and underside of separate 5 cm x 5 cm antistatic carbon filament polyester fabric squares than an increased contact period of either 30 or 60 seconds. A five second contact time resulted in 2.78 % of the bacteria from the topside of the fabric being recovered. This was found to be a significantly higher than that recovered from the topside of fabric squares tested using a 30 second (1.45 %) or 60 second (1.67 %) agar contact time (*** $p < 0.001$). The same pattern was observed with contact plates used to recover the same bacteria from the underside of separate fabric squares, pre - inoculated on the topside with *S. aureus* NCTC 6571 at the same density. A five second agar contact time was found to recover 2.97 % of bacteria, whereas only 1.63 % were recovered using a 30 agar second contact period and 2.18 % using a 60 second agar contact period (*** $p < 0.001$). From the underside of the fabric only there was also found to be a significant increase between the percentage of bacteria recovered between a 30 second and 60 second agar contact time (** $p < 0.01$). In fact, percentage bacterial recovery in all cases was found to be greater from the underside of the fabric when compared to the topside of the fabric,

although this was not found to be significant. Due to the increased recovery efficiency observed with a 5 second agar contact time, compared to that achieved with a 30 or 60 second contact time, for the remainder of this part of the study the shorter contact period was employed.

Aside from bacterial seeding density and agar contact time, previous research studies have also shown that surface moisture content can also effect the efficiency of surface sampling methods (Sattar *et al.* 2001; Moore and Griffith 2002b; Frawley *et al.* 2008). Therefore, a comparison of the percentage of *S. aureus* NCTC 6571 recovered from antistatic carbon filament polyester fabric squares dried for 30 minutes (to replicate moist clean room garments) and 60 minutes (to replicate dry clean room garments) was undertaken. The percentage of *S. aureus* NCTC 6571 recovered from the topside and underside of separate fabric squares, pre – inoculated on the topside with 100 µL of the bacterium at a density of 1 x 10^4 cfu / mL, using the direct agar contact method, is shown in Figure 2 – 13.

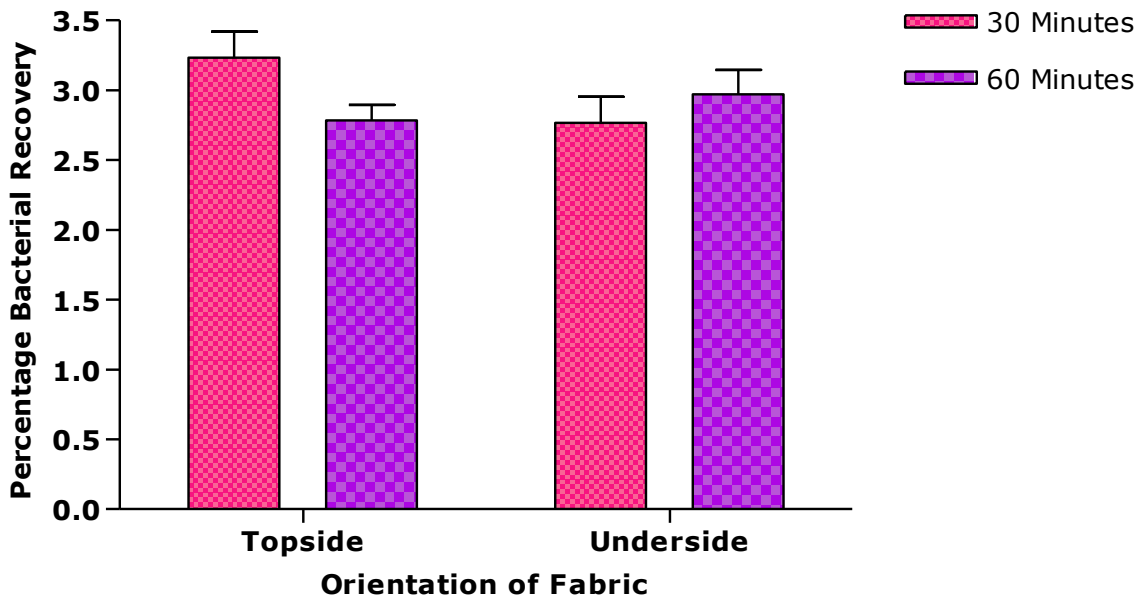


Figure 2 – 13: A comparison of fabric drying time (30 minutes or 60 minutes) against the percentage of *S. aureus* NCTC 6571 recovered from the topside and underside of separate 5 cm x 5 cm antistatic carbon filament polyester fabric squares pre – inoculated on the topside with 100 µL of the bacteria at a density of 1 x 10^4 cfu / mL (1000 cfu per fabric square).

As shown in Figure 2 – 13, there was found to be no statistically significant difference between the percentage of *S. aureus* NCTC 6571 recovered from the topside and underside of separate fabric square pre – inoculated on the topside with the culture and dried for either 30 minutes or 60 minutes. As previously discussed (Figure 2 – 8), contact plates used to test fabric squares pre – inoculated with the culture and dried for 60 minutes recovered 2.78 % of the bacteria from the topside of the fabric and 2.97 % from the underside of separate fabric squares. In comparison, 3.23 % of bacteria pre - inoculated onto the topside of fabric and dried for only 30 minutes were recovered, as well as 2.77 % recovered from the underside of fabric squares inoculated on the topside with the same culture and dried in the same manner. As previously discussed (Figure 2 – 8), a higher percentage of bacteria were recovered from the underside of fabric squares pre – inoculated and dried for 60 minutes than the topside of fabric treated in the same manner. However, interestingly, during the comparison of fabric drying time, a higher percentage of bacteria were recovered for the topside of fabric pre – inoculated and dried from only 30 minutes compared to that recovered from the underside of separate fabric squares. This suggests that bacteria will not only permeate antistatic carbon filament polyester fabric but that this infiltration will increase over time.

2.5 Discussion

The capacity of a surface sampling method to recover microbes from a surface is a measure of its effectiveness (Moore and Griffith 2002b). Initially, in this part of our study, the efficiencies of both a direct agar contact and swabbing methods of bacterial recovery, undertaken on pre - inoculated antistatic carbon filament polyester fabric squares, were found to be very poor, with efficiency values below 3 % being obtained (Figure 2 – 8). However, the direct agar contact method was shown to recover statistically more *S. aureus* NCTC 6571 (topside – 2.78 % / underside 2.97 %) from the fabrics' surface than the two swabbing methods of recovery employed, using a swab with either a moist (topside – 0.84 % / underside – 1.43 %) or dry bud (topside - 0.07 % / underside – 0.46 %) (*** $p < 0.001$) (Figure 2 – 8). Previously, there have been a number of research studies which have also compared the recovery efficiencies of both of these surface sampling methods (Tebbutt 1991; Salo *et al.* 2000; Foschino *et al.* 2003; Obee *et al.* 2007).

However, the results of these previous studies have proved highly inconsistent and in some case the results far outweigh the percentage recovery observed with our study. Furthermore, it is difficult to draw comparison between our current study and most of these previous studies because they examine bacterial recovery from hard, non - porous materials such as stainless steel or glass, which do not harbour variables such as depth, material composition and porosity, all of which can potentially impede recovery and need to be taken into consideration when recovering bacteria from antistatic carbon filament polyester fabric. It is important to note that whilst a sampling method may recover a high percentage of bacteria from one surface, it may recover significantly less from another (Edmonds 2009). Despite this, as per the findings of our study, the majority of these have reported contact plates to be more effective at recovering bacteria from a surface than their swabbing equivalent. Obee *et al.* (2007) observed the recovery efficiency of swabbing to be between 0.47 - 3.85 %, with recovery increasing significantly to 72.50 % using a direct contact method. Such findings are also supported by Foschino *et al.* (2003) who recovered between 57 - 80 % of pre - inoculated *E. coli* from eight different surfaces using contact plates, compared to 0 - 4 % recovery using a swabbing method. In addition, Kusumaningrum *et al.* (2003) found a single contact plate to recover between 7 – 46 % of bacteria inoculated onto a stainless steel surface. However, this was found to be dependent upon the species of organism initially pre - inoculated onto the surface. Interestingly, greatest percentage recovery (46 %) during this previous study was with *S. aureus*, the organism investigated in the current study.

Determining the bacterial bioburden of a surface is difficult (Foschino *et al.* 2003) and currently there is no consensus to a general accepted protocol for either of the methods assessed in this chapter. In fact, this lack of standardised method is one of the biggest problems associated with the swabbing method of bacterial recovery, with a number of factors associated with this method having been shown to lead to extreme variability in results (Moore and Griffith 2002a; Edmonds 2009) including sampling surface, swab bud moisture content and composition, substances used to improve bacterial detachment, the fluid used for bacterial re - suspension, as well as operator dependant variables such as swabbing pattern and velocity, the pressure applied to the swab handle and the vigour generated between the swab bud and surface (Moore and Griffith 2002a; Moore and Griffith

2002b). During our study the recovery efficiency of the swabbing method with respect to swab bud moisture content was examined, and subsequently shown to affect bacterial recovery. A significantly higher percentage of bacteria were recovered and transferred onto the agar surface using a swab with a moist bud compared to the percentage recovered and transferred onto agar using a swab with a dry bud (topside – 0.84 % vs. 0.07 % (* $p < 0.05$) / underside 1.43 % vs. 0.46 % (** $p < 0.01$ %)) (Figure 2 – 8). Moistened swab buds, like those compared during our study, have previously been shown to have an increased recovery efficiency when compared to their dry counterparts, with swabbing with a moisten swab bud being the preferred surface swabbing method (Foschino *et al.* 2003; Rose *et al.* 2004; Moore and Griffith 2007; Frawley *et al.* 2008). The results of a number of previous studies suggest that this is due to moisture aiding bacterial transmission (Sattar *et al.* 2001; Moore and Griffith 2002b). Moore and Griffith (2002b), found that bacterial recovery increased when the surface or the swab bud was wet rather that dry, thought to be due to the decreased adhesion between bacteria and a wet surface. This would suggest that bacterial recovery in this current study may have been impeded by drying the pre – inoculated fabric for 60 minutes prior to sampling, potentially increasing bacterial adhesion to the fabric. However, this factor was investigated by comparing the percentage recovery of *S. aureus* NCTC 6571 from fabric dried for 60 minutes (to replicate dry clean room garments) against that recovered from fabric dried for only 30 minutes (to replicate moist clean room garments) using the direct agar contact method. The results showed that drying time and moisture content of the fabric did not significantly affect the recovery of bacteria. Bacterial recovery from the "moist" fabric was found to be between 2.77 – 3.23 % and recovery from the "dry" fabric between 2.78 – 2.97 % (fabric orientation dependant) (Figure 2 – 13). It could be argued that the poor bacterial recovery rates obtained during our study could be due to the decline in viability of bacterial cells during the surface drying stage undertaken during our study. However, it is important to consider that the sampling of dry surfaces, compared to moist surfaces, is more relevant in real life scenarios (Obee *et al.* 2007), especially during this study when considering the sampling of clean room operator's garments, which are obviously dry in nature.

The poor recovery efficiencies obtained during our study, using swabbing, are thought to be the result of the number of stages adherent to the procedure. Firstly, recovery relies upon the swab bud's ability to collect microorganisms from the antistatic carbon filament polyester fabric, secondly these bacteria are required to be effectively released from the swab bud onto the agar and finally these organisms are required to grow on the agar during incubation (Figure 2 – 3). Following the agar transfer stage of the swabbing method employed in this study bacteria were found to adhere to the swab buds (Table 2 – 1). In each case, moist and dry swab buds, the observation of growth was made in nutrient broth used for swab bud suspension. The visual observation for growth in serially diluted bottles suggests that dry swab buds used to sample both the topside and underside of fabric squares retained < 10 colonies each (< 1 % of bacteria pre – inoculated onto the fabric square) whereas moist swab buds, used to test separate fabric squares of the same orientations, retained between 10 – 90 colonies each (1 – 9 % of bacteria pre – inoculated onto the fabric square). This would again suggest that moist swab buds recover more bacteria than their dry counterparts. However, this could also suggest that either the majority of *S. aureus* NCTC 6571, pre – inoculated onto the fabric square, remained adhered to the fabric following swabbing, or that *S. aureus* NCTC 6571 remained adhered to the swab bud following its suspension in diluent. Bacterial retention on swab buds is thought to be due to bacteria adhering to the naturally absorbent cotton cellulose fibres of a cotton swab tip (Moore and Griffith 2007), the type of swab used in this current study. This could be due to the water molecules, along with any bacteria, becoming attracted to and entrapped within the cellulose rings located between the lamellae of the cotton bud (Moore and Griffith 2007). A study by Rose *et al.* (2004) found that 6.1 – 16.2 % of colonies pre – inoculated onto swab buds were retained. However, this may be swab type dependant, a study by Dalmaso *et al.* (2008) found that rayon swabs realised ~ 21 % of bacteria recovery from a surface whereas flocked swabs realised ~ 92 % of cells. In addition, as previously discussed, bacteria are found to adhere to cotton fibres in greater proportions than other fibre types studied (Bajpai *et al.* 2011). These findings along with the results of our study suggests that using a swab with a bud constructed of a less absorbent material may be a more effective alternative to cotton buds during surface sampling of clean room operators garments.

Given all the factors associated with the swabbing method discussed above, further consideration to an effective swabbing procedure and materials for practical application to recover bacteria from clean room operator's clothing would need to be undertaken during future work to ensure swabbing is an effective method of recovering bacteria from clean room garments constructed of antistatic carbon filament polyester fabric. Not only could a moist swab head constructed of a less absorbent material potentially improve bacterial recovery but this may also be enhanced by adapting a number of other variables associated with the method. Firstly, the energy generated between the swab bud and the fabric could be increased. Moore and Griffith (2002b) showed that an increase in such energy significantly increased the number of bacteria recovered from a surface. This could also be achieved in our study by using a swab head made of coarse foam rather than softer cotton fibres. Increased energy could also be achieved using a swab with a wooden shaft (Moore and Griffith 2002b), instead of a polypropylene shaft, as also used in this current study. This less flexible shaft may allow an increased pressure to be applied to the swab, increasing the energy generated between the swab head and the fabric or agar. However, it is important to remember that such an increase in energy is operator subjective and depends solely on the pressure applied to the shaft. It is also important to note that swab handle design allows only a limited pressure to be applied to a test surface (Moore and Griffith 2002a) and even forceful swabbing may only detach a small percentage of the contaminating microorganisms (Salo *et al.* 2000). Furthermore, after microbes become attached to a surface harsh methods of removal can damage cells (Moore and Griffith 2002a) which may then be unable to grow on agar. Unfortunately, due to time constraints encountered during this part of our study these factors could not be fully assessed and are certainly issues which require further investigation.

Overall, due to the significantly higher percentage of *S. aureus* NCTC 6571 being recovered from antistatic carbon filament polyester fabric using the direct agar contact method of sampling, compared to the percentage recovered using swabbing, as well as the number of variables associated with the swabbing process, leading to extreme variability in results, a number of variables associated with the agar contact method were further investigated during this part of our study. Over the years various research studies have reported that the number of

bacteria on a surface can significantly affect the recovery efficacy of a method (Salo *et al.* 2000; Edmonds *et al.* 2009; Probst *et al.* 2011). Therefore, percentage bacterial recovery from antistatic carbon filament polyester fabric seeded with *S. aureus* NCTC 6571 at varying densities using direct agar contact was investigated. The results of this stage of the study found there is a correlation between bacterial seeding density and percentage bacterial recovery from antistatic polyester carbon filament fabric using this method (Figure 2 – 11). As shown, as seeding density increased the percentage of bacteria recovered from the fabric square also increased. This increase was shown to be significant between contact plates used to recover bacteria pre – inoculated on fabric at a density of 1 x 10^3 cfu / mL and those used to test fabric pre – inoculated at a density of 1 x 10^4 cfu / mL (** $p > 0.01$) and 1 x 10^5 cfu / mL (*** $p > 0.001$) from both the topside (1.63 % vs. 2.78% vs. 2.85 %) and underside (1.30 % vs. 2.97 % vs. 3.16 %) of separate fabric squares. Although an increase in recovery was also shown between contact plates used to test fabric inoculated at a density of 1 x 10^4 cfu / mL and those used to test fabric inoculated with the bacteria at a density of 1 x 10^5 cfu / mL this was not shown to be statistically significant. This correlation between seeding density and percentage bacterial recovery is supported by a number of previous surface sampling research studies, each of which have shown there to be a direct relationship between surface bacterial seeding concentration and the percentage efficiency of sampling methods, with recovery efficiency reduced when bacterial numbers on a surface are low (Edmonds *et al.* 2009; Probst *et al.* 2011). One of these previous studies by Edmonds *et al.* (2009) found that when using swabbing percentage bacterial recovery increased as the concentration of bacteria pre - inoculated onto the surface increased, with 42.1 % of bacteria recovered when seeded at a density of 1 x 10^4 cfu / mL, whereas 92.7 % were recovered when a seeding density of 1 x 10^7 cfu / mL was employed. This was also found by Probst *et al.* (2001), who demonstrated that 6.5 % of *B. atrophaeus* spores inoculated on fabric at a concentration of 2,500 cfu / m^2 were recovered, whereas when the fabric was seeded at a higher concentration (40,000 cfu / m^2) 13.2 % of spores were recovered. The results of this part of our study would suggest a greater percentage recovery would be obtained by sampling fabric inoculated with a higher density of bacteria, however, as shown in Figure 2 – 10, when this was undertaken contact plates were considered too dense to count following their incubation. Salo *et al.* (2000) found that bacteria seeded at a high concentration (16,250 cfu / mL)

resulted in a 21.2 % recovery efficiency, whereas a lower density (325 cfu / mL) resulted in a higher recovery, at 55.5 %. As previously discussed, this may be due to greater numbers of bacteria adhering to fibres at increased concentrations (Hsieh and Merry 1986). Furthermore, high levels of contamination would not be representative of those found on clean room garments. Such conflicting results have led to difficulties in comparing and evaluating the efficiency of methods used in previous studies with the results obtained in this current study, as previously discussed, in comparison to our study these works have employed different variables such as sample technique and surface. To the author's knowledge our research is thought to be the first such study.

It could be argued that the overall low recovery efficiencies observed during our study, both with the swabbing and direct agar contact method, are a result of an insufficient agar incubation period, as well as the composition of agar used. However, the 24 hour agar incubation period adopted during this part of the study was the same as that previously used in direct agar contact (Tebbutt 1991; Foschino *et al.* 2003; Obee *et al.* 2006; Pinto *et al.* 2008) and swabbing studies (Moore and Griffith 2002a). In fact, a 24 hour contact plate incubation followed by counting was historically recommended by Hall and Hartnett (1964). Agar composition has also been shown to have an effect on bacteria recovery, therefore the use of only nutrient agar in this current study may have limited recovery. General purpose biological tryptone soya agar (TSA) has been proven to recover higher percentages of bacteria when compared to selective agars such as Violet Red Bile agar or MacConkey agar, which each recovered less than 1 % of bacteria pre - inoculated onto a surface (Foschino *et al.* 2003). Therefore, bacterial recovery in the present study may have been improved by using TSA filled contact plates instead of nutrient agar filled plates. However, it is thought that the difference in bacterial recovery would be minimal as both are general purpose bacteriological agars. However, due to the concerns raised, the issue of agar composition in recovering bacteria and supporting their subsequent growth (in Chapter 3) and agar incubation time will be investigated (in Chapter 4).

Foschino *et al.* (2003) suggests that an increase in contact time between agar and a surface can significantly increase the percentage of bacteria recovered using the

direct agar contact method. Their study found that a one minute contact time between agar and a stainless steel surface resulted in a bacterial recovery efficiency of 17 %, whereas an increase in contact time to one hour improved recovery to 35 % and four hours to approximately 100 %. This would suggest that initial bacteria recovery in our current study was impeded by the 5 second period of contact time between the agar and the fabric sample. However, a significant increase in contact time would not be representable of using contact plates to screen operators' clothing in the clean room environment. Furthermore, it is important to note that over a long period of contact time, such as the four hours investigated by Foschino *et al.* (2003), cells could grow and develop into a biofilm and if this was the case at least one cell would be recovered during sampling. This would not be representative of the initial bacterial load. Therefore, a moderate increase in contact time during our present study was investigated. It was initially though that an increase in contact time would proportionally increase percentage bacterial recovery, as had previously been shown. However, on further investigation, percentage recovery efficiency from the top and underside of separate fabric squares was found to significantly decrease with an increase in agar contact time to 30 seconds, as opposed to 5 seconds, as initially employed (topside - 1.45 % vs. 2.78 % / underside – 1.63 % vs. 2.97 % (*** $p < 0.001$)) (Figure 2 – 12). This was thought to be due to the agar in the contact plate losing its initial adhesive properties. As also shown in Figure 2 – 12, the percentage recovery of bacteria was shown to increase between a contact time of 30 – 60 seconds (topside – 1.45 % vs. 1.67 % / underside – 1.63 % vs. 2.18 %). However, this was only shown to be statistically significant in plates used to test the underside of the material (** $p < 0.01$). This could be due to the increase in moisture observed between the fabric and plate over this time frame, aiding bacterial recovery. It is important to note that following the incubation of contact plates used to test fabric at the 60 second time point most of the colonies were observed around the edge of the contact plates. This suggests that the pressure applied onto the plate over that time period forced the bacteria to migrate to the edge of the fabric and potentially onto the petri – dish, out with the recovery scope of the contact plate. Obviously, this would lead to an underestimation of the surface bacterial load. Furthermore, there was still significantly more bacteria recovered with an agar contact time of 5 seconds compared to that recovered with a 60 second contact time (topside - 2.78 % vs. 1.67 % / underside – 2.97 vs. 2.18

%) (*** $p < 0.001$) (Figure 2 – 12). Clean room operators work under considerable pressure, sometimes to time constraints. Therefore, if using the direct agar contact method to recover bacteria from their garments, a 5 second contact time, over a considerably longer period, would be preferable.

The poor recovery efficiencies observed during our study would suggest that the majority of bacteria remained on the fabric following testing. Maunz and Kanz (1969) found that the recovery efficiency obtained with contact plates depends on the uniformity of the surface being examined, however, the weft and weave of antistatic carbon filament polyester fabrics offers a fairly small surface area to be tested. Furthermore, as previously discussed by Hoborn and Nyström (1985), bacteria will penetrate deeper into the structure of the fabric and therefore may not be recovered during sampling. Kusumaningrum *et al.* (2003) investigated the presence of bacteria on stainless steel surfaces following initial contact plate recovery during their study using 5 consecutive contact plates. Forty six % of *S. aureus* were initially recovered from the surface using a single contact plate, and a total of 88 % using 5 consecutive plates, with each contact plate's recovery efficiency decreasing. This percentage recovery was significantly higher than that observed during our study, however, this previous study cannot be directly compared to our research due to differences in the surface composition. During our study, ten consecutive contact plates were found to recover a cumulative total of 40.4 % of bacteria from the topside of fabric squares, with the initial contact plate recovering 2.8 % of pre – inoculated *S. aureus* NCTC 6571 and each subsequent plate recovering between 3.0 – 6.1 % of the remaining cells. From the underside of a separate fabric square a total of 30.5 % of bacteria were recovered with the 10 successive plates, the initial plate recovered 2.98 % of bacteria with each of the subsequent plates recovering between 2.0 – 4.0 % of the bacteria initially inoculated onto the fabric (Figure 2 – 9 and Table 2 - 2). As shown, contact plate recovery efficiency did not decrease with each consecutive plate, as was found by Kusumaningrum *et al.* (2003). In fact, in each case (topside and underside of the fabric square) the 10^{th} plate was shown to recover more bacteria than its predecessors, significantly so in some cases (** $p < 0.01$; * $p < 0.05$) (Figure 2 – 13). This may be due to the increase in fabric moisture over time due to the agar, promoting bacterial recovery. Although previously our study showed

there to be no significant difference in bacterial recovery from 'moist' and 'dry' fabric (Figure 2 – 13), this increase may be due to moisture originating from the recovery tool (contact plate) rather than the surface, as previously demonstrated using a moist swab head. The results of our study suggest that moist contact plates may be more effective at recovering bacteria from fabric than dried plates (as used in this part of the study). This is an area worthy of future investigation, which due to time constraints, could not be achieved during the scope of this study.

Not only do the results of this study show that bacteria remain on the fabric following surface sampling but they also show that *S. aureus* NCTC 6571 will permeate antistatic carbon filament polyester fabric (and therefore clean room garments of this composition). In fact, the results of the current study show there to be no significant difference in the percentage of *S. aureus* recovered from the topside and underside of the polyester fabric squares (Figure 2 – 8), as well as suggesting that bacterial penetration will increase over time (Figure 2 – 13). Although in this part of our study the bacteria were present in their uni – cellular form (0.8 – 1.0 µm) Grangè *et al.* (2010) have previously shown that bacteria will penetrate clean room garments during wear, contaminating the outer surface of the garment. Migration of bacteria through the fabric should be of concern to the clean room industry, with bacteria having been shown to survive for days and even months on commonly used hospital fabrics, with *S. aureus* surviving for between 1 – 56 days on polyester (Neely and Maley 2000). The ability of bacteria to bind to polyester (Hsieh and Merry 1986; Neely and Maley 2000; Takashima *et al.* 2004; Schmidt – Emrich 2016) suggests that clean room garment could potentially act as a mode of bacterial transmission, spreading microorganisms and potentially contaminating the environment and subsequently products. Previous studies have shown that that *S. aureus* can be transferred from fabrics onto other surfaces (Sattar *et al.* 2001). However, the bacterium has also been shown to bind with polyester fibres (Takashima *et al.* 2004) and may account for the low recovery efficiency found in this current study. High percentages of *S. aureus* have been shown to adhere to polyester fibres when compared to numbers binding to cotton fibres (Takashima *et al.* 2004). Therefore, *S. aureus* attached to desquamated operator's skin could irreversibly bind to the fibres within polyester clean room clothing and be retained, reducing the quantity of bacteria transferring from operators into the environment. Therefore, the use of polyester fabric to

manufacture clean room clothing over other fabrics such as cotton could also be advantageous, but is a factor which would require considerable research.

The results of this chapter suggest that contact plates and swabbing methods of surface sampling may be significantly underestimating the bacterial bioburden of clean room surfaces and it could be argued that these are inefficient at recovering and enumerating bacteria on surfaces harbouring low bacteria densities, inaccurately representing the levels of contamination within the clean room environment, resulting in contamination limits being exceeded without knowledge. The problem is that low levels of contamination on a surface, such as in a clean room environment, are difficult to detect (Dalmaso *et al.* 2008) and surface sampling techniques such as direct agar contact and swabbing may not fully represent the bacterial bioburden of a surface (Salo *et al.* 2000). Furthermore, as in the case of this thesis, sometimes maximum microbial recovery is not the end user's primary aim (Hall and Hartnett 1964) but rather to use the recovery efficiency of a method obtained to enumerate and compare the bacterial population on a surface. The results of our study contribute towards the knowledge and ability to estimate the number of *S. aureus* NCTC 6571 on clean room garments constructed of antistatic carbon filament polyester fabric, known to harbour low levels of contamination (i.e. recovery represents only ~ 1 – 3 % of bacteria actually on the fabric surface). Something which, prior to this study, was unknown. Due to the superior recovery efficiency of bacteria from antistatic carbon filament polyester fabric obtained using the direct agar contact method of bacterial recovery as opposed to the swabbing method employed during our study, as well as the simplicity, availability, cost and time efficiency of contact plate use it was decided that this method would be used to recover, enumerate and compare the levels of contamination of the surface of clean room operators garments during the remainder of this study. Furthermore, contact plates have previously been shown to be effective in monitoring the bacterial bioburden of the surface of clean room garments during their wear (Grangè *et al.* 2010).

2.1 Conclusion

In this chapter the efficiency of a direct agar contact and swabbing methods of surface sampling at recovering *S. aureus* NCTC 6571 from antistatic carbon

filament polyester fabric were compared. Recovery of this organism from the underside of fabric squares, pre - inoculated on the topside, proves that this bacterium will permeate antistatic carbon filament polyester fabric, with rates increasing over time. Poor recovery was observed with both of the methods studied, with < 3 % of the pre - inoculated bacterium being recovered from the topside and underside of separate fabric squares. Moist swab buds were found to recover significantly more bacteria than their dry counterparts, however, swab buds (both moist and dry) were found to retain bacteria following the agar transfer stage of swabbing. Moreover, the direct contact method of surface sampling was found to recover significantly more *S. aureus* 6571 NCTC from antistatic carbon filament polyester fabric than its swabbing equivalent. The recovery efficiency of the direct agar contact method was found to rise as the density of bacteria on the fabric increased. However, an increase in agar contact time from 5 seconds to either 30 or 60 seconds was shown to significantly decrease the number of bacteria recuperated. Despite a continuous direct contact assay showing that bacteria were still present on the pre - inoculated fabric following initial testing using a single contact plate, the direct agar contact method was deemed a sufficient tool to recover, enumerate and compare the levels of bacteria on the surface of clean room operators' garments during the remainder of this study.

Chapter 3:

A Comparison of the Efficiency of Out – Sourced Specialist Garment Laundering, with and without Terminal Gamma Sterilisation, upon the Resultant Bacterial Bioburden of the Surface of Reusable Antistatic Carbon Filament Polyester Clean Room Suits

3.1 Introduction

Clean room operators can wear either reusable garments, disposable garments or a combination of both clothing types whilst working in the clean room environment (Bryant 2010). However, there are a number of factors which must be considered when choosing which clothing type to wear, with the most important factor being that the arrangement of garments meets the requirements of the clean room class in which it will be worn (European Commission 2008). There must also be consideration to facility requirements, the number of garments required, as well as the number of visiting operatives (Bryant 2010). In addition, garment management must also be considered from an economic view point. Disposable clean room garments are considered the most cost effective clothing system (Larkin 2009), whereas reusable garments eliminate any charges associated with the storage, shipping and the disposal of single use garments (Hobson 2007). However, research has shown that during wear, the surface of reusable clean room garments can become contaminated with microorganisms (Grangé *et al.* 2010), therefore, these must undergo decontamination following wear, with subsequent processing, which does not increase the surface bacterial bioburden of the garment (European Commission 2008). Therefore, the costs associated with the decontamination of reusable garments must also be taken into consideration, including not only the laundering and sterilisation of the garments but additional fees such as delivery (Larkin 2009). Worryingly, there have previously been concerns raised over the lack of sterility of reusable clean room garments returning from out – sourced laundering facilities (Larkin 2009). Despite this, there is a severe lack of published literature which assesses the efficiency of laundering / sterilisation processes upon the resultant bacterial bioburden of reusable clean room garments. Therefore, in this part of our study the efficiency of two clean room garments laundering processes (one with and one without terminal gamma sterilisation) on reusable antistatic carbon filament polyester clean room garments were assessed and compared using the direct agar contact method examined in Chapter 2.

3.1.1 Out - Sourced Laundering Facilities

It is recommended that reusable clean room garments to be worn in pharmaceutical clean rooms are processed by out – sourced laundering facilities,

using standard operating procedures (European Commission 2008), ensuring garments are laundered to the levels set by the European and British Standard entitled BS EN 14065: 2016 Textiles - Laundry Processed Textiles - Bio Contamination Control System (Clayton and Eaton 2011). Such establishments can typically offer three different contract types - laundering of customer owned garments, laundering of garments purchased from the laundering company or laundering of clothing rented from the facility itself (English 1996). Typically, if the number of garment changes per week exceeds 20 most facilities opt to use the latter contract type with an out - sourced facility, covering all aspects of the system (Bryant 2010). Particulate and microbial decontamination of reusable clean room garments is usually undertaken by the specialist laundering company using a linear flow, closed loop process (Eudy 2014), through a series of distinct stages including garment cleaning, removal of particles, terminal sterilisation, checking and inspection, and secondary packaging following processing (Clayton and Eaton 2011). The basic layout of such a typical closed – loop laundering facility and the general material flow is shown in Figure 3 – 1.

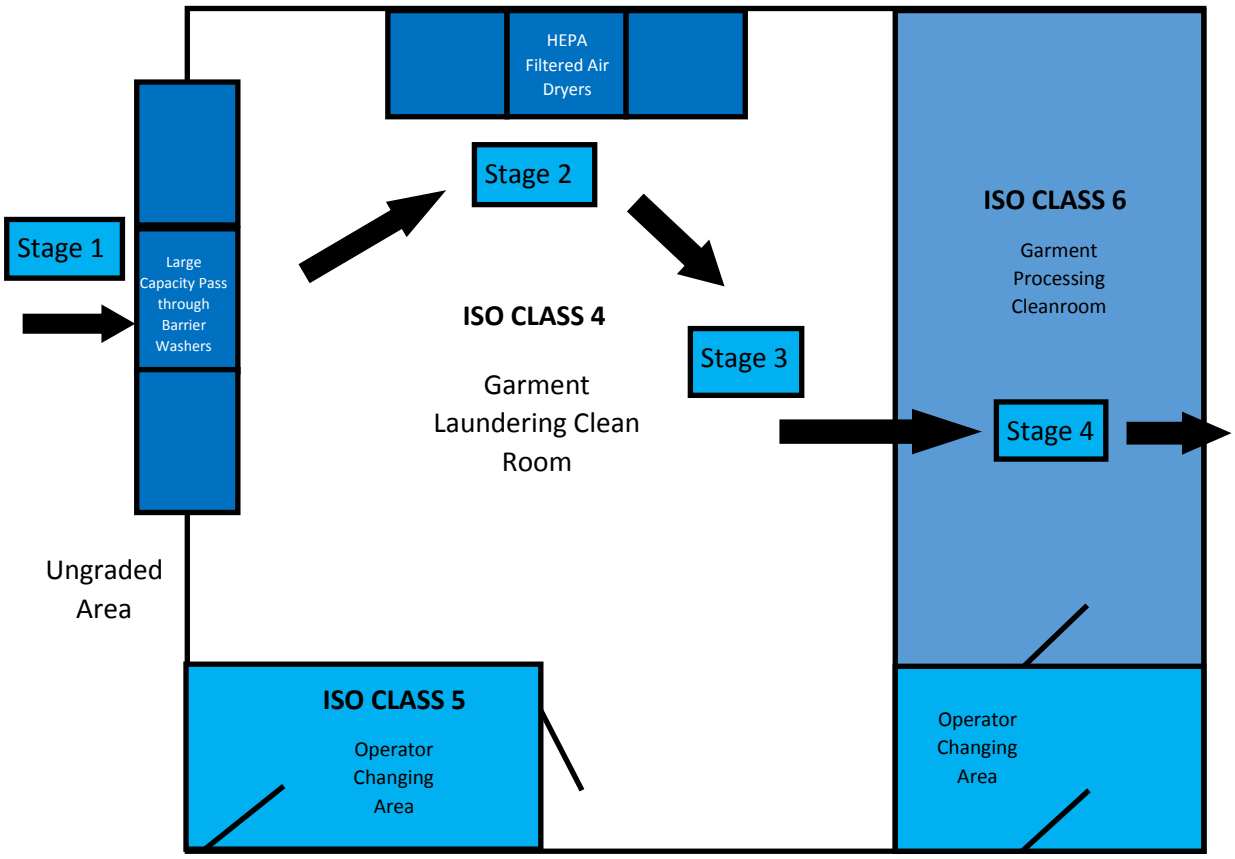


Figure 3 – 1: Diagram showing the typical layout, room classifications and general material flow of a closed - loop clean room laundering facility.

Stage one of the garment laundering process (Figure 3 – 1) begins when contaminated garments arrive into a non – classified area of the laundering facility, where within the sorting area each batch is sorted into groups of similar items and inspected (Hobson 2007). During this part of the procedure each individual piece of clothing is processed so it can latterly be identified (English 1996), allowing garments to be tracked throughout the laundering process (Marshall 2015). It is at this point in the system that damaged garments are taken out for repair or replacement (English 1996). From the sorting area, garments are then loaded into large capacity pass through barrier washers (English 1996) where, to remove particles, clothing is rinsed using 0.22 μm filtered softened 18 megohm deionised water, along with ion free detergents (Eudy 2014). These pass through washers allow the garments to enter the washer on the non - classified side of the facility and be removed out into a clean room environment (built and maintained to at least an ISO Class 5 standard (Clayton and Eaton 2011) (Table 1 – 1)), following rinsing (Hobson 2007). During stage 2 of the process garments are then passed through HEPA filtered air dryers (English 1996). Following drying, in stage 3, each piece of clothing is inspected (English 1996), folded and individually packaged into bags (Eudy 2014). Once sealed these maintain the subsequent sterility of the garments until their use (Larkin 2009). In stage 4, decontaminated garments are transferred into a lower grade of clean room, built and maintained to at least an ISO Class 8 environment, for secondary packaging and sorting prior to their delivery (Clayton and Eaton 2011). To avoid any mix ups and to prevent contamination the facility should clear the area between batches as well as clean down surfaces (Bryant 2010).

To ensure quality assurance and that particulate levels have been achieved, checks are carried out on garments from each batch (English 1996). This is achieved using the Helmke Drum Test or the American Society for Testing and Materials International (ASTM) F51 / 00 Microscopic Particle and Fibre test (Clayton and Eaton 2011). During the Helmke Drum Test a garment from a laundered batch is rotated in a drum which replicates particle shedding from operators through movement. The drum contains a particle counter which determines the density of particles at ≥ 0.3 μm and ≥ 0.5 μm over a 10 minute period (English 1996). In comparison, the ASTM F51 / 00 microscopic test,

quantifies the number of detachable particulate and fibre contaminants ≥ 5 μm on the surface of cleanroom garments, using vacuum filtration and microscopy (Clayton and Eaton 2011). In each case particle values are analysed and tabulated to determine if the number of particles on garments are within the defined limits (McIlvaine and Tessien 2006).

3.1.1 Terminal Sterilisation of Reusable Clean Room Garments

Following packaging, garments to be worn in clean rooms which manufacture products susceptible to microbial contamination can undergo additional terminal sterilisation. This is used to destroy any microorganisms which may remain on the garment following laundering and / or processing (Eudy 2007). However, sterilisation is a matter of degree or probability, with microbial death expressed as an exponential function (Eudy 2007). Therefore, sterilisation of clean room garments is only achievable to an acceptable sterility assurance level (SAL). For example, if the SAL of clean room garment is 10^{-6} this equates to a probability that there is only a one in a million chance that a microorganism will remain on the garment following sterilisation (Larkin 2009). Therefore, although the probability of microorganisms remaining on clean room garments following their sterilisation remains low, this number can never reach zero.

Terminal sterilisation of clean room clothing following laundering is usually achieved using one of three commonly applied methods - steam autoclaving, ethylene oxide (EtO) or gamma radiation (Eudy 2007). Steam autoclaving involves subjecting the sealed laundered garments to a temperature of 121 ^{o}C for 15 minutes (Eudy 2007). The steam and heat penetrate the garment killing any viable microorganisms and spores. However, this process has been shown to cause clean room garments to shrink significantly (McIlvaine and Tessien 2006), this not only creates a problem with regard garment fit but also causes seam deformation, which may allow the passage of microorganisms. Furthermore, steam sterilisation has been reported to cause degradation of the fabric (Eudy 2007), which can result in reduced fabric filtration efficiency - an essential clean room garment property. Gamma sterilisation is considered the preferred (Eudy 2007) and cheapest option to terminally sterilise clean room garments (Larkin 2009). During this process garments are subjected to destructive gamma

irradiation, emitted by the nucleus of the radioactive cobalt 60 atom during decay, in a controlled manner, at a minimum validated sterilising dose, necessary to reach the required acceptable SAL (Martin 2012). The high energy photons emitted from the atom ionise organisms, causing their covalent bonds in their DNA to break, resulting in organism death or their inability to spread (Martin 2012). To calculate the dose of gamma irradiation required to reach the necessary SAL the bacterial bioburden of the sample item proportion (SIP) (usually 10 % of a medium clean room suit) must be determined. From this the bacterial bioburden of the whole garment is then assessed and the dose calculated to reach the required SAL (Eudy 2007). There are a number of methods which can be used to calculate the required sterilisation dose, one of these is the VDmax method, where a dose of 25 kGy is predetermined to achieve a SAL of 10^{-6} (Martin 2012). The full garment laundering process is summarised in Figure 3 – 2.

To the author's knowledge there are no previous research studies which evaluate the bacterial bioburden of the surface of clean room garments following their laundering either with or without terminal sterilisation, with this being the first such study. The majority of previous garment laundering studies which examine the efficiency of such processes on textiles relate to those fabrics found in the hospital setting (Fijan *et al.* 2007; Nordstrom *et al.* 2012) and therefore cannot be directly compared to our study due to differences in fabric and laundering processes. Furthermore, previous research studies which focus on the effect laundering and sterilisation have on clean room garments do so with respect to fibre degradation and the barrier efficiency of the garment rather than the effectiveness of the process at achieving garment sterility (Ljungqvist and Reinmüller 2003; Lee *et al.* 2012; Galvin and Vyas 2016).

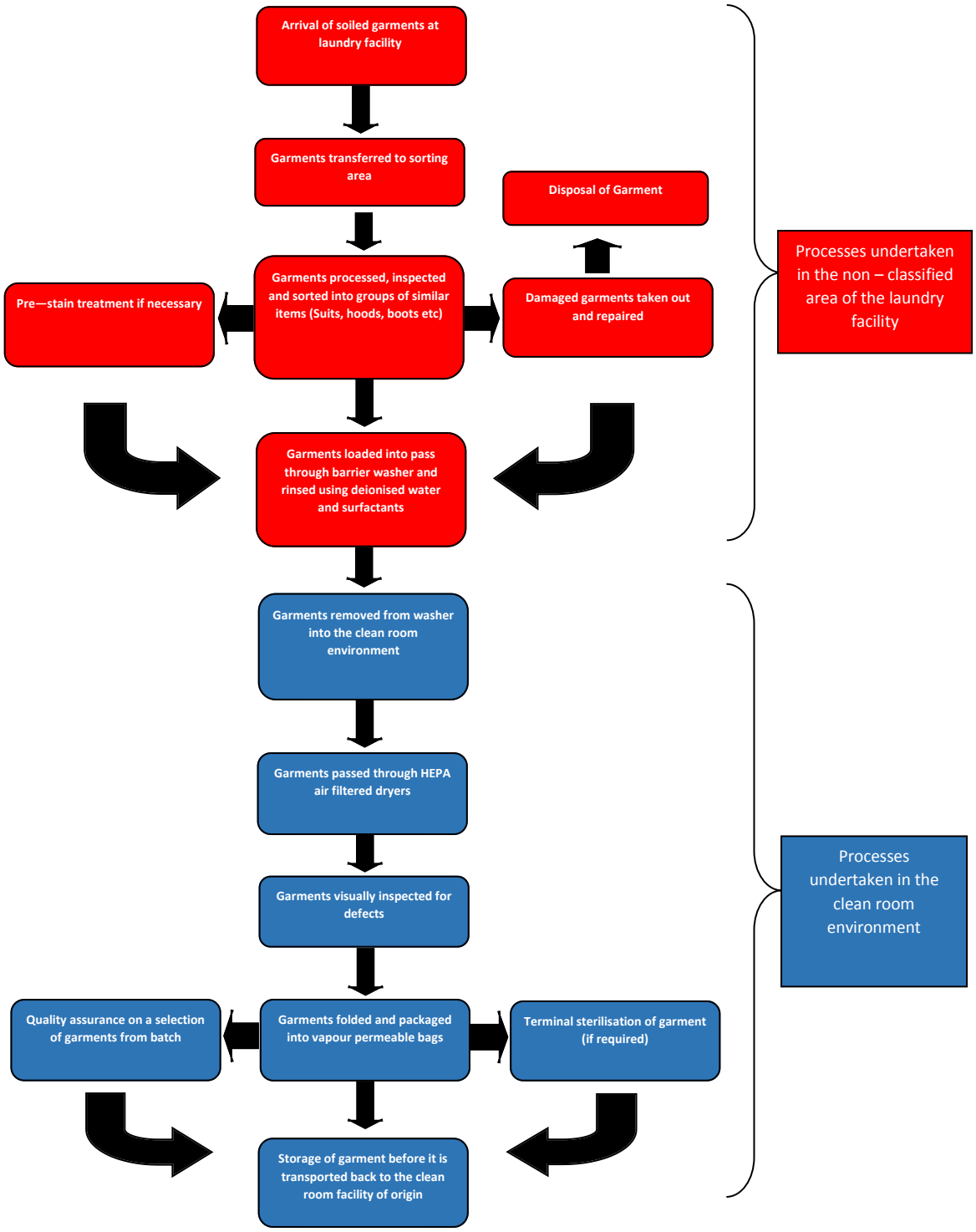


Figure 3 – 2: Schematic diagram of the laundering process undertaken on reusable clean room garments within an out - sourced laundering facility. Boxes in red dictate processes undertaken in the non - classified area of the facility and boxes in blue describe the processes undertaken in the clean room environment (Adapted from: English 1996; Eudy 2005; Hobson 2007; Bryant 2010; Clayton and Eaton 2011; Marshall 2015).

3.2 Aim & Objectives

The aim of this part of the project was to compare the surface bacterial bioburden of clean room suits following their laundering either with or without terminal sterilisation. In order to achieve this a series of specific objectives were developed:

- To use the direct agar contact method, developed in Chapter 2, to recover, enumerate and compare the levels of bacteria on the surface of clean room garments laundered either with or without terminal gamma sterilisation.
- With respect to laundering process, either with and without terminal gamma sterilisation, use the direct agar contact method to compare the surface bacterial bioburden of 13 clean room suit sites.
- With respect to agar composition, NA or TSA, assess the percentage of bacteria recovered from clean room garments laundered either with or without terminal gamma sterilisation.

3.3 Materials & Methodology

In order to achieve the specified aim and objectives in this study the following materials and methods were used.

3.3.1 Preparation of Contact Plates

Fifty five mm tryptone soya agar (TSA) and nutrient agar (NA) filled contact plates were prepared and stored as per the method discussed in Chapter 2 - Section 2.3.4. Contact plates were removed from the fridge and left at room temperature for two hours prior to use to eliminate condensation on the surface of the agar.

3.3.2 Clean Room Suits

Reusable polyester clean room suits interwoven with carbon anti – static material were purchased by Clean Room Facility A from Chemsplash ((Manchester, UK) (Formerly CCA Products)). Clean room suits of the same composition were supplied to Clean Room Facility B on a rental contract by Laundering Facility B.

3.3.3 Laundering & Sterilisation of Clean Room Suits

Clean room suits owned by Clean Room Facility A were laundered by Laundering Facility A. Clean room suits rented by Clean Room Facility B were owned, laundered and sterilised by Laundering Facility B. In each case thermal disinfection (75 ^{o}C for 6 minutes) was provided by rinsing the clothing in a barrier washing machine within an ISO Class 4 cleanroom (Table 1 – 4) using ultrapure water. Clothing was then dried using HEPA filtered air prior to packaging in vapor permeable bags. In addition, Clean Room Facility B's garments were terminally sterilized using gamma irradiation at a validated dose.

3.3.4 Direct Agar Contact Method of Bacterial Recovery

Clean room suits from each facility were tested under different conditions. This was because clean room suits from Clean Room Facility B could not leave their facility, nor could clean room suits from Clean Room Facility A enter into their facility. Therefore, suits owned by Clean Room Facility A were tested within a Grade A laminar airflow cabinet (Hepaire Products Corporation, Kanata, Canada) within an ungraded room at the Robert Gordon University (RGU) Garthdee Campus, Aberdeen. Suits rented by Clean Room Facility B were tested on an open bench top within a Grade C clean room at Clean Room Facility B. In both cases operators donned a hair net, face mask and sterile clean room gloves before donning a sterile clean room suit. The laminar airflow cabinet or bench top were disinfected using 70 % ethanol wipes (Critical Environment Solutions Ltd, Wiltshire, UK) and this was repeated between suits. Eight suits from each facility were tested at 13 different sites (Figure 3 – 3), 4 suits were tested with TSA filled contact plates and 4 suits were tested with NA filled contact plates. Testing was carried out in triplicate (in total 24 suits were tested from each facility - 12 suits were tested with TSA filled contact plates and 12 suits were tested with NA filled contact plates). Prior to testing, the outer packaging of each suit was disinfected using 70 % ethanol wipes before being cut open using sterile scissors and facing upwards the suit was slid from the packaging into either the cabinet or onto the bench for testing. In a systematic fashion, to avoid contamination of the suits, the direct contact method was undertaken on 13 areas of each suit (Figure 3 – 3) in numerical order. The lid of the contact plate was removed and the agar surface was applied to the test surface for 5 seconds with constant pressure. The lid of the contact plate was replaced and the contact plates were

inverted and incubated. Contact plates used for testing clean room suits at Clean Room Facility B were re - packaged in plate sleeves post testing to ensure their integrity and transported at a constant temperature back to RGU for incubation within one hour. All plates were examined for growth after 24 and 48 hours incubation at 37 ^{o}C. All results are reported as those at 48 hours due to an increase in the percentage of contact plates displaying low, moderate and high levels of growth following a further 24 hour incubation period at 37 ^{o}C (taking the total incubation period to 48 hours) observed during Chapter 4 of our study. To remove residual agar from the suits following testing, these were sponged with a 70 % ethanol impregnated wipe and the clothing sent to be laundered as per usual procedure.

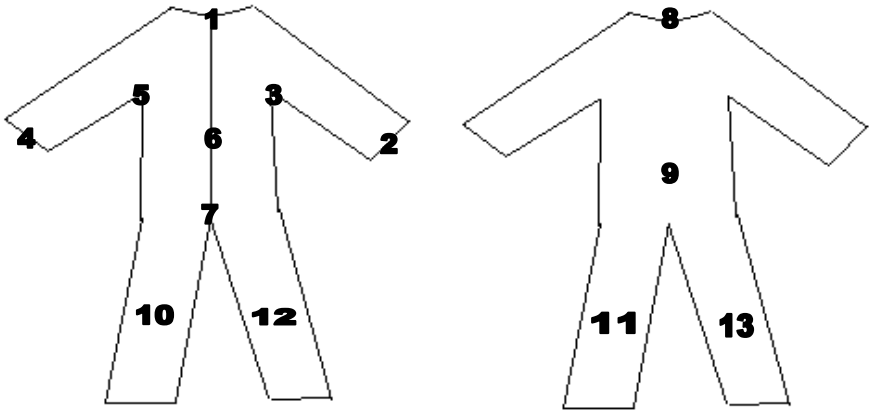


Figure 3 – 3: Diagram representing the front and back of a clean room suit. Numbers on the suit represent areas tested using the direct contact method. The areas are; 1 – chest, 2 – left carpus (wrist), 3 – left axilla (underarm), 4 – right carpus (wrist), 5 – right axilla (underarm), 6 – umbilicus (umbilical), 7 – pelvis (groin), 8 – posterior cervicis (nape of neck), 9 – lumbus (lower back) , 10 – right crus (shin), 11 – left sura (calf), 12 – left crus (shin), 13 – right sura (calf).

3.3.5 Statistical Analysis of Results

All results were analysed using GraphPad Prism 4.0 (GraphPad Software Inc., La Jolla, CA). Two – Way Analysis of Variance (ANOVA) at a 95 % confidence level was used to statistically analyse the results of the study.

3.4 Results

The direct agar contact method assessed in Chapter 2, using a total of 624 contact plates, was undertaken on 13 exterior sites of 48 clean room suits - 24 laundered with terminal gamma sterilisation and 24 laundered without terminal gamma sterilisation. Bacteria were recovered from 9 of the 24 suits tested which had undergone laundering without gamma sterilisation, as well as from 4 of the 24 suits tested which had undergone laundering with gamma sterilisation. Initially, in Chapter 2, the agar composition of contact plates were thought to have potentially underestimated the percentage of *S. aureus* NCTC 6571 recovered from the pre – inoculated antistatic carbon filament polyester fabric squares. Therefore, a comparison of the efficiency of two different agar types at recovering and supporting the growth of bacteria from the surface of reusable clean room suits laundered with and without terminal sterilisation was assessed in this stage of the study. Contact plates filled with either NA or TSA agar (156 of each - representing 100 %) were used to evaluate the surface bacterial bioburden of 24 clean room suits having undergone laundering without sterilisation and 24 clean room suits having undergone laundering with terminal gamma sterilisation (12 suits per agar composition). The percentage of contact plates displaying growth under each of the laundering process against agar composition can be seen in Figure 3 – 4. To further address the issue of bacterial recovery efficiency from clean room garments against agar composition a comparison was also drawn between agar type and the level of growth displayed on these contact plates following their incubation. The percentage of contact plates displaying either no growth (0 cfu / plate), low level growth (1 – 9 cfu / plate), moderate level growth (10 – 20 cfu / plate) or high level growth (> 20 cfu / plate) against agar composition, used to test the surface of suits laundering without terminal gamma sterilisation, can be seen in Figure 3 – 5 (a) and those plates used to test suits laundered with terminal gamma sterilisation can be seen in Figure 3 – 5 (b).

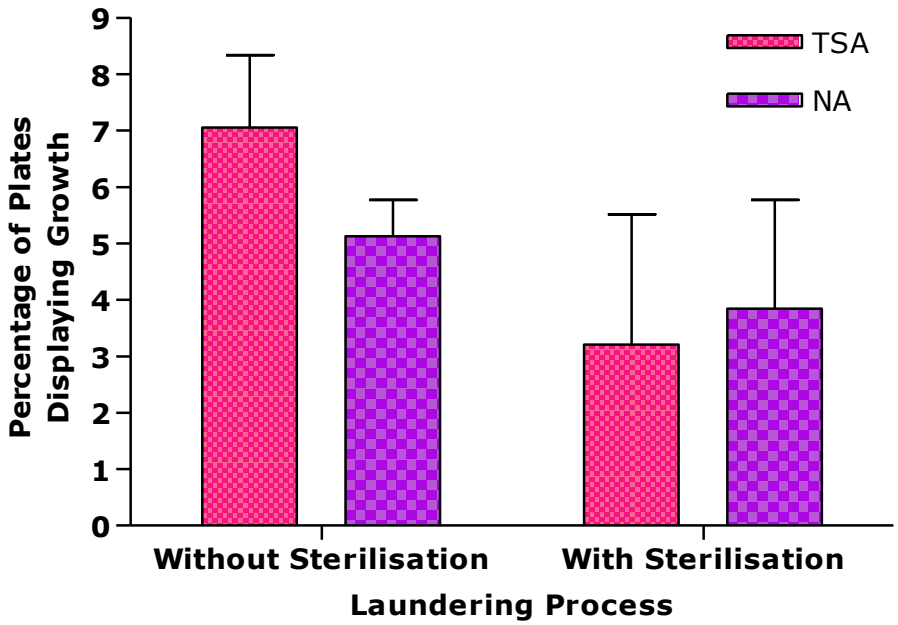


Figure 3 – 4: A comparison of agar composition (TSA or NA) against the percentage of contact plates displaying growth following a 48 hours incubation period at 37 °C, used to test the surfaces of clean room suits laundered with and without terminal gamma sterilisation.

Following a 48 hour agar incubation period, 7.1 % [11 / 156] of TSA filled contact plates and 5.1 % [8 / 156] of NA filled contact plates, used to test the exterior surface of suits laundered without gamma sterilisation, displayed growth. Furthermore, 3.2 % [5 / 156] of TSA filled contact plates and 3.8 % [6 / 156] of NA filled contact plates, used to test the surface of suits having undergone laundering with gamma sterilisation, displayed growth (Figure 3 – 4). Statistically, there was found to be no significant difference between the percentage of contact plates displaying growth against agar composition, for either laundering process - with or without gamma sterilisation.

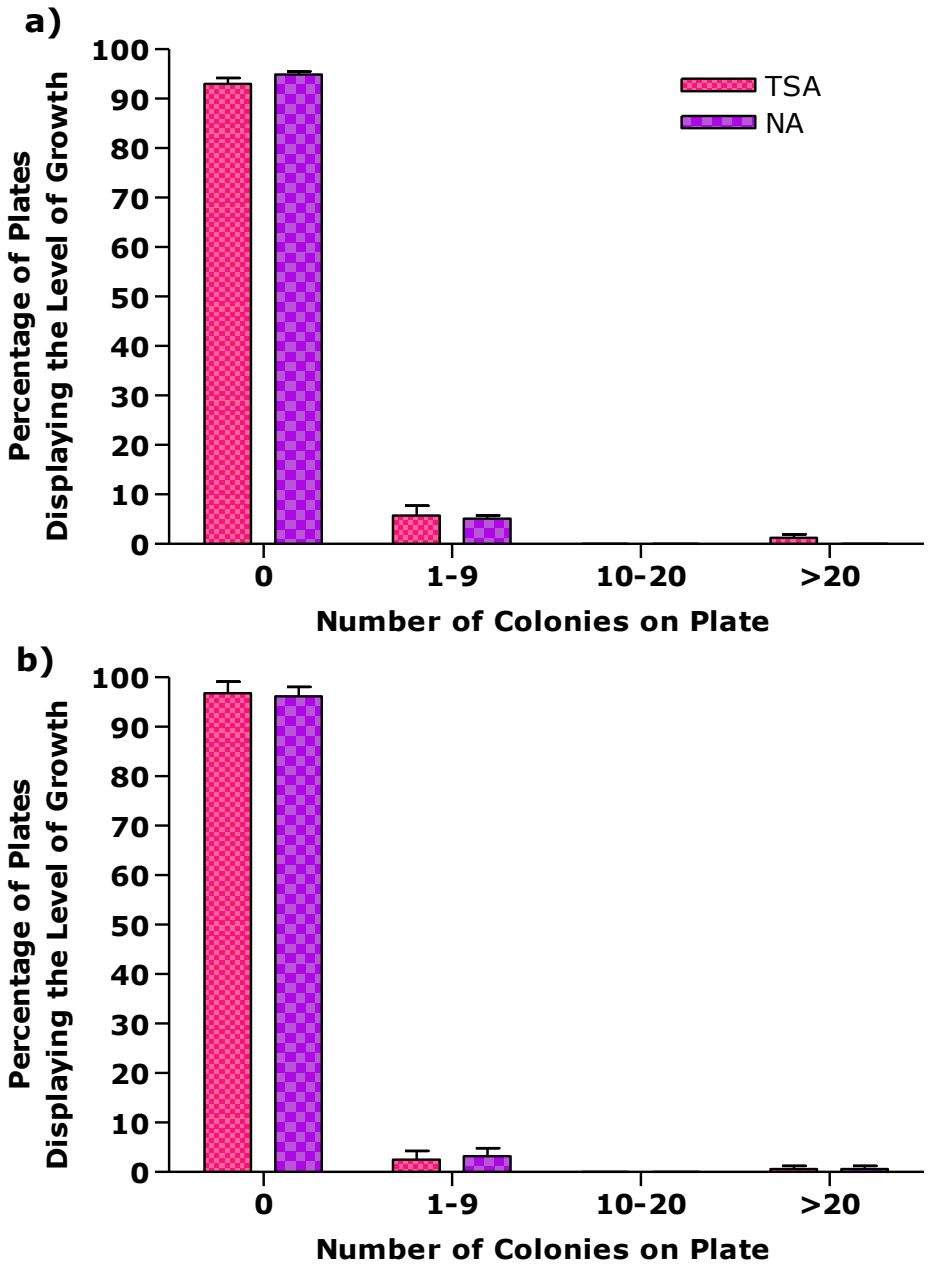


Figure 3 – 5: A comparison of agar composition (TSA or NA) against the percentage of contact plates displaying either 0 (no growth), 1 – 9 (low level growth), 10 – 20 (moderate level growth), or > 20 (high level growth) cfu / plate following a 48 hours incubation period at 37 °C, used to test (a) the surface of clean room suits laundered without terminal gamma sterilisation and (b) the surface of clean room garments laundered with terminal gamma sterilisation.

Of the 5.1 % [8 / 156] of NA plates which displayed growth following incubation, used to test the surface of suits laundered without gamma sterilisation, these all displayed low level growth. In comparison, of the 7.1 % [11 / 156] of TSA plates displaying growth following incubation, used to test the surface of garments

laundered under the same conditions, 5.8 % [9 / 156] of these showed low level growth and 1.3 % [2 / 156] high level growth (Figure 3 – 5 (a)). Of the 3.8 % [6 / 156] of NA contact plates displaying growth, used to test the surface of suits laundered with gamma sterilisation, 3.2 % [5 / 156] of these plates presented low level growth and 0.6 % [1 / 156] high level growth. In comparison, of the 3.2 % [5 / 156] of TSA contact plates displaying growth used to test suits laundered under the same condition, 2.6 % [4 / 156] showed low level and 0.6 % [1 / 156] high level growth (Figure 3 – 5 (b)). Statistically, there was shown to be no significant difference between the percentage of contact plates displaying the levels of growth and agar composition, either in plates used to test the surface of suits laundered without gamma sterilisation or those laundered with gamma sterilisation. Therefore, for the remainder of this study no comparison was drawn between the percentage of contact plates displaying growth and agar composition.

The efficiency of the two laundering processes, one without and one with terminal gamma sterilisation, were then compared (312 contact plates were used to test the surface of suits laundered under each process – representing 100 %). The percentage of contact plates displaying growth, used to test clean room suits laundered either with or without terminal gamma sterilisation, is shown in Figure 3 – 6. A further comparison was drawn between each process and the levels of growth displayed on these contact plates following their incubation. The percentage of contact plates displaying either 0 (no growth), 1 – 9 (low level growth), 10 – 20 (moderate level growth) or > 20 (high level growth) cfu / plate against laundering process is shown in Figure 3 – 7.

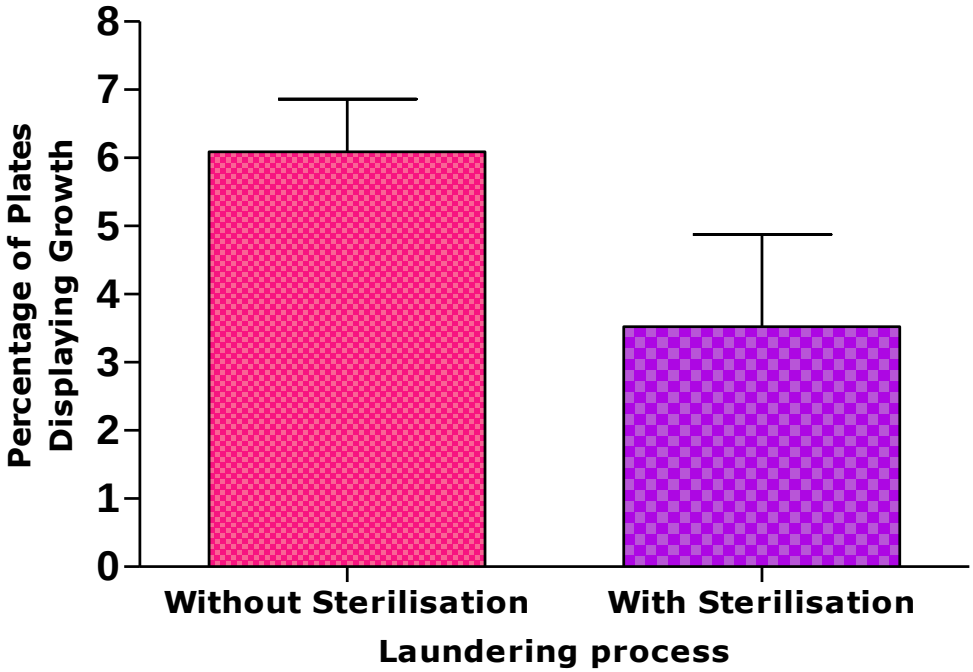


Figure 3 – 6: A comparison of laundering process (with or without terminal gamma sterilisation) against the percentage of contact plates displaying growth following a 48 hour incubation period at 37 ^{o}C, used to test the surface of clean room suits laundered with and without terminal gamma sterilisation.

Following a 48 hour incubation period, 6.1 % [19 / 312] of contact plates used to test the surface of clean room suits laundered without gamma sterilisation and 3.5 % [11 / 312] contact plates used to test the surface of suits laundered with gamma sterilisation displayed growth. Statistically, there was shown to be no significant difference between the percentage of plates displaying growth and laundering process. However, this difference equates to a 43 % reduction in the percentage of plates displaying growth between the two laundering processes (with gamma sterilisation - 11 contact plates (3.5 %) vs. without gamma sterilisation - 19 contact plates (6.1 %).

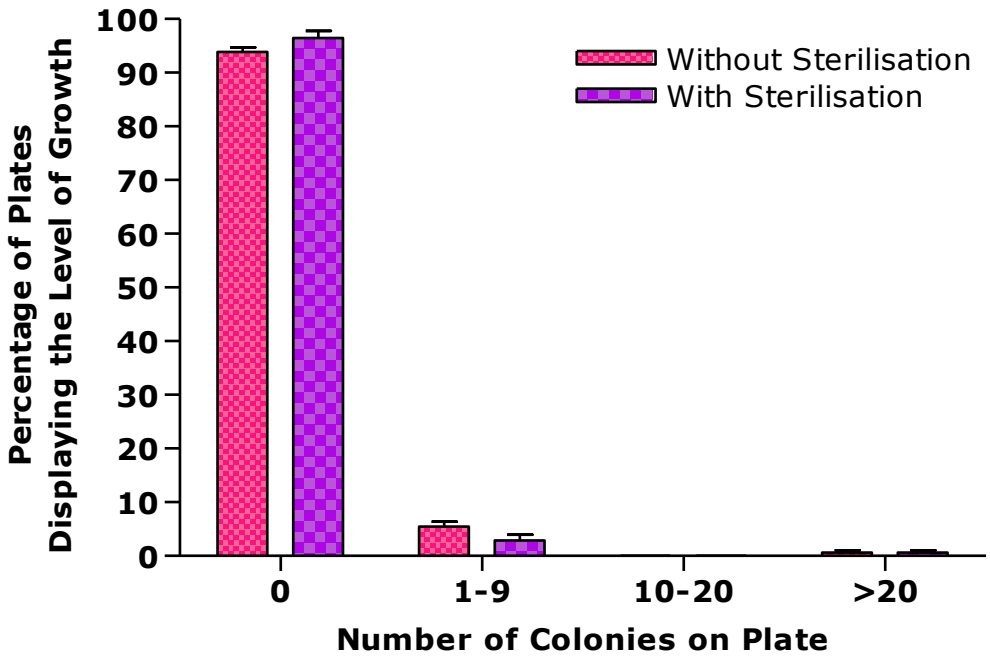
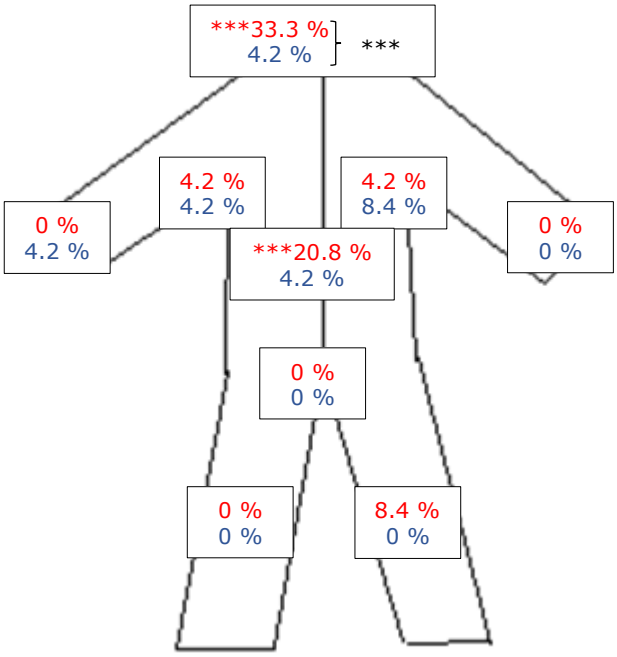


Figure 3 – 7: A comparison of laundering process (with or without terminal gamma sterilisation) against the percentage of contact plates displaying either 0 (no growth), 1 – 9 (low level growth), 10 – 20 (moderate level growth) or >20 (high level growth) cfu / plate following a 48 hour incubation period at 37 °C, used to test the surface of clean room garments laundered with and without terminal gamma sterilisation.

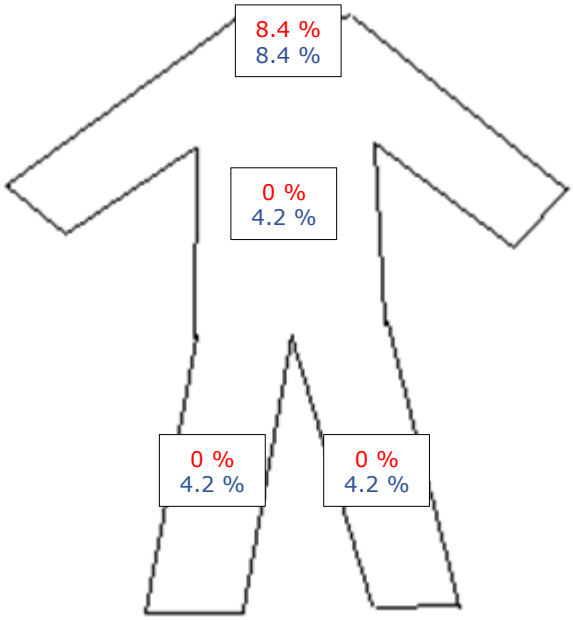
Of the 6.1 % [19 / 312] of contact plates displaying growth, used to test the surface of suits laundered without gamma sterilisation, 5.5 % [17 / 312] of these displayed low level growth and 0.6 % [2 / 312] high level growth. In comparison, of the 3.5 % [11 / 312] of contact plates displaying growth, used to test the surface of suits having undergone additional gamma sterilisation, 2.9 % [9 / 312] of these plates showed low level growth and 0.6 % [2 / 312] high levels of growth. Statistically, there was found to be no significant difference in the levels of growth displayed on contact plates against the variable, with and without gamma sterilisation.

In order to fully investigate the bacterial bioburden of clean room suits following laundering with and without terminal gamma sterilisation a further comparison was drawn between laundering process and the percentage of contact plates displaying growth at each of the garment sites tested (24 contact plates were used to test each site per laundering process – representing 100 %) (Figure 3 – 3). The percentage of contact plates

displaying growth at each suit site tested for each laundering process is compared in Figure 3 – 8. To further compare the bacterial bioburden of each suit site tested with respect to laundering process an additional comparison was drawn between laundering process and the levels of growth displayed on these contact plates at each site. The percentage of contact plates displaying either 0 (no growth), 1 – 9 (low level growth), 10 – 20 (moderate level growth) or > 20 (high level growth) cfu / plate at each site of clean room garments laundered with and without gamma sterilisation (Figure 3 – 9). Additional analysis was also undertaken to assess the levels of growth displayed on contact plates and suit sites for each of the laundering processes individually. The percentage of contact plates displaying either 0 (no growth), 1 – 9 (low level growth), 10 – 20 (moderate level growth) or > 20 (high level growth) cfu / plate at each suit site for garments laundering without gamma sterilisation can been seen in Figure 3 – 10 (a) and those laundered with gamma sterilisation can be seen in Figure 3 – 10 (b).



Front of Clean Room Suit



Back of Clean Room Suit

Figure 3 – 8: Diagram representing the total percentage of plates displaying growth at each if the 13 suit sites tested, under each laundering process, without (red) and with (blue) gamma sterilisation, following a 48 hour incubation period at 37 ºC. (*** $p < 0.001$) represents the percentage of plates displaying growth used to test the chest of suits laundered without gamma sterilisation vs. the percentage of plates displaying growth used to test the chest of suits laundered with gamma sterilisation)(*** $p < 0.001$ represents the percentage of contact plates displaying growth at the umbilicus and chest of suits laundered without gamma sterilisation vs. the percentage of plates displaying growth at the other sites of suits laundered under the same condition).

Following incubation, 33.3 % [8 / 24] of contact plates used to test the chest and 20.8 % [5 / 24] of those used to test the umbilicus (umbilical) regions of suits laundered without gamma sterilisation displayed growth. Furthermore, 8.4 % [2 / 24] of those used to test the posterior cervicis (nape of neck) and left crus (shin) and 4.2 % [1 / 24] of those used to test the left and right axilla (underarm) also displayed growth. Contact plates used to test the left and right carpus (wrist), pelvis (groin), lumbus (lower back) or right and left sura (calf) and right crus of suits laundered under the same condition did not display bacterial growth. In comparison, 8.4 % [2 / 24] of contact plates used to test the left axilla and posterior cervicis regions of suits laundered with sterilisation displayed growth, as well as 4.2 % [1 / 24] of those used to test the chest, umbilicus, right axilla, lumbus, right carpus and right and left sura region of suits laundered under the same condition. The was no growth present on contact plates used to test the pelvis, left and right crus, and left carpus regions of the same suits. Statistically, a significantly higher percentage of plates used to test the chest region of suits laundered without gamma sterilisation displayed growth compared to the percentage of plates displaying growth used to test the same region of suits laundered with gamma sterilisation (*** $p < 0.001$). Additionally, a significantly higher percentage of plates used to test the chest and umbilicus of suits laundered without gamma sterilisation displayed growth compared to the percentage of plates displaying growth used to test the other suit sites laundered under the same condition (*** $p < 0.001$). There was shown to be no significant difference between the percentages of plates displaying growth and suit sites in those garments laundered with gamma sterilisation.

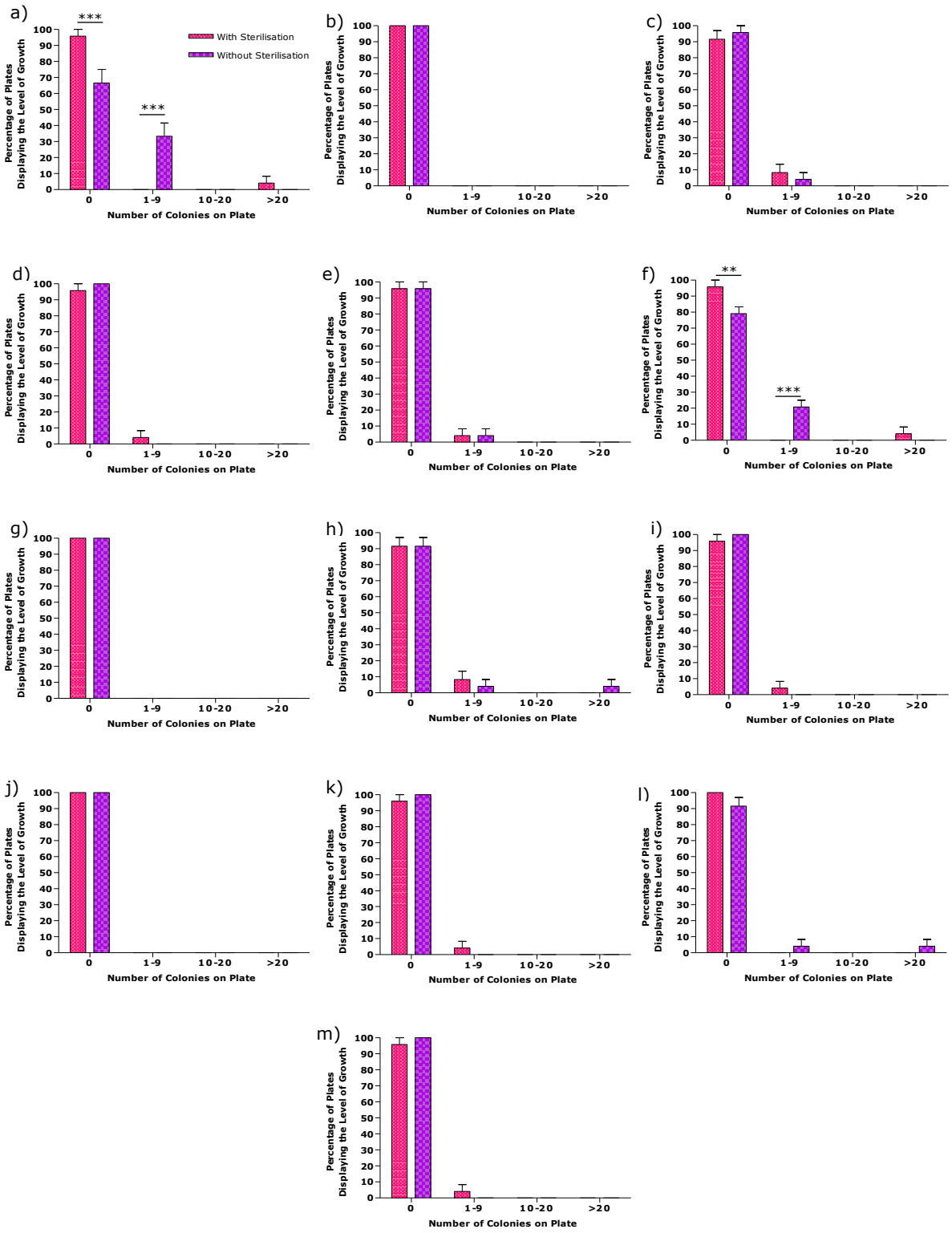


Figure 3 – 9: A comparison of laundering process (with and without terminal gamma sterilisation) against the percentage of contact plates displaying either 0 (no growth), 1 – 9 (low level growth), 10 – 20 (moderate level growth) or > 20 (high level growth) cfu / plate, following a 48 hour incubation period at 37 °C, used to test the – (a) chest, (b) left carpus, (c) left axilla, (d) right carpus, (e) right axilla, (f) umbilicus, (g) pelvis, (h) posterior cervicis, (i) lumbus, (j) right crus, (k) left sura, (l) left crus, (m) right sura regions of clean room garments (*** $p < 0.001$; ** $p < 0.01$).

As shown in Figure 3 – 9 (a), a significantly higher percentage of plates used the test the chest region of suits laundered with gamma sterilisation (95.8 % [23 / 24]) displaying no growth following incubation compared to the percentage of plates displaying growth used to test the same region of suits laundered without gamma sterilisation (66.7 % [16 / 24]) (*** $p < 0.001$) (as also shown in Figure 3 – 8). Additionally, there was found to be a significantly higher percentage of plates used to test the chest of garments laundered without gamma sterilisation displaying low levels of growth (33.3 % [8 / 24]) compared to the percentage of plates displaying growth the same levels of growth, used to test garments laundered with gamma sterilisation (0 % [0 / 24]) (*** $p < 0.001$). A statistical significance was also shown between the percentage of contact plates displaying growth used to test the umbilical region and laundering process (Figure 3 – 9 (f)). A significantly higher percentage of plates used to test the umbilicus region of suits laundered with gamma sterilisation displayed no growth (95.8 % [23 / 24]) compared to the percentage displaying no growth used to test the same region of suits laundered without gamma sterilisation (79.20 % [19 / 24]) (** $p < 0.01$). Additionally, there was found to be a significantly higher percentage of plates displaying low level growth used to test the umbilicus region of suits laundered without gamma sterilisation (20.8 % [5 / 24]) compared to the percentage of plates displaying the same level of growth used to test the same region of garments laundered with gamma sterilisation (0 % [0 /24]) (*** $p < 0.001$). However, there was shown to be no significant difference between laundering process and the percentage of contact plates displaying either no, low, moderate or high levels of growth and the other sites tested.

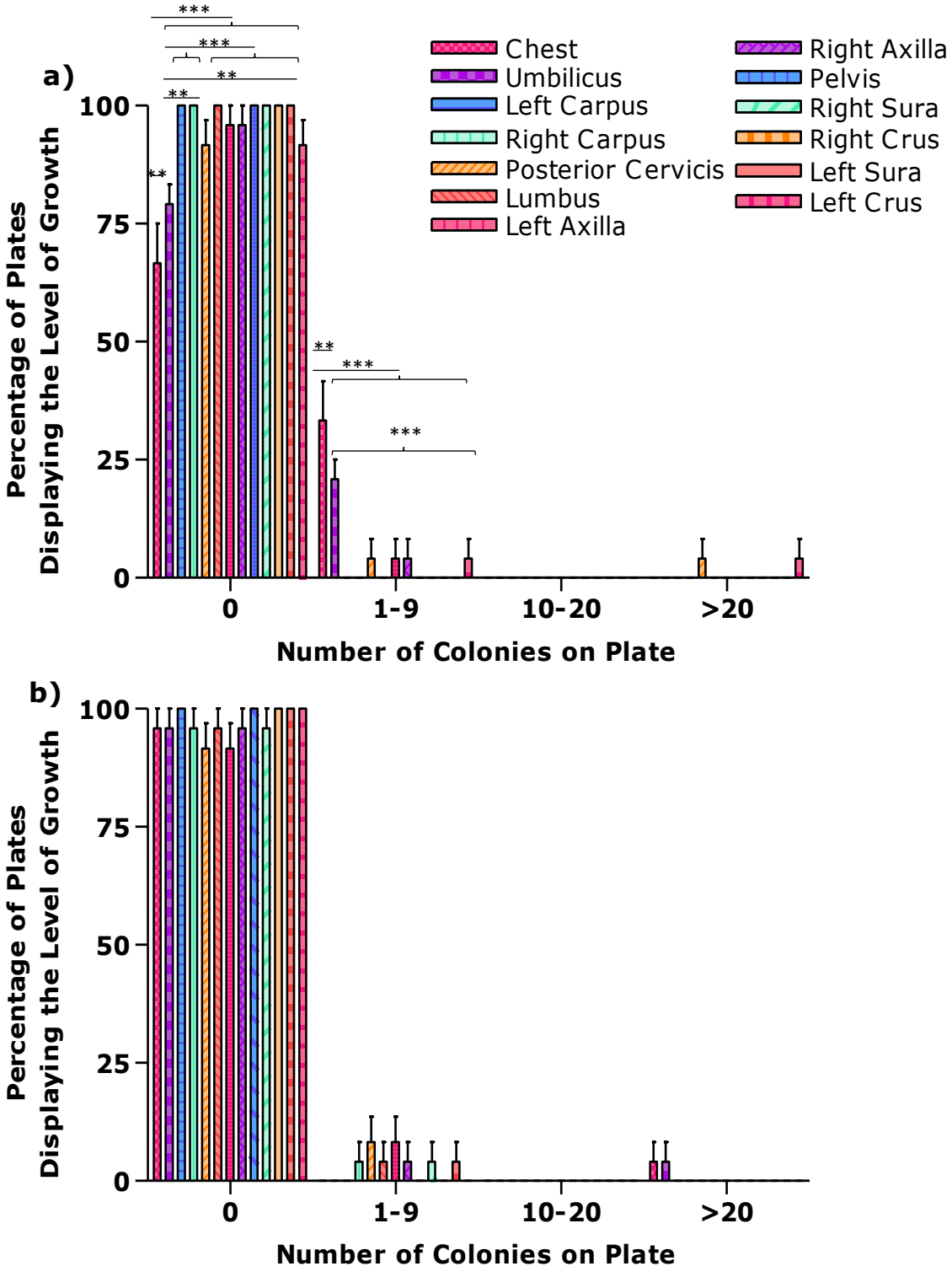


Figure 3 – 10: A comparison of suit site against the percentage of contact plates displaying either 0 (no growth), 1 – 9 (low level growth), 10 – 20 (moderate level growth) or >20 (high level growth) cfu / plate following a 48 hour incubation period at 37 ^{o}C, used to test 13 sites of clean room suits (a) laundered without terminal gamma sterilisation and (b) laundered with terminal gamma sterilisation (** $p < 0.01$; *** $p < 0.001$).

As shown in Figure 3 – 10 (a), a significantly lower percentage of contact plates used to test the chest (66.7 % [16 / 24]) displayed no growth following incubation when compared to the percentage of plates displaying no growth used to test the umbilical (79.2 % [19 / 24]) (** $p < 0.01$), posterior cervicis and left crus (both 91.8 % [22 / 24]), right and left axilla (both 95.8 % [23 / 24]) and lumbus, right and left carpus, pelvis, right and left sura and right crus (all 100 % [24 / 24]) (all *** $p < 0.001$). Furthermore, a significantly lower percentage of contact plates used to test the umbilicus region of suits laundered under the same conditions displayed no growth compared to the percentage displaying growth used to test all other sites, aside from the chest (** $p < 0.01$ - posterior cervicis and left crus; *** $p < 0.001$ – right and left axilla, left and right carpus, lumbus, pelvis, left and right sura and right crus).

In addition, of the plates used to test the chest that displayed growth all showed low levels (33.3 % (8 / 24)), this was found to be a significantly higher percentage than the percentage of plates displaying low level growth used to test the umbilicus region (20.8 % [5 / 24]) (** $p < 0.01$) and the posterior cervicis, right and left axilla and left crus (4.2 % [1 / 24]), as well as the left and right carpus, lumbus, pelvis, right and left sura and right crus (all 0 % [0 / 24]) (all *** $p < 0.001$). Aside from the chest, a higher percentage of plates used to test the umbilicus region of suits laundered without gamma sterilisation also displayed low levels of growth compared to those plates used to test the other 11 sites (20.8 % [5 / 24] vs. 4.2 % [1 / 24] (posterior cervicis, left and right axilla, and left crus) and 0 % [0 / 24] (left and right carpus, lumbus, pelvis, right and left sura and right crus) (all *** $p < 0.001$) (Figure 3 – 10 (a)). Statistically, the percentage of plates displaying either no, low, moderate or high levels of growth did not vary significantly between suit sites in those garments laundered with gamma sterilisation (Figure 3 – 10 (b)).

3.5 Discussion

Clean room garments should not contribute towards the bacterial bioburden of the clean room environment. In a bid to prevent the transmission of microorganisms via clean room operators' specialist garments the NHS Pharmaceutical Quality Assurance Committee recommend surface monitoring of such garments following the donning process. The board recommend that

garment testing is carried out in the changing area of the clean room facility following dressing by the operator. Both the right and left hand side of the front hood, collar cuff, wrist, leg and boots should be monitored using contact plates or wetted swabs and investigative and corrective action taken if any sample achieves levels greater than 5 cfu / plate (Rhodes 2006). However, bacterial monitoring of clean room garments post laundering (with / without gamma sterilisation) and prior to the donning process is an area not routinely documented and is one which severely lacks in published literature. This is of concern as the results of this part of our study confirm that that microorganisms were still present on the surface of reusable clean room suits despite these having undergone out – sourced laundering either with or without terminal gamma sterilisation (Figure 3 – 6). Furthermore, with the recovery of bacteria from antistatic carbon filament fabric being found to be less than 3 % using the direct agar contact method (Chapter 2), this suggests that levels of bacteria present on the surface of clean room suits following laundering and / or sterilisation may be well in excess of what is reported in the study. The results of our study suggest that reusable clean room clothing may increase the number of microorganisms being introduced into the clean room environment, having a potentially detrimental impact upon the environment and product(s) manufactured within the room. It also reiterates previous concerns raised over the sterility of reusable garments returning from out – sourced facilities (Larkin 2009). However, as previously discussed, no laundering and / or sterilisation process is 100 % reliable and even gamma irradiation of clean room garments is only achievable to an acceptable sterility assurance level (SAL) (Eudy 2007). Furthermore, although a gamma irradiation dose of 25 kGy is traditionally recommended for the sterilisation of medical devices (Nguyen *et al.* 2007), bacterial resistance has previously been reported in various materials at this dosage (Rainey *et al.* 2005; Shathele 2009), as also suggested by the presence of bacteria on contact plates having been used to test clean room garments having previously undergone gamma sterilisation in this study.

Despite the recovery of bacteria from suits having undergone each of the laundering processes in this study, a 43 % reduction in the number of contact plates displaying growth was observed between those used to test garments having undergone laundering without gamma sterilisation against those used to

test garments having undergone gamma sterilisation (6.1 % [19 / 312] vs. 3.5 % [11 / 312]), as shown in Figure 3 – 6. This suggests that reusable garments to be worn in cleans rooms in which the product is at risk of microbial contamination should undergo additional gamma sterilisation following laundering to reduce their bacterial bioburden. Furthermore, the results of our study also suggest that gamma sterilisation of clean room garments can significantly reduce the bacterial bioburden of clean room garment sites prone to higher levels of contamination during garment wear. The chest region of clean room suits have previously been reported as acquiring higher levels of contamination during wear. This is primarily thought to be due to the billowing or pumping actions of the clean room suit during wear, with particles and microorganisms travelling up the suits in the direction of the head and feet (Eudy 2003) and becoming contained within the airflow out of the garment (Clark and de Calcina - Goff 2009). In addition, this area may be more highly contaminated than other sites as there may be no everyday clothing barrier between it and the skin. Furthermore, this region is close to the mouth and may become contaminated by bacteria exiting the oral cavity, which has previously been identified on skin (Dewhirst *et al.* 2010). As shown in Figure 3 – 8, a significantly higher percentage of contact plates used to test the chest and umbilicus region of suits laundered without gamma sterilisation displayed growth compared to those used to test the other sites (*** $p < 0.001$). Upon investigation of the levels of growth displayed on these plates, as shown in Figure 3 – 10, significantly more plates used to test the chest and umbilicus regions displayed low level growth than the other sites tested (*** $p < 0.001$), with significantly more used to test the chest region of suits also displaying low level growth than those used to test the umbilicus region (** $p < 0.01$). Interestingly, a previously unpublished study by Tayside Pharmaceuticals Quality Assurance Department (2007), testing the surface bacterial bioburden of clean room operators garments following donning, recovered higher numbers of bacteria from the neck region of suits (correlating to the chest region in our study) than the other sites tested when investigated using swabbing. Furthermore, in a study by Grangè *et al.* (2010), investigating the presence of bacteria on cleanroom suits during their wear, the neck of the garments were found to be more highly contaminated than the other sites tested following 3, 4 and 5 hours wear.

It is unknown why the umbilical region of suits, having undergone laundering without gamma sterilisation, were found to be more contaminated than other sites tested during this study, but this may be due to the close proximity of this region to the direct working area and potential contact with hands and work surfaces. However, one theory to consider is that both of these sites may have become contaminated by hand borne transfer during the processing phase of laundering post drying. At this point in the process the zip of the suit is pulled up across the umbilicus and chest region, additionally, a hand is used to secure the stud fastening at the chest. In fact, recontamination of clean room garments post washing is of concern, and therefore additional gamma sterilisation of garments may mitigate bacteria associated with the recontamination of garments during the process and packaging phase of laundering, post washing and drying. Therefore, it is critical that if garments are to be laundered without terminal gamma sterilisation that a high standard of caution is maintained during the processing and packaging phase to mitigate recontamination of garments. Therefore, along with the results of our study it is suggested that clean room garments should undergo terminal sterilisation post laundering. The results of our study also suggest that gamma sterilisation is a means by which to decontaminate areas of clean room garments prone to contamination during wear. This was observed in Figure 3 – 8, where a significant reduction was observed between the percentage of contact plates displaying growth used to test the chest of suits laundered without and with gamma sterilisation (33.3 % [8 / 24]) vs. 4.2 % [1 / 24]) (*** $p < 0.001$). Additionally, in Figure 3 – 9, a higher percentage of plates used to test the chest (95.8 % [23 / 24] vs. 66.7 % [16 / 24]) (*** $p < 0.001$) (Figure 3 – 9 (a)) and umbilicus (95.8 % [23 / 24] vs. 79.2 % [19 / 24] (** $p < 0.01$) (Figure 3 – 9 (f)) regions of suits laundered with gamma sterilisation displayed no growth compared to those used to test the same region of suits laundered without gamma sterilisation following incubation. Also in Figure 3 – 9, a significant reduction in the percentage of plates displaying the low levels of growth, used to test the chest and umbilicus of suits laundered without gamma sterilisation was also observed ((chest 33.3 % [8 / 24] vs. 0 % [0 / 24] (*** $p < 0.001$) (Figure 3 – 9 (a)) and ((umbilicus - 0 % [0 / 24] vs. 20.8 % [5 / 24] (** $p < 0.01$) (Figure 3 – 9 (f)).

The results of this part of our study suggest that clean room garments to be worn in pharmaceutical clean rooms where the product is at risk of microbial contamination should undergo gamma sterilisation following laundering. However, multiple laundering cycles with and without gamma sterilisation have previously been shown to effect the physical properties of reusable clean room garments (Galvin and Vyas 2016). In fact, repeated gamma sterilisation of garments has been found to decrease the fabric strength and barrier efficiency of the clothing, furthermore, there was found to be an increase in the number of particles being disseminated from the garments (Galvin and Vyas 2016). Therefore, although gamma sterilisation of reusable clean room clothing may significantly decrease the microbial load of the garment post laundering, as was shown in our study, over time and numerous washing / sterilisation cycles, degradation of the polyester may occur, resulting in deterioration of the garment performance and significantly decreasing the barrier efficiency of the garment, which is critical in a pharmaceutical clean room. In addition, Lee *et al.* (2012), studying the effect of washing and abrasion on cleanroom garment failure, found that as clean room garments are abraded and washed there is an increased pattern of airborne particles. However, Ljungqvist and Reinmüller (2003) found that the barrier efficiency of clean room garments was not proportional to their number of laundering and sterilisation cycles, with operators dispersing less particles when wearing garments having undergone 50 cleaning and sterilisation cycles than those wearing garments having undergone 25 rounds (Ljungqvist and Reinmüller 2003). Additionally, Leonas (1998), studying the effect of industrial laundering on surgical garments, showed that pore size of fabrics significantly increased following 25 laundering cycles, however, there was shown to be no significant difference between pore sizes after 25 and 50 cycles. These studies show that not only could the initial bacterial bioburden of the suit pose a risk to the product and patient, but such defects in the fabric caused by laundering processes could reduce the barrier filtration efficiency of the garment and in turn increase the passage of bacteria from the operator into the environment and product.

There are a number of ways in which the microbial contamination and the reduced barrier efficiency associated with reusable garments may be mitigated. Firstly, reusable clothing could be substituted for single - use disposable attire.

Disposable clean room garments are usually manufactured using non - woven flash – spun polyethylene fabric. According to clean room analysts, garments constructed in this manner can filter particles and microorganisms at the sub - micron level, whilst protecting the operator from non – hazardous fluids (Larkin 2009). However, previous studies have shown that wearing garments constructed of such occlusive fabrics can reduce the air permeability between the operator's skin and the environment, making the garment uncomfortable to wear by increasing the body temperature and stress of the operator (Hao *et al.* 2004; Chen *et al.* 2013). Despite this, analysts in the clean room industry confirm that most facilities will opt for their operators to wear disposable clean room clothing due to concerns that reusable clothing returning from out - sourced laundering facilities may be contaminated (Larkin 2009). Such disposable clothing assures a predictable and consistent performance, eliminating variability in the barrier filtration efficiency of reusable garments and potentially improving contamination control (Larkin 2012), which is especially important in pharmaceutical clean rooms. Furthermore, disposable clean room clothing can provide an easier gowning process. Unlike reusable suits the design of disposable suits can help reduce the potential of contamination during the donning process (Larkin 2012). However, despite the apparent advantages of disposable garments, these can prove to be expensive, with not only the cost of the garments to consider but their shipping and storage (Hobson 2007). Furthermore, disposal of the garments can not only prove expensive but can have a considerable environmental impact (Hobson 2007). However, it is also important to consider the costs associated with the laundering of reusable clothing, which can also prove expensive. Charges can mount up and can include services such as transportation fees and lost garment charges, as well as fees for the actual laundering process itself and any additional sterilisation procedures (Larkin 2009). Despite this, reusable clothing is still reported to be nearly a third cheaper than a disposable garment system (Hobson 2007). Therefore, although disposable clothing mitigates problems associated with garment contamination and fabric barrier filtration efficiency, reusable clothing can be cost effective, whilst also reducing the waste and environmental impact associated with disposable clothing. It may also be more comfortable for the operator to wear (McIlvaine and Tessien 2006). However, before a facility considers the use of disposable clean room garments in place of reusable garments, further work

should be undertaken to determine the bacterial bioburden of disposable clean room garments prior to donning and a comparison drawn between these and reusable garments – something which to the author's knowledge has not been studied.

Microbial contamination of reusable clothing may also be mitigated by manufacturing garments using an antimicrobial treated fabric, which can either be applied to the surface of the fabric or incorporated into the fibres (Clayton and Eaton 2011). There are a number of anti – microbial fabric available which can be used to manufacture clean room garments (Clayton and Eaton 2011), with such a finish reducing the number of viable organisms adhering to the fabric (Bajpai *et al*. 2011) and therefore reducing the bacterial bioburden of clean room garments prior to their laundering (McIlvaine and Tessien 2006). Furthermore, it may also decrease the release of microorganisms from the garment into the environment (Hobson 2007). An additional benefit of such finishes is they can reduce odour causing microbial growth (McIlvaine and Tessien 2006). Despite a rise in the use of antimicrobial treated fabrics (Bajpai *et al.* 2011) the use of such fabrics to manufacture clean room garments, as well as their efficiency, is not well documented in the literature. Lastly, confidence in laundering / sterilisation processes may be increased by clean room facilities opting to install their own on - premises laundry as an alternative to using an out - sourced facility. However this option has to be cost effective, as well as the initial outlay, the cost of maintaining such a facility is substantial (Eudy 2005). Therefore, on site laundering would not be a viable option for most clean room facilities.

Ideally, to ensure reproducibility of the results obtained during this part of our study further investigation of the bacterial bioburden of clean room garments following laundering with or without gamma sterilisation should be undertaken. This should include the sampling of larger garment pools from a greater number of out – sourced laundering establishments. Furthermore, garments should be tested under the same conditions. This is a factor which in our study could not be controlled due to access issues at facility B including the inability to remove or bring garments into or out of their premises. The sampling of garments during our study had to be undertaken at two different locations and therefore under different conditions. However, in order to counteract this effect steps were taken

to minimise the impact upon the results of our study. During this study contamination risk was minimised by undertaking all analysis under clean conditions, ensuring all surfaces and packaging was fully sterilised using 70 % ethanol prior to and between suits being tested. The operator also took every precaution to avoid cross contamination during the sampling process, including donning clean room garments and accessories appropriate for the task. This also helps mitigate the argument that contamination of the suits arose during their testing. Determining the identification of the bacteria recovered from the suits during this study would help to establish their source, therefore phenotypic and genotypic methods of bacteria identification were undertaken on a representative selection of colonies recovered during this part of our study and the results will be presented in Chapter 6.

It was previously reported in Chapter 2 of our study that agar composition may have been a contributing factor towards the low bacterial recovery efficiencies observed using the direct agar contact method. Agar composition has previously been shown to have an effect on bacteria recovery using surface sampling techniques (Foschino *et al.* 2003) and using contact plates filled with TSA instead of NA may have increased recovery as it has been shown to recover higher percentages of bacteria compared to selective agars such as Violet Red Bile agar or MacConkey agar (Foschino *et al.* 2003). However, both NA and TSA are general purpose agars used for the cultivation of a wide range of microorganisms, and are both comprised of nutrients suitable to sustain bacterial growth. Therefore, any difference in bacterial recovery would be minimal. During this part of our current study this issue was investigated by testing suits with contact plates filled with both TSA and NA. There was shown to be no statistically significant difference between the percentage of contact plates displaying growth and agar composition for both laundering processes (Figure 3 – 4). Furthermore, there was found to be no significant difference in the levels of growth recovered and agar composition (Figure 3 – 5). These results support the argument raised in Chapter 2 - Section 2.6, that differences in TSA and NA at supporting microbial growth should be minimal because both are general purpose bacteriological agars. Therefore, the poor recovery efficiencies obtained in Chapter 2 of this current study are not thought to be a result of agar composition.

3.6 Conclusion

In this chapter the bacterial bioburden of the surface of reusable antistatic carbon filament polyester clean room suits laundered with or without terminal gamma sterilisation were compared using the direct contact method assessed in Chapter 2. Microorganisms were recovered from suits having undergone either process, with a higher percentage of plates used to test suits laundered without gamma sterilisation displaying growth. A higher percentage of plates used to test the chest and umbilicus regions of suits laundered without gamma sterilisation displayed low levels of growth when compared to the other 11 sites tested in suits laundered under the same conditions. Terminal sterilisation of clean room garments following laundering was found to reduce the percentage of contact plates displaying growth by 43 %, with a significant reduction in the percentage of plates used to test the chest and umbilical regions displaying low levels of growth. In addition, bacterial recovery using contact plates filled with two different agar compositions, TSA and NA, was studied. These agar types were shown to have no significant effect upon the percentage of contact plates displaying growth, as well as the levels of growth exhibited.

Chapter 4:

An Investigation into the Surface Bacterial Bioburden of Reusable Antistatic Carbon Filament Polyester Clean Room Garments Donned by Operators Dressing Wearing Either No Gloves, Non – Sterile Gloves or Sterile Clean Room Gloves

4.1 Introduction

The polyester fibres of the antistatic carbon filament fabric commonly used to manufacture reusable clean room garments can provide a suitable substrate for bacteria to adhere to and grow upon (Hsieh and Merry 1986; Neely and Maley 2000; Schmidt – Emrich *et al.* 2016). In turn, these specialist garments can become a vehicle for the transmission of microorganisms, having a potentially detrimental impact upon the sterility and integrity of a clean room environment and its products. Inadequate garment donning processes can lead to the outer surface of clean room clothing becoming contaminated with bacteria prior to these entering the clean room environment (Rhodes 2010), therefore, such garments should be handled in a manner which does not increase their bacterial bioburden (European Commission 2008), ensuring the outer surface of the garments remain sterile following their donning (Rhodes 2010) and do not acquire microorganisms which can be introduced into the clean room and potentially shed from the garment at a later date. However, despite contaminated hands having been reported as a primary transmission mode for microorganisms (World Health Organisation 2009), there is no standardised approach to glove use during the clean room garment donning process. This was an issue deemed worthy of investigation due to the differing approaches undertaken within various clean room facilities. For example, clean room operators at both RGU and Tayside Pharmaceuticals wear gloves whilst donning clean room clothing - operators at RGU wear non - sterile gloves, whilst operators at Tayside Pharmaceuticals wear sterile clean room gloves (C. Alexander 2012, Personal Communication). In comparison, clean room operators at Aberdeen Royal Infirmary opt to omit glove use during the donning process (C. Dawson 2012, Personal Communication). To the author's knowledge there are no published research studies which compare the surface bacterial bioburden of clean room garments following their donning under this variable. Therefore, in this part of our study, the bacterial bioburden of the surface of clean room garments donned by operators dressing wearing either no gloves, non – sterile gloves or sterile clean room gloves was compared and assessed using a direct agar contact plate method (as examined previously in Chapters 2 and used to successfully compare the resultant bacterial bioburden of the surface of antistatic carbon filament polyester clean room garments laundered with and without gamma sterilisation in Chapter 3).

4.1.1 Microbial Transmission via the Hand Borne Route

The spread of an infectious organism from one site to another is reliant upon a number of key stages. Also known as the chain of infection (Taylor 2001), this process is illustrated in Figure 4 – 1.

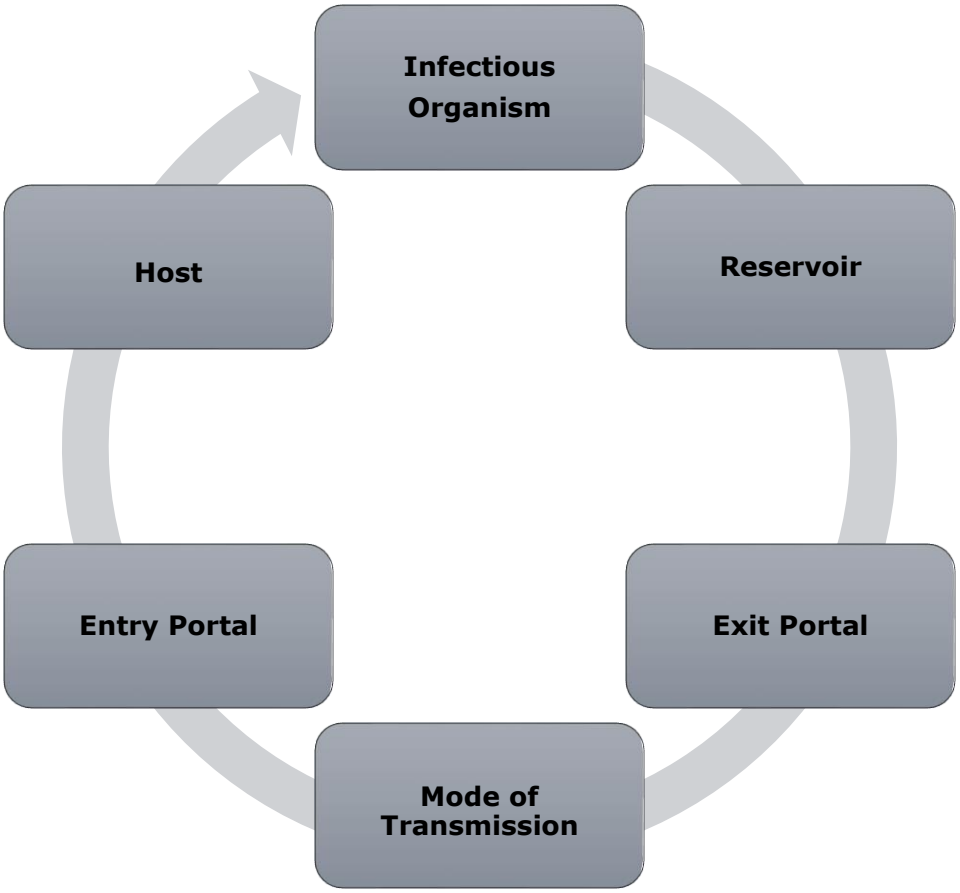


Figure 4 - 1: A systematic diagram of the key stages of the chain of infection.

The chain of infection begins when a microorganism with the ability to cause disease exits its host's reservoir, where it survives and grows, through an exit portal. It is then transported to and enters the new host using a mode of transmission (Centers for Disease Control and Prevention 2012). There are a number of transmission modes which exist including environmental factors such as wind and water (Hsu 2014), as well as food and insects (Taylor 2001) and direct and indirect contact methods between people which can include microbial transfer between their hands and objects (Koenig *et al.* 2015). Some examples of

transmission modes, including examples of human to human vectors can be seen in Figure 4 - 2.

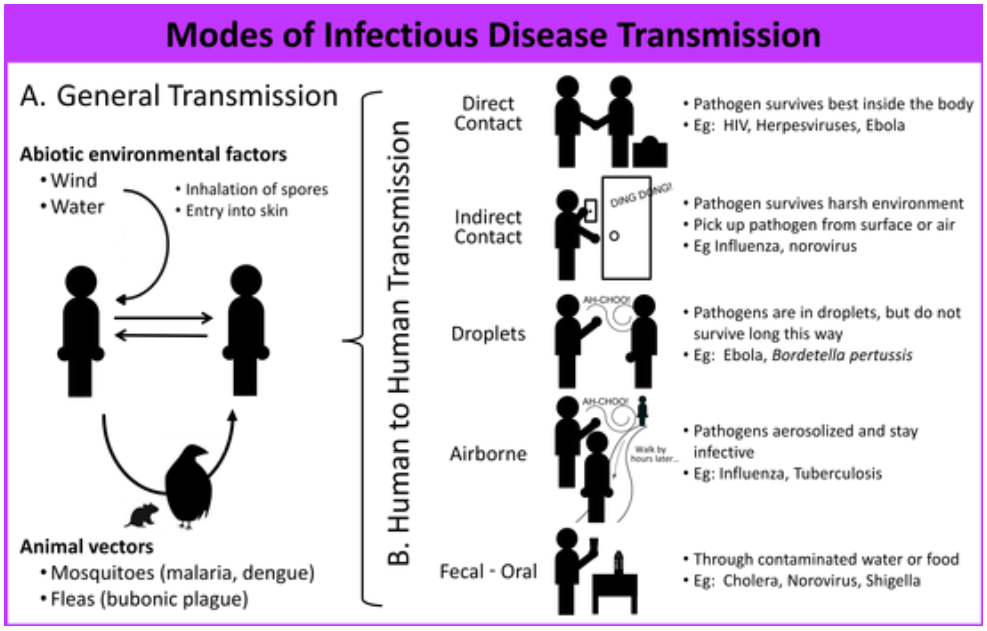


Figure 4 - 2: Examples of human to human modes of microbial transmission (Taken from: Hsu 2014, Science in the News, Harvard University. Open source, available from: http://sitn.hms.harvard.edu/flash/specialeditiononinfectiousdisease/2014/anintroduction-to-infectious-disease/).

Since the middle of the 19th Century human hands have been recognised as one of the main transmission modes for disease (World Health Organisation 2009). Hand resident bacteria exist on the superficial cells of the stratum corneum, as well as on the skin surface. In general these resident bacteria do not cause infection unless they permeate a sterile body cavity, or gain entry through damaged skin. However, in contrast, transient microorganisms, acquired on the hands during direct and indirect contact, and further spread through hand borne transmission, have been shown to play a major role in the spread of disease (World Health Organisation 2009). In fact, hands contaminated with transient bacteria have been shown to transfer these organisms onto several further surfaces (Baker *et al.* 2004). Ease of bacterial transmission via the hand borne route is dependent upon a number of factors including organism specific features such as density and species, as well as particular skin characteristics including moisture content (World Health Organisation 2009). To date there have been a number of previously published studies which have investigated the use of sterile and / or non - sterile

gloves as a barrier method to prevent the spread of microorganisms via the hand borne route (Bodiwala and George 1982; Perelman *et al.* 2004; Hemsworth *et al.* 2007). However, these past studies relate to infection control between healthcare workers and patients within the hospital setting and do not consider the transfer of hand borne bacteria with respect to clean room garments. To the author's knowledge this is the first such study.

4.1.2 Gloves

Randers - Pehrson (1960) (cited in Eisen 2011) reported that the first glove type, developed in the mid - 18th Century, only partially concealed the hand and was made of sheep intestines. Over the years various glove types made of cotton, leather or silk followed (Eisen 2011) before rubber gloves were introduced into the hospital setting in the late 19th Century (Osman and Jensen 1999). However, it wasn't until the late 20th Century that glove use became common place in the healthcare setting (Rego and Roley 1999). Nowadays, the majority of sterile and non – sterile gloves are manufactured in South East Asia using latex from natural rubber trees due to its properties which make gloves constructed of this material ideal for working with fine dexterity (Galatowitsch 1996). These are manufactured using a dipping process, as shown in Figure 4 – 3. During this process a hand shaped former is gently lowered into a tank containing a stirred suspension of rubber, before being slowly raised. Prior to packaging, water – soluble material is extracted before the gloves are dried and vulcanized in hot air ovens (Dolez *et al.* 2012).

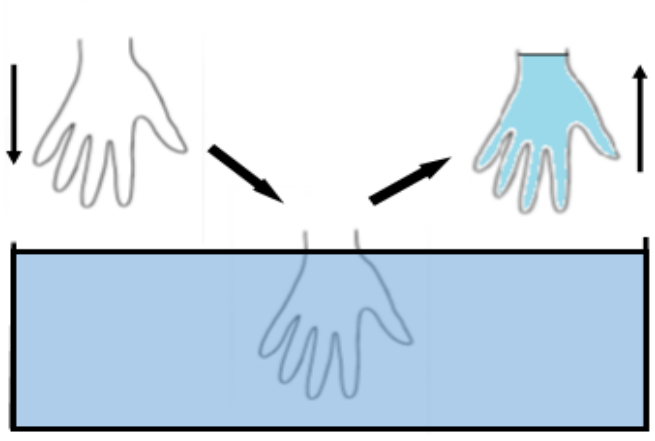


Figure 4 - 3: A diagram depicting the glove dipping process.

However, many industries have become cautious of latex glove use due to concerns over commonly occurring allergic reactions caused by the proteins in the natural rubber (Casser 2011b). Nevertheless, there are a number of common synthetic rubber alternatives available such as polychloroprene / neoprene, nitrile, vinyl and polyisoprene (Watts 2014). The preferred substitute for natural rubber latex is nitrile due to its resistance, static diffusion and flexibility (Galatowitsch 1996). In fact, a study by Rego and Roley (1999), comparing the barrier efficiency of both latex and nitrile gloves, found both materials to be comparable in performance.

4.1.2.1 Non – Sterile Gloves

Non - sterile gloves present in an ambidextrous, flat form (Watts 2010) and are packaged in boxes which contain multiple single gloves, as shown in Figure 4 – 4 (a). These gloves are deemed adequate for general purpose use and are commonly used in the hospital environment to prevent cross contamination between practitioners and patients (Wilson *et al.* 2015). However, limitations of non – sterile glove use exist. For example, a study by Hughes *et al.* (2013) found that open boxes of non – sterile gloves can become contaminated with bacteria. In addition, hand movement can be limited by their ambidextrous shape (Watts 2010).

Figure 4 – 4: (a) an example of a box of non – sterile gloves containing multiple single gloves, (b) a pair of sterile gloves packaged in a sterile paper wallet and (c) a pair of sterile clean room gloves packaged in a non – linting sterile wallet.

4.1.2.2 Sterile Gloves

Sterile gloves are manufactured in the same manner as their non – sterile equivalents, however, to kill any bacteria present on the glove, as well as remove any powder, these are subsequently dipped in a chlorine bath (Watt 2010), before being packaged in pairs within paper wallets (Watts 2014) (Figure 4 – 4 (b)).

Sterile gloves are manufactured in accordance with the existing British and European standards, which are a series of documents which define glove characteristics such as size, strength and the maximum number of pinholes permitted (Watts 2010). Sterile gloves are considered the most effective method at preventing the transmission of bacteria via the hand borne route in the hospital setting (Harnoβ *et al.* 2010). However, sterile medical gloves are wrongly perceived as being clean (Watts 2014). Although sterile, these gloves are covered in potentially contaminating particles including human detriment, fibres from garments, as well as non - viable bacteria (Watts 2014). Therefore, the use of sterile medical gloves in the clean room environment could prove to be at the detriment of the product (Watts 2010). In addition, the paper wallets in which medical sterile gloves are packaged may contaminate the gloves and environment during opening (Watts 2014). If such non – viable particles are introduced into the clean room environment these could potentially impact upon the integrity of the product being manufactured, which may result in on of a number of diverse health conditions or even patient death (Tran *et al.* 2006).

4.1.2.3 Sterile Clean Room Gloves

In the clean room environment gloves are used to provide a barrier between the operator and the product, which is crucial in controlling contamination (Gardener 2011). Following the manufacture process, clean room gloves are subsequently rinsed with deionised water to remove any extractable particles (Galatowitsch 1996), these are then packaged in non – particulate shedding polyethylene pouches (Watts 2014) (Figure 4 – 4 (c)). Choosing the right clean room glove depends on a number of factors, not only its cleanliness, but also its fit, feel and grip (Watts 2014). There are a number of tests used to evaluate clean room gloves - as well as tests to identifying any particulate, microbial and extractable material on the glove there are also tests to determine material permeability, diffusion, resistance and static dissipation (Plamthottam 1996). Twenty years ago it was common for operators working within clean rooms to wear sterile medical gloves. However, over time, and in an attempt to reduce the levels of particles being introduced into their clean room environment, the technological advanced sectors adopted the use of sterile clean room gloves. However, the pharmaceutical industry have been slow to pursue this change (Gardner 2011).

4.2 Aim & Objectives

The aim of this aspect of the project was to compare the surface bacterial bioburden of reusable antistatic carbon filament polyester room garments donned by operators dressing wearing either no gloves, non - sterile gloves or sterile clean room gloves. In order to achieve this a number of specific objectives were developed:

- To use the direct agar contact method, developed in Chapter 2, to recover, enumerate and compare the levels of bacteria on the surface of clean room garments donned by operators dressing wearing either no gloves, non – sterile gloves or sterile clean room gloves.
- With respect to glove type worn during the donning process, no gloves, non – sterile gloves or sterile clean room gloves, compare the surface bacterial bioburden of clean room garments at 7 different sites.
- With respect to agar incubation time, 24 and 48 hours, use the direct agar contact method to assess the percentage of bacteria recovered from clean room garments donned by operators dressing wearing either no gloves, non – sterile gloves or sterile clean room gloves.

4.3 Materials & Methodology

In order to achieve the specified aim and objectives the following materials and methods were used.

4.3.1 Clean Room Facility

This investigation was carried out within the grey changing area of the clean room facility based within the Technical Building at the Robert Gordon University Schoolhill Campus, Aberdeen, the layout of which can be seen in Figure 4 - 5. The facility was originally installed by Bassaire Ltd (Southampton, UK) in 1988 and comprised of a Grade C clean room and a Grade A clean room with a Grade B background. Clean air conditions within the facility were supported by Bassaire fan filter modules and the ambient conditions controlled by an Airdale VA6P air handling unit and two condensing units. The air - conditioning unit was installed by Chillforce Ltd (Dundee, UK) and was serviced on a biannual basis. The facility was also serviced biannually by Bassaire Ltd (Southampton, UK). Access to the clean room was via the changing area, which was accessed from the main building.

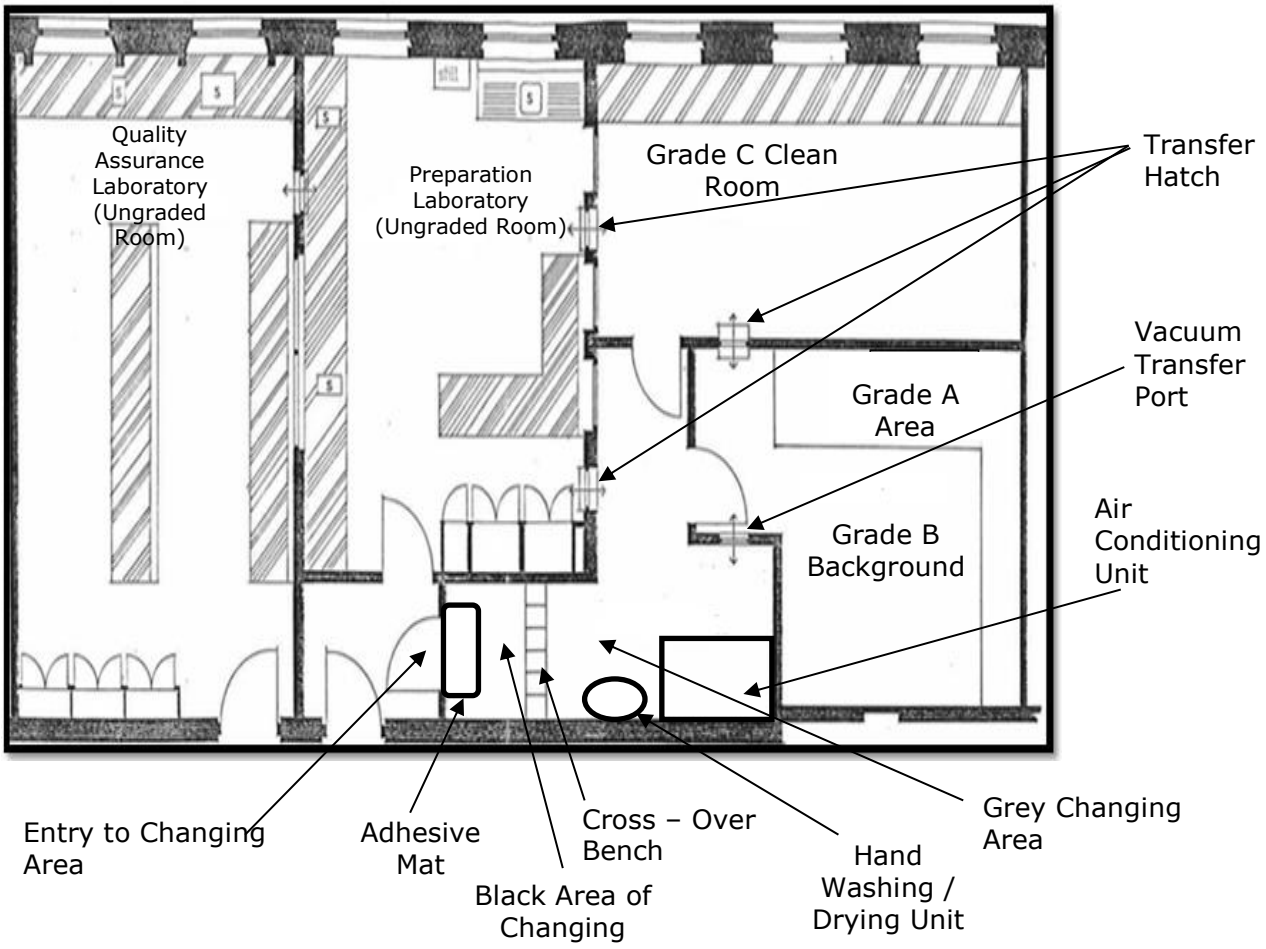


Figure 4 – 5: The layout of the clean room facility at the Robert Gordon University Schoolhill Campus, Aberdeen. This comprised of a Grade C clean room and Grade A clean room with a Grade B background. These rooms were accessed via the changing area, which was accessed from the main building.

4.3.2 Clean Room Maintenance

Once weekly the floor and wall surfaces within the facility were thoroughly vacuumed (using the built in vacuum cleaner system). These were then cleaned with general detergents A and B (Critical Environmental Solutions Ltd, Wiltshire, UK) which in conjunction with one another provided a rotational disinfection program for the facility. General detergent A contained 15 % isopropanol and general detergent B contained 30 % isopropanol, 5 % anionic detergent and 5 % phenolic. The detergents were diluted with sterile double distilled water which had been filtered through a 0.2 µm pressure filter (Fisher Ltd, Loughborough UK). Each room was cleaned using a sterile mop and head (Critical Environment Solutions Ltd, Wiltshire, UK) in an outwardly direction starting at the far end of the room

cleaning in the direction of the door. Equipment and working surfaces were wiped daily using Hydrex HS hard surface disinfectant (chlorhexidine gluconate 0.02% w/v in denatured ethanol 70% v/v) (Nu-Care Products Ltd, Bedford, UK) and Azowipes bactericidal wipes (70 % isopropyl alcohol) (Nu-Care Products Ltd, Bedford, UK) following each working period.

4.3.3 Ethical Approval

A research ethics self - assessment (RESA) form was submitted in respect to the University's Research Ethics Policy. This application was reviewed by the ethical review panel of the School of Pharmacy and Life Sciences at the Robert Gordon University and approved on the 5th November 2012 - no changes were deemed necessary.

4.3.4 Clean Room Garments & Laundering

Reusable antistatic carbon filament polyester clean room garments were supplied by Chemsplash (Manchester, UK (formerly CCA Products)). These garments were laundered by Fishers Laundry Group (Aberfeldy, UK). In each case thermal washing was undertaken by rinsing the garments in a barrier washing machine within an ISO Class 4 clean room (Table 1 – 4) using ultra - pure water. Garments were then dried using HEPA filtered air prior to being packed in vapour permeable bags. Hair nets and over shoes were supplied by Critical Environment Solutions Ltd (Wiltshire, UK). KIMTech G5 sterile latex clean room gloves were supplied by Basan UK (Basingstoke, UK) and non - sterile boxed nitrile gloves were supplied by Fisher Scientific (Loughborough, UK).

4.3.5 Entering the Clean Room

Prior to entering the clean room changing area operators removed all jewellery and makeup. Operators then entered the changing area of the facility, stepping firstly onto an adhesive mat (Critical Environment Solutions Ltd, Wiltshire, UK), then into the black area of the changing area (Figure 4 – 5). The operator then removed their outdoor shoes and donned a hair net, ensuring full coverage of hair and ears. The operator sitting on the cross – over bench facing the external door placed an over shoe over their right foot and without allowing the foot to touch the ground swung their foot across the bench onto the grey side of the changing area.

This was repeated with the left foot. The operator then washed their hands using HiBiScrub® cleanser (Fisher Scientific Ltd, Loughborough, UK) before drying them using a hot air hand dryer. The operator then donned either no gloves, disposable non - sterile gloves, or disposable KIMTECH pure G5 sterile latex clean room gloves. Next the operator donned a sterile face mask; the packaging of the mask was torn open and the mask removed, touching only the inside of the mask. The mask was placed over the nose and mouth and tied behind the head so that it was firmly in place. Operators then donned a sterile hood (Section 4.3.6) and a sterile clean room suit (Section 4.3.7).

4.3.6 Donning a Clean Room Hood

The operator tore open the bag containing the hood and touching only the inside of the fabric it was removed from the packaging. Holding the bottom of the hood it was placed over the operators head and pulled into place so that the spaces were over the eyes. The tapes were then tied behind the head so that the hood was firmly in place. The base of the hood was enclosed within the suit at the neck, once this was donned.

4.3.7 Donning a Clean Room Suit

The operator tore open the packaging containing the suit and touching only the inside of the fabric at the nape of the neck removed it from the bag. Whilst making sure it did not touch the floor, the operator undid the zip of the suit and rolled the suit down to the waist touching only the inside of the fabric. The operator carefully placed one leg into the suit, ensuring no part touched any surfaces, and then stepped into the other leg whilst holding the suit at waist level. After unrolling, one arm was then placed into the suit followed by the other, at all times touching only the inside of the fabric. The zip was pulled up using the pull and studs fastened at the neck, wrists and ankles.

4.3.8 Preparation of Contact Plates

Fifty five mm nutrient agar contact plates were prepared and stored as per the method discussed in Chapter 2 - Section 2.3.4. Contact plates were removed from the fridge and left at room temperature for two hours prior to use to eliminate condensation on the surface of the agar.

4.3.9 Direct Agar Contact Method of Bacterial Recovery

To determine the bacterial bioburden of the surface of clean room garments, immediately following dressing the direct contact method was undertaken on six areas of the clean room suit, as well as the hood, by the operator wearing the attire (Figure 4 – 6). This was undertaken on the exterior surface of clean room garments donned by 3 operators wearing no gloves, 3 operators wearing non – sterile gloves and 3 operators wearing sterile clean room gloves, all in triplicate.

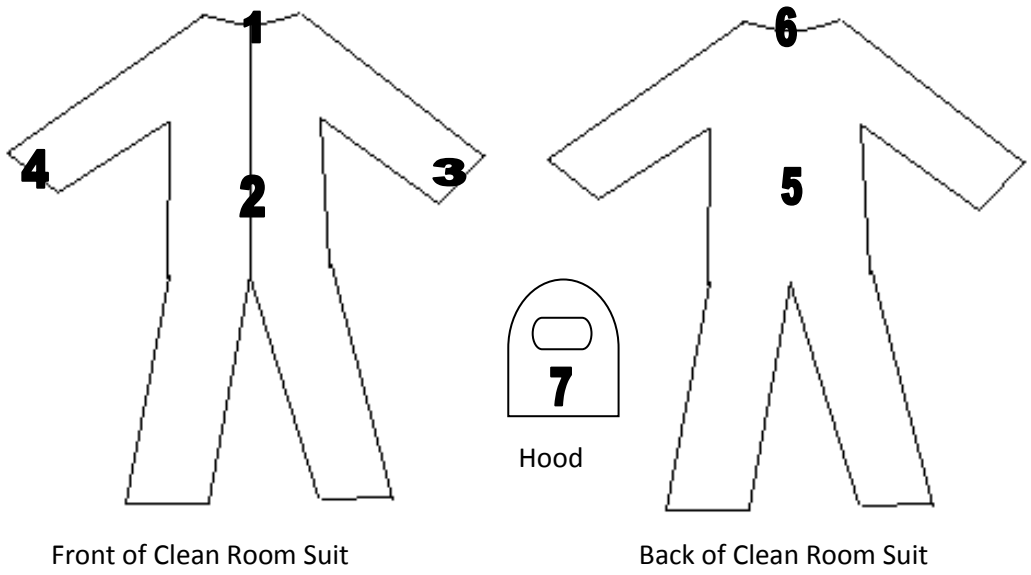


Figure 4 – 6: The front and back of a clean room suit. Numbers on the suit represent areas to be tested using the direct contact method; 1 – chest, 2 – umbilicus (umbilical), 3 - left carpus (wrist), 4 - right carpus (wrist), 5 – lumbus (lower back), 6 – posterior cervicis (nape of neck) and 7 – oral cavity (mouth).

During testing the lid of the contact plate was removed and the surface of the agar was applied to the test surface for five seconds at constant pressure. The lid of the contact plate was replaced and the plates were inverted and incubated at 37 ^{o}C. To examine the effect of agar incubation time on the total percentage of plates displaying specific levels of growth the numbers of colonies per plate were recorded following both 24 and 48 hour incubation periods. Following testing, the areas of the garments tested were wiped with a 70 % ethanol impregnated wipe (Critical Environment Solutions Ltd, Wiltshire, UK) and the clothing sent to be laundered as described in Section 4.3.4.

4.3.10 Statistical Analysis of Results

The results of this part of the study were analysed using GraphPad Prism 4.0 (GraphPad Software Inc., La Jolla, CA) and statistically analysed using Two – Way Analysis of Variance (ANOVA) at a 95 % confidence level.

4.4 Results

The bacterial bioburden of the surface of antistatic carbon filament polyester clean room garments donned by operators dressing wearing either no gloves, non – sterile gloves or sterile clean room gloves was assessed using the direct agar contact method developed in Chapter 2. A total of 189 contact plates were used to test the exterior surface of garments donned by three clean room operators wearing no gloves, non – sterile gloves and sterile clean room gloves, all in triplicate (63 plates represent 100 %). Furthermore, a comparison of the efficiency of two agar incubation periods at 37 ^{o}C, 24 and 48 hours, was assessed.

A comparison of agar incubation time against the total percentage of contact plates displaying growth, used to test the surface of clean room garments donned by operators wearing no gloves, non – sterile gloves or sterile clean room gloves is shown in Figure 4 – 7. To further assess the issue of agar incubation time with respect to bacterial recovery from clean room garments an additional comparison was drawn between agar incubation time and the total percentage of these contact plates displaying either 0 (no growth), 1 - 9 (low level growth), 10 - 20 (moderate level growth) or > 20 (high level growth) cfu / plate under the variable no gloves, non – sterile glove or sterile clean room gloves (Figure 4 – 8).

Figure 4 – 7: A comparison of agar incubation time, 24 and 48 hours, against the total percentage of contact plates displaying growth, used to test the surface of clean room operators' garments donned by operators dressing wearing either no gloves, non – sterile gloves or sterile clean room gloves.

As displayed in Figure 4 – 7, although not found to be statistically significant, an increase in the percentage of contact plates, used to test the surface of garments donned by operators wearing either no gloves, non – sterile gloves or sterile clean room, displaying growth was observed following a further 24 hour incubation period (taking the total incubation period to 48 hours). As shown, 48 % [30 / 63] of contact plates used to test the surface of clean room garments donned by operators wearing no gloves, 43 % [27 / 63] of plates used to test the surface of garments donned by operators wearing non – sterile gloves and 33 % [21 / 63] of those used to test the surface of garments donned by operators wearing sterile clean room gloves displayed growth following a 24 hour incubation period. In comparison, following a further 24 hour incubation period (taking the total incubation period to 48 hours) the percentage of plates displaying growth increased to 64 % [40 / 63], 57 % [36 / 63] and 59 % [37 /63] respectively. Furthermore, Figure 4 – 7 shows there to be no significant difference between the percentages of plates displaying growth and the variable no gloves, non – sterile gloves and sterile clean room gloves following either a 24 or 48 hour incubation period.

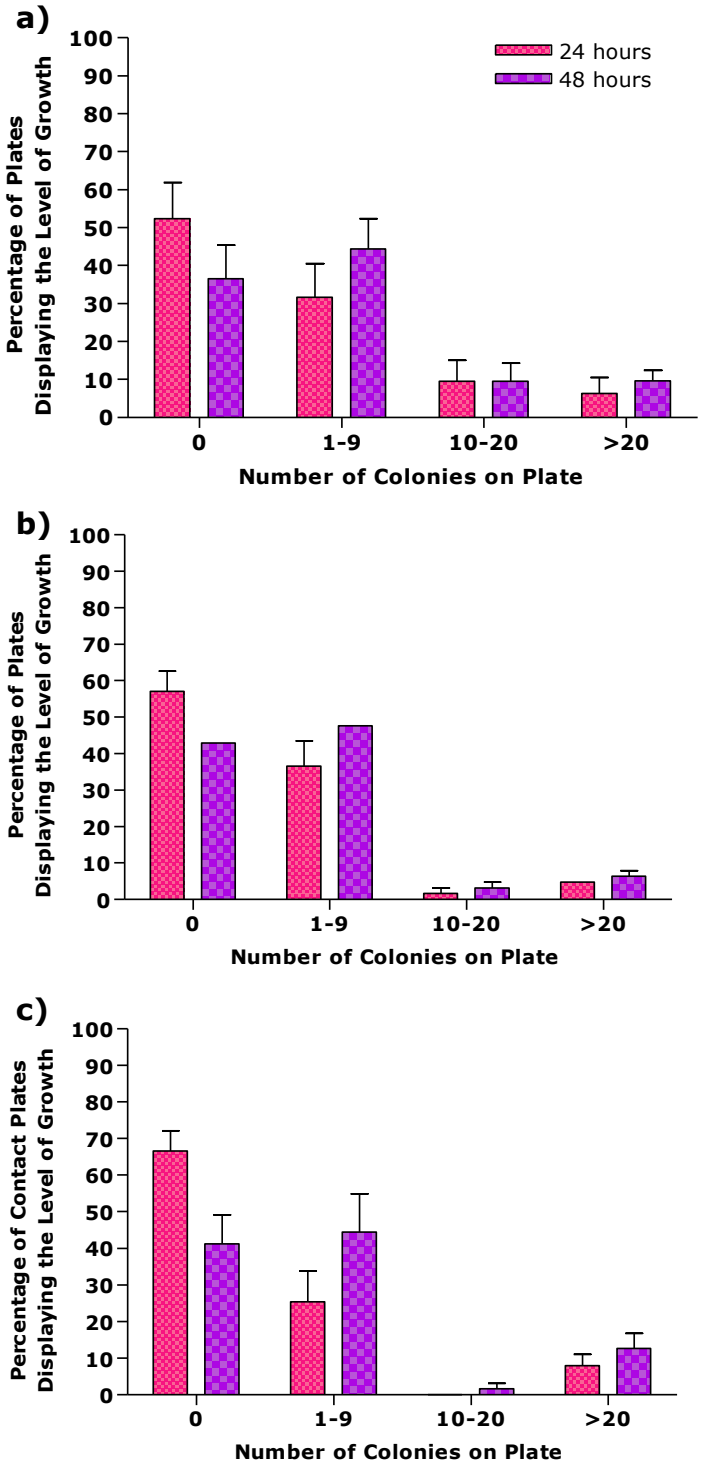


Figure 4 – 8: A comparison of agar incubation time, 24 and 48 hours, against the total percentage of contact plates displaying either 0 (no growth), 1 - 9 (low level growth), 10 - 20 (moderate level growth) or > 20 (high level growth) cfu / plate, used to test the surface of clean room garments donned by operators dressing wearing either (a) no gloves, (b) non – sterile gloves or (c) sterile clean room gloves.

Overall, although not shown to be statistically significant, an increase in the percentage of contact plates displaying low, moderate and high levels of growth following a further 24 hour incubation period (taking the total incubation period to 48 hours) was observed with plates used to test the surface of garments donned by operators wearing either no gloves, non – sterile gloves or sterile clean room gloves. As shown in Figure 4 – 8, of the 48 % [30 / 63] of contact plates, used to test the surface of garments donned with no gloves, which displayed growth following the 24 hours agar incubation period (Figure 4 – 7), 32 % [20 / 63] displayed low level growth, 10 % [6 / 63] moderate level growth and 6 % [4 / 63] high level growth. Of the 64 % [40 / 63] of plates which displayed growth following an additional 24 hour agar incubation period the percentage of plates displaying the levels of growth were 44 % [28 / 63], 10 % [6 / 63] and 10 % [6 / 63] respectively (Figure 4 – 8 (a)). Of the 43 % [27 / 63] of contact plates, used to test garments donned with non - sterile gloves, which displayed growth over a 24 hour agar incubation period 37 % [23 / 63] of these showed low level growth, 2 % [1 / 63] moderate level growth and 5 % [3 / 63] high level growth. Following a 48 hour agar incubation period the percentage of plates displaying each of these level of growth (57 % [36 / 63]) increased to 48 % [30 / 63], 3 % [2 / 63] and 6 % [4 / 63] in turn (Figure 4 – 8 (b)). The percentage of plates displaying either low, moderate and high levels of growth, used to test the surface of garments donned by operators wearing sterile clean room gloves, following 24 hours agar incubation period (33 % [21 / 63]), was found to be 25 % [16 / 63] low level growth and 8 % [5 / 63] high level growth. No moderate levels of growth were displayed on these plates. Following a 48 hour agar incubation period, of the 59 % [37 / 63] of plates which displayed growth, 44 % [28 / 63] of these showed low level growth, 2 % [1 / 63] moderate level growth and 13 % [8 / 63] high level growth. This along with the increase in the total percentage of plates displaying growth at 48 hours compared to a 24 hour agar incubation period, observed in Figure 4 – 7, suggests that nutrient agar contact plates should be incubated for 48 hours at 37 ^{o}C to allow viable cells sufficient time to grow. Therefore, for the remainder of this current study only the percentage of plate displaying the levels of growth following a 48 hour incubation period are considered.

As previously shown in Figure 4 – 7, statistically, there was shown to be no significant difference between the percentage of contact plates displaying growth

and the variable no gloves, non – sterile gloves or sterile clean room gloves following a 24 hours (48 % [30 / 63] vs. 43 % [27 / 63] vs. 33 % [21 / 63] respectively) or 48 hour agar incubation period (64 % [40 / 63] vs. 57 % [36 / 63] vs. 59 % [37 / 63] respectively). Therefore, to further investigate this issue a comparison was drawn between glove type (no gloves, non – sterile gloves or sterile clean room gloves) worn during the donning process and the total percentage of these contact plates displaying either 0 (no growth), 1 - 9 (low level growth), 10 - 20 (moderate level growth) or > 20 (high level growth) cfu / plate following the 48 hour incubation period.

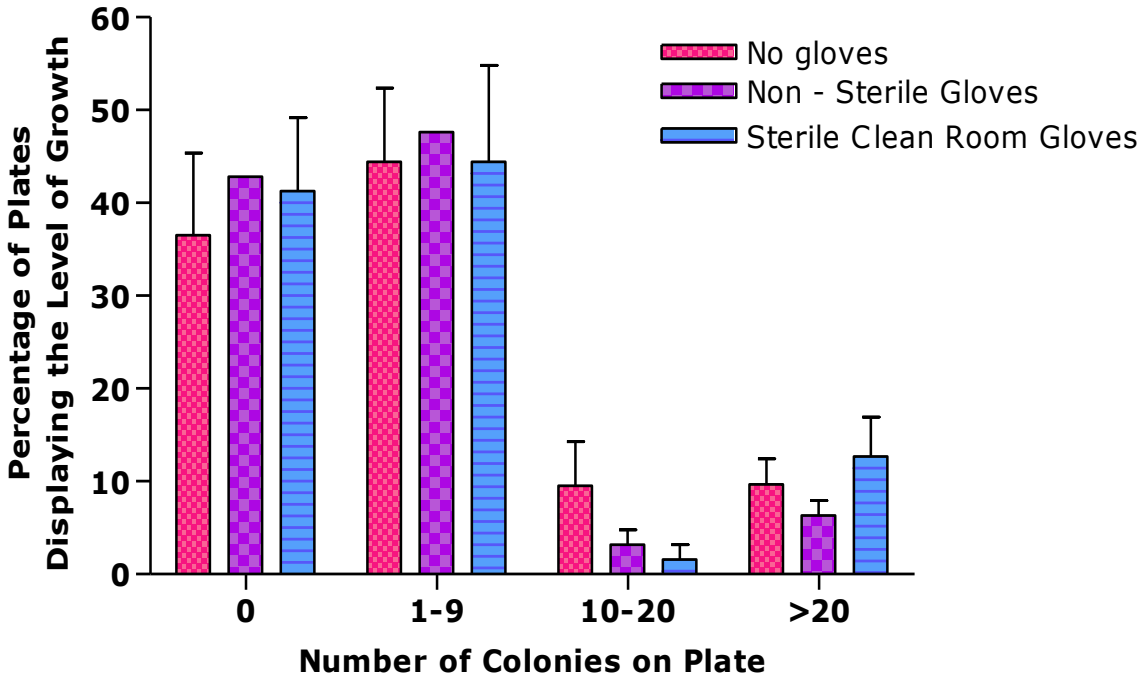


Figure 4 – 9: A comparison of glove type (no gloves, non – sterile gloves or sterile clean room gloves) worn during the donning process against the percentage of contact plates displaying either 0 (no growth), 1 - 9 (low level growth), 10 - 20 (moderate level growth) or > 20 (high level growth) cfu / plate following a 48 hour incubation period at 37 ^{o}C.

As shown in Figure 4 – 9, of the 64 % [40 / 63] of contact plates which displayed growth following a 48 hour agar incubation period, used to test the surface of clean room garments donned by operators wearing no gloves (Figure 4 – 7), 44 % [28 / 63] of these displayed low level growth, 10 % [6 / 63] moderate levels of growth and 10 % [6 / 63] high levels of growth. Of the 57 % [36 / 63] of contact plates displaying growth, used to test the surface of garments donned by

operators wearing non – sterile gloves, the percentage of plates displaying either low, moderate or high levels of growth were found to be 48 % [30 / 63], 3% [2 / 63] and 6 % [4 / 63] respectively and of the 59 % [37 / 63] plates used to test garments donned with sterile clean room gloves displaying growth 44 % [28 / 63], 2 % [1 / 63] and 13 % [8 / 63] respectively. Statistically, these results show that there is no significant difference between the percentage of contact plates displaying the levels of bacterial growth and the variable no gloves, non – sterile gloves or sterile clean room gloves. Further analysis was then undertaken to compare the bacterial bioburden of the 7 garments sites tested (Figure 4 – 6) with respect to glove use during the donning process. The total percentage of plates displaying growth at each of the sites of garments donned by operators wearing either no gloves, non – sterile gloves or sterile clean room gloves is shown in Figure 4 – 10 (9 plates were used to test each site per the variable - no gloves, non – sterile gloves or sterile clean room gloves - representing 100 %).

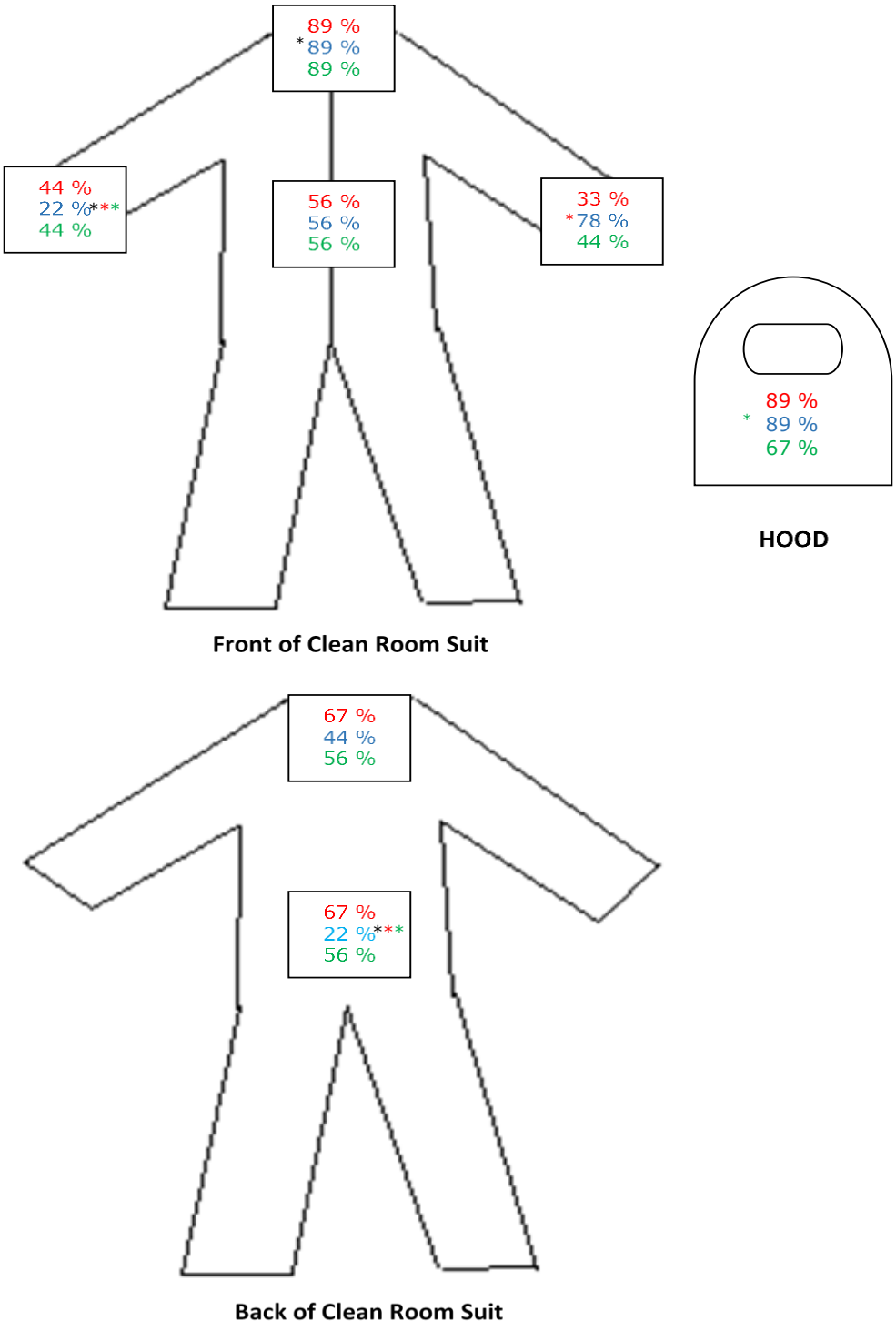


Figure 4 – 10: A comparison of glove type, no gloves (red), non – sterile gloves (blue) or sterile clean room gloves (green), against the total percentage of contact plates displaying growth for each of the garments sites tested following the donning process (*p < 0.05 represents the percentage of plates displaying growth used to test the chest, *p < 0.05 represents the left carpus (wrist) and * p < 0.05 represents the oral cavity (mouth) of suits donned with non – sterile gloves vs. the percentage of plates displaying growth used to test the lumbus (lower back) and right carpus of suits donned under the same condition.

As shown in Figure 4 – 10, statistically, there was found to be no significant difference between the percentages of plates displaying growth at each site tested against the variable no gloves, non – sterile gloves or sterile clean room gloves. As shown, in each case, 89 % [8 / 9] of contact plates used the test the chest region of garments donned with no gloves, non - sterile gloves or sterile clean room glove displayed growth. In addition, 56 % [5 / 9] of plates used to test the umbilicus of garments donned under all 3 conditions also displayed growth. Eighty nine % [8 / 9] of plates used to test the oral cavity of hoods donned by operators wearing no gloves and non – sterile gloves, as well as 67 % [6 / 9] of those used to test the same area of hood donned by operators wearing sterile clean room gloves displayed growth. Respectively, 33 % [3 / 9], 78 % [7 / 9] and 44 % [4 / 9] of plates used to test the left carpus, 44 % [4 / 9], 22 % [2 / 9] and 44 % [4 / 9] of those used to test the right carpus, 67 % [6 / 9], 44 % [4 / 9] and 56 % [5 / 9] of those used to test the posterior cervicis and 67 % [6 / 9], 22 % [2 / 9] and 56 % [5 / 9] of plates used to test the lumbus region of garments donned with no gloves, non – sterile gloves and sterile clean room gloves all displayed growth following incubation.

A further comparison was drawn between glove type worn and the total percentage of these plates displaying either 0 (no growth), 1 - 9 (low level growth), 10 - 20 (moderate level growth) or > 20 (high level growth) cfu / plate for each of the garment sites tested (9 plates represent 100 %) (Figure 4 – 11).

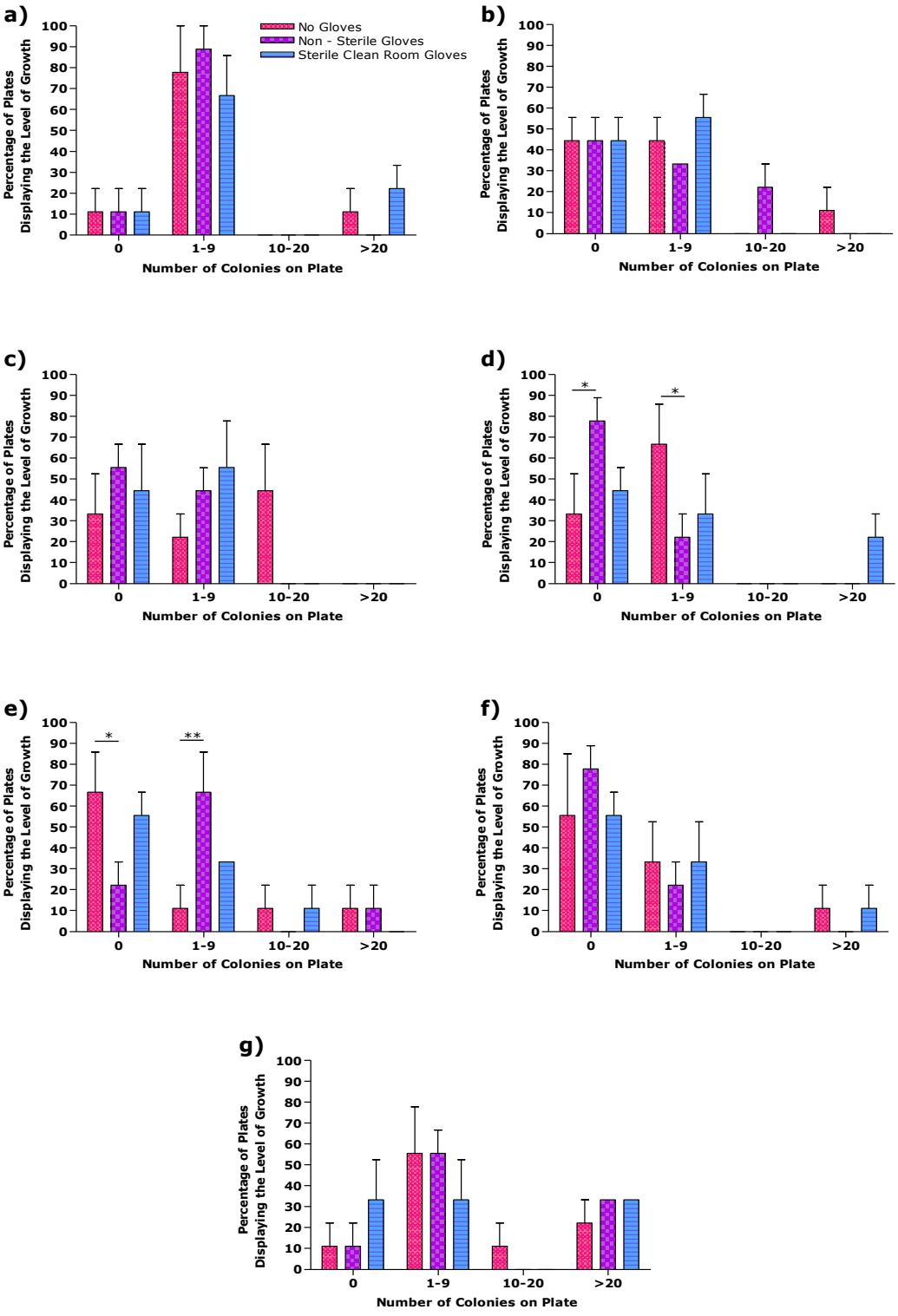


Figure 4 – 11: A comparison of glove type, no gloves, non – sterile gloves or sterile clean room gloves, against the percentage of contact plates displaying either 0 (no growth), 1 - 9 (low level growth), 10 - 20 (moderate level growth) or > 20 (high level growth) cfu / plate following a 48 hour incubation period at 37 ^{o}C, used to test the (a) chest, (b) umbilicus, (c) posterior cervicis, (d) lumbus, (e) left and (f) right carpus and (g) oral cavity regions of garments donned by operators wearing either no gloves, non – sterile gloves or sterile clean room gloves (* $p < 0.05$; ** $p < 0.01$).

As shown in Figure 4 – 11, statistically there was found to be no significant difference between gloves type and the levels of growth displayed on plates used to test the chest (Figure 4 – 11 (a)), umbilicus (Figure 4 – 11 (b)), posterior cervicis (Figure 4 – 11 (c)), right carpus (Figure 4 – 11 (f)) and oral cavity (Figure 4 – 11 (g)) of clean room garments donned with no gloves, non – sterile gloves and sterile clean room gloves. However, as shown in Figure 4 – 11 (d), a significantly higher percentage of contact plates used to test the lumbus region of garments donned with non – sterile gloves displayed no growth following incubation compared to the percentage of plates displaying no growth used to test the same region of garments donned without gloves (77.7 % [7 / 9] vs. (33.3 % [3 / 9]) (* $p < 0.05$). This was due to a significantly higher percentage plates used to test the lumbus region of garments donned without gloves displayed low levels of growth compared to the percentage displaying low level growth, used to test garments donned with non – sterile gloves (66.7 % [6 / 9] vs. 22.2 % [2 / 9]) (* $p < 0.05$). However, as shown in Figure 4 – 11 (e), a significantly higher percentage of plates used to test the left wrist of garments donned with no gloves displayed no growth following incubation compared to the percentage displaying no growth, used to test the same region of garments donned using non – sterile gloves (66.7 % [6 / 9] vs. 22.2 % [2 / 9]) (* $p < 0.05$). This was due to a higher percentage of plates used to test the lumbus of garments donned with non – sterile gloves displaying low levels of growth following incubation compared to the percentage of plates displaying the same level of growth in garments donned using no gloves (66.7 % [6 / 9] vs. 11.1 % [1 / 9]) (** $p < 0.01$).

A further comparison was drawn between garment site tested and the total percentage of these contact plates displaying either 0 (no growth), 1 - 9 (low level growth), 10 - 20 (moderate level growth) or > 20 (high level growth) cfu / plate following the 48 hour incubation period for each of the glove types tested (Figure 4 – 12). Despite there being found to be no significant difference between the total percentage of contact plates displaying growth at each of the sites tested against the variable – no gloves, non – sterile gloves or sterile clean room gloves (Figure 4 – 10) there was shown to be a statistically significant difference between the percentage of plates displaying growth and the garment site in suits donned with non – sterile gloves. As shown in Figure 4 – 10, significantly more contact plates used to test the chest and oral cavity (both 89 % [8 / 9]) and left carpus (78 %

[7 / 9]) of garments donned with non - sterile gloves displayed growth compared to those used to test the other 5 sites. This increase was found to be statistically significant between the percentage of plates used to test the right carpus and lower back, which in each case 22 % [2 / 9] of contact plates used to test these sites displayed growth following incubation (* $p < 0.05$).

As shown in Figure 4 – 12, a lower percentage of plates used to test the chest and oral cavity region of garments donned with (a) no gloves, (b) non – sterile gloves or (c) sterile clean room gloves displayed no growth following incubation when compared to the percentage of plates displaying growth used to test the other sites investigated under the same conditions. This was shown to be significant between the chest and oral cavity (both 11.1 % [1 / 9] and the left carpus (66.6 % [6 / 9]) of suits donned with no gloves (Figure 4 – 12 (a)). This was also found to be significant between the chest and oral cavity (both 11.1 % [1 / 9]) and umbilicus (44.4 % [4 / 9]) (* $p < 0.05$), posterior cervicis (55.5 % [5 / 9]) (** $p < 0.01$), right carpus and lumbus (both 77.7 % [7 / 9]) (both *** $p < 0.001$) of garments donned with non – sterile gloves. In addition, there was also found to be a significantly lower percentage of plates used to test the umbilicus (44.4 % [4 / 9]) regions of suits donned with non – sterile gloves displaying no growth compared to those used to test the right carpus and lumbus (both 77.7 % [7 / 9]) (* $p < 0.05$). As well as between those used to test the left carpus (22. 2 % [2 / 9]) and those used to test the right carpus, lumbus (both 77.7 % [7 / 9]) (both *** $p < 0.001$) and posterior cervicis (55.5 % [5 / 9]) (* $p < 0.05$) region of garments donned under the same condition (Figure 4 – 14 (b)).

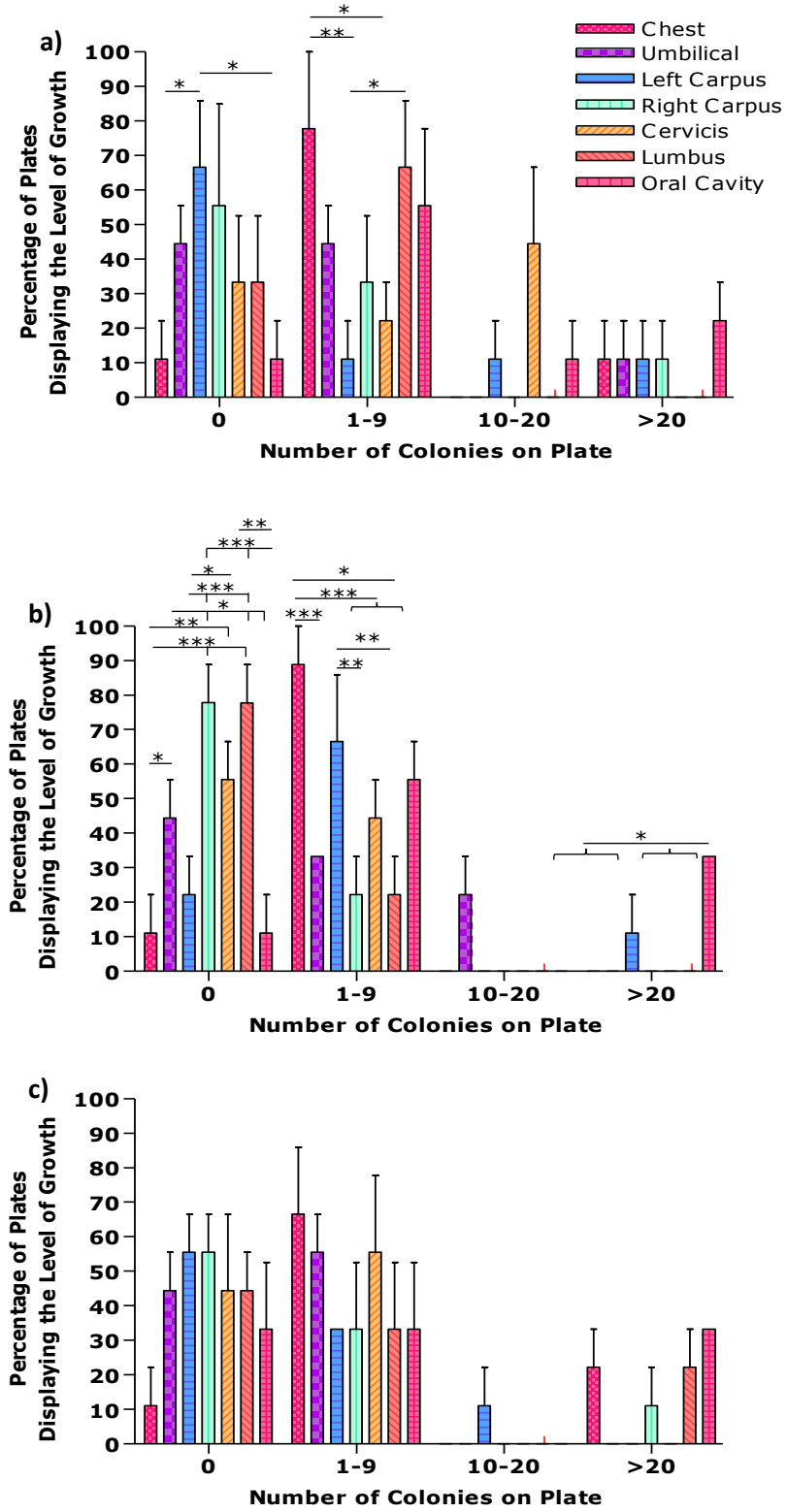


Figure 4 – 12: A comparison of garment site against the percentage of contact plates displaying either 0 (no growth), 1 - 9 (low level growth), 10 - 20 (moderate level growth) or > 20 (high level growth) cfu / plate following 48 hours incubation at 37 ^{o}C, used to test the surface of garments donned by operators wearing either (a) no gloves, (b) non – sterile gloves or (c) sterile clean room gloves (*** $p < 0.001$; $p < 0.01$; * $p < 0.05$).

As shown in Figure 4 – 12 (a), a higher percentage of plates used to test the chest and lumbus region of garments donned with no gloves displaying low levels of growth compared to the percentage of plates displaying the same level of growth used to test the 5 other sites. Seventy eight % [7 / 9] of contact plates used to test the chest region of suits donned without gloves displayed low levels of growth, this was found to be significantly more than the percentage displaying low levels of growth used to test the left carpus (11.1 % [1 / 9]) (** $p < 0.01$) and posterior cervicis (22.2 % [2 / 9]) (* $p < 0.05$) of suits donned under the same conditions. In addition, 66.7 % [6] of contact plates used to test the lumbus region of suits donned without gloves also displayed low levels of growth this was significantly more than the percentage displaying low level growth used to test the left carpus (* $p < 0.05$). There was found to be no significant difference between the percentage of contact plates displaying moderate and high levels of growth and suit site in garments donned under the same conditions.

As shown in Figure 4 – 12 (b), there was also found to be a significant difference between the percentage of plates displaying low level and high levels of growth and site tested in garments donned with non – sterile gloves. A higher percentage of plates used to test the chest and left carpus regions of suits donned with non – sterile gloves displayed low levels of growth compared to the percentage displaying the same level of growth used to test the 5 other sites. Eighty nine % [8 / 9] of contact plates used to test the chest of garments donned with non – sterile gloves displayed low levels of growth, this was found to be statistically higher than the percentage of plates displaying the same level of growth used to test the posterior cervicis (44 % [4 / 9]), umbilicus (33 % [3 / 9]), right carpus, lumbus (22 % [2 / 9]) (all *** $p < 0.001$) and oral cavity (56 % [5 / 9]) (* $p < 0.05$) of garments donned under the same conditions. Additionally, the 67 % [6 / 9] of plates displaying low levels of growth used to test the left carpus was found to be significantly higher than the percentage displaying low levels of growth used to test the right carpus and lumbus of the same suits (both 22 % [2 / 9] (** $p < 0.01$). Furthermore, a higher percentage of plates used to test the oral cavity (33 % [3 / 9]) of hoods donned by operators wearing non – sterile gloves displaying high levels of growth, this was found to be significantly more than those used to test the chest, umbilicus, right carpus, cervicis and lumbus of suits donned under the same conditions (all 0 % [0 / 9] (* $p < 0.001$)). There was found to be no

significant difference between the percentage of contact plates displaying moderate levels of growth and garment site (Figure 4 – 12b). As shown in Figure 4 – 12 (c), there was found to be no significant difference between the percentage of contact plates displaying the levels of growth and suit site in garments donned with sterile clean room gloves.

4.4 Discussion

Despite human hands being recognised as one of the primary transmission modes for disease (World Healthcare Organisation 2009), communication with clean room specialists has established a lack of a standardised protocol for the use of gloves as part of the garment donning procedure (C. Alexander 2012, Personal Communication; C. Dawson 2012, Personal Communication). Furthermore, to the author's knowledge there are no published research studies which assess the bacterial bioburden of clean room garments with respect to glove use during the donning process, this being the first such study. During this part of our study bacteria were recovered from the surface of clean room garments immediately following their donning by operators dressing wearing with either no gloves, non – sterile gloves or sterile clean room gloves, using the direct agar contact method examined in Chapter 2 of this project. The recovery of bacteria from clean room garments following their donning, as observed, is of concern as the surface of clean room garments should remain sterile following this process (Rhodes 2006) to ensure the garments themselves do not contribute towards the bacterial load of the clean room environment. Furthermore, with the recovery efficiency of the direct agar contact method from antistatic polyester carbon filament fabric being found to be less than 3 % (Chapter 2), this suggests that the levels of bacteria present on the surface of clean room garments, as observed during this study, may well be in excess of what is reported here.

During this stage of our study there was shown to be no significant difference between the percentage of contact plates displaying bacterial growth at either 24 and 48 hour incubation periods (Figure 4 – 7) and the variable dressing wearing either no gloves, non – sterile gloves or sterile clean room gloves. As well as no significant difference between the levels of growth displayed on these contact plates (Figure 4 – 8 and Figure 4 - 9). Furthermore, there was shown to be no significant difference between the percentages of contact plates displaying growth

between the variable for each of the 7 garment sites tested (Figure 4 – 10). Overall, this suggests that sterile clean room gloves can be substituted for either their non - sterile equivalents or no gloves during clean room garment donning process without subsequently increasing the surface bacterial bioburden of the garments. The results of our study could be significant for the clean room industry, where they could not only have a potentially positive economic impact but also provide clean room operators with a quicker more comfortable donning process, which in return may result in greater productivity. Firstly, substituting sterile clean room gloves for their non – sterile equivalents or omitting to wear gloves during the clean room garment donning process would considerably reduce the overall cost output of a clean room facility. For example, the average price of a pair of non - sterile boxed gloves used within the clean room facility at RGU is approximately £0.18 (Fisher Scientific Ltd 2018a), in comparison sterile clean room gloves are considerably more expensive at approximately £1.47 per pair (Fisher Scientific Ltd 2018b). In addition to the financial advantage of omitting sterile clean room glove use, opting to dress without gloves or with non - sterile gloves instead of sterile clean room gloves during the donning process could also be time saving. The clean room garment donning process should be kept as simple as possible (Clayton and Eaton 2011) to ensure the clothing doesn't become contaminated throughout the process. However, the donning of sterile gloves can be time consuming due to the arrangement of the gloves within their sterile packaging, as well as having to don the glove without touching the exterior surface of the rubber to avoid contamination. Another issue to consider is the operators' comfort whilst dressing. Opting to dress without gloves may prove more comfortable and more productive for the operator. However, the use of non – sterile gloves instead of clean room gloves may have the opposite effect. Whilst sterile clean room gloves are manufactured to fit the hand's contour (Watts 2014), non - sterile gloves are restricted by their ambidextrous shape (Watts 2010). Therefore, some operators may be uncomfortable with their fit and find this hinders the garment donning process during use. When considering the type of glove to use during the donning process it is also important to consider the length of time for which the glove will be worn. Previous research has shown an increase in the incidence of glove perforations over wearing time (Partecke *et al.* 2009). Furthermore, increased wear can cause gloves to act as a second skin (Girou *et al.* 2004), enabling the spread of microorganisms onto the product or into the

environment. However, gloves used to don clean room clothing are worn for only a short period of time and therefore comfort and the risk of perforations which respect to time should not pose too much a concern to the operator.

Unfortunately, the results of this part of our study cannot be directly compared to any previous research studies due to a general lack of literature associating the efficiency of sterile clean room gloves against other glove types with respect to hand - borne microbial transmission. However, there are a number of previous studies which have compared the use of sterile or non - sterile gloves (Perelman *et al.* in 2004; Hemsworth *et al.* 2007), as well as comparing the use of sterile gloves with no gloves (Bodiwala and George 1982), all with respect to bacterial transmission. Nevertheless, these were undertaken in the hospital setting and are related to infection control in patients and therefore do not draw comparison between sterile clean room gloves and other glove types. Despite this, these earlier studies suggest there is no significant difference between the hand borne bacterial transfer levels of healthcare workers wearing no gloves, non – sterile gloves and sterile gloves, as also shown in this study. Bodiwala and George (1982) found there to be no significant difference in the subsequent infection rates of 418 accident and emergency patients who had previously received sutures by healthcare workers wearing either no gloves or sterile gloves. Perelman and colleagues (2004) further observed there to be no significant difference of the subsequent rate of infection in patients following the repair of uncomplicated traumatic lacerations by healthcare workers wearing either sterile or non - sterile gloves and Hemsworth *et al.* (2007), studying the incidence of septicaemia in children having had central venous catheters inserted with and without sterile gloves, found no significant difference in consequent infection rates. Although there are a number of variables between these previous studies and this current study, these findings help support the results of our study, that sterile gloves don't significantly reduce the transfer of hand borne bacteria when compared to their non – sterile equivalents or omitting to wear gloves.

However, if considering substituting sterile clean room gloves for their non - sterile equivalents it is important to note that there can be variability in performance between brands, with the number of defects per box varying (Zinner 1994). Therefore, if an organisation considers non – sterile glove use during the donning

process a thorough investigation would be helpful to assess the defect rate per brand. Furthermore, bacteria have been identified in 81.6 % of opened non - sterile glove boxes; thought to be introduced via the hand borne route during glove retrieval, with 50 % of these isolates being identified as species associated with the human skin (Hughes *et al.* 2013). Hughes *et al.* (2013) suggests that box design, location and retrieval (including a hand washing protocol) should be considered to minimise bacterial contamination entering the boxes, this could include reviewing the area in which boxes are stored, or developing a glove box design which would potentially reduce the number of bacteria entering via the hand borne route. When assessing the effectiveness of glove type during the donning process another factor which should be considered is the failure rate of different styles (Zinner 1994). Multiple research studies have highlighted problems associated with sterile and non - sterile glove use (Zinner 1994; Osman and Jensen 1999; Harnoβ *et al.* 2010) but draw no comparison to clean room gloves. In one of these previous studies non – sterile gloves were found to exhibit a higher percentage of holes and / or tears than their sterile equivalents, 5.7 % and 0.3 % respectively (Zinner 1994). Such glove perforations can lead to reduced barrier effectiveness (Osman and Jensen 1999) which may cause an increased spread of pathogens. A study into the passage of bacteria through glove perforations found that 5 % of defective gloves transferred bacteria, in particular *Staphylococcus, Micrococcus* and *Enterococcus* species, from the wearer (Harnoβ *et al.* 2010). Furthermore, during our study, despite significantly more plates used to test the lumbus of garments donned without gloves displaying low level growth when compared to the percentage displaying the same level of growth used to test the same area of garments donned with non – sterile gloves (* $p < 0.05$) (Figure 4 – 11 (d)), as shown in Figure 4 – 11 (e), a significantly higher percentage of plates used to test the left carpus of suits donned with non – sterile gloves displayed low levels of growth compared to the percentage of plates displaying the same level of growth and used to test the same region of garments donned by operators wearing no gloves (** $p < 0.01$). Additionally, there was shown to be no significant difference between the percentage of contact plates displaying either low, moderate or high levels of growth used to test the sites of garments donned with no gloves and non – sterile gloves and the percentage of contact plates displaying the same levels of growth, used to test garments donned with sterile clean room gloves. Therefore, despite our earlier suggestion that sterile clean room can be

substituted for either their non – sterile equivalents or the omission of gloves during the donning process, the results of our study in conjunction with the points discussed above suggests that donning clean room garments without gloves and in conjunction with a standardised hand washing protocol may be equally effective at minimising hand borne transfer of bacteria to clean room clothing than using potentially contaminated non - sterile gloves.

The results of our current study show that a higher percentage of plates used to test the chest and oral cavity of garments donned by operators wearing no gloves, non – sterile gloves or sterile clean room gloves displayed growth compared to the other sites tested (Figure 4 – 12), significantly so in those use to test garments donned with no gloves (Figure 4 – 12 (a)) and non – sterile gloves (Figure 4 – 12 (b)). This suggests that these sites were more highly contaminated that the other sites tested. A higher percentage of plates used to test the chest, lumbus and oral cavity regions of garments donned without gloves displayed low level growth compared to those used to test the 4 other sites (Figure 4 – 12 (a)). This was shown to be significant between the chest and left carpus (** $p < 0.01$) and cervicis (* $p < 0.05$), as well as between the lumbus and left carpus (* $p < 0.05$). In addition, a higher percentage of plates used to test the chest, left carpus and oral region of suits donned with non – sterile gloves displayed low levels of growth compared to the other sites tested (Figure 4 – 12 (b)). This was shown to be significant between the chest and umbilical, right carpus, posterior cervicis, lumbus (all *** $p < 0.05$) and oral cavity (* $p < 0.05$), as well as between those plates used to test the left carpus and right carpus and lumbus (both ** $p < 0.01$). The higher percentage of plates displaying low level growth used to test the chest region of suits encountered during this stage of our study were similar to the results observed with plates used to test the same region of suits laundered without sterilisation during Chapter 3 of our study (the same method of laundering used on garments in this part of the study). However, although some bacteria at the chest region may be due to bacteria on the suits following their laundering and / or recontamination of the garments during their processing, as previously discussed, it is thought that the majority of this bacteria were transferred onto the chest region of the suits via the hand borne route during the donning process. This is thought to be due to the complexity of retrieving the garments from their sterile packaging and their subsequent donning. A previous study by Kimberly Clark

Professional evaluating the feature and functions of clean room garments found that over one third of clean room operators thought that the donning of a clean room suit was the most difficult part of the overall clean room dressing process, taking nearly a third of the overall time (Larkin 2009). In addition, once removed from their sterile packaging, clean room suits are unzipped prior to donning from the chest region. They are then held at the neck and chest region and rolled downwards towards the waist prior to donning. Therefore, the chest region of the suit is in contact with the rest of the suit as it is being rolled down. Again, following donning, the zip is pulled up across the chest area and the hand used to secure the stud fastening at the chest. Furthermore, as previously discussed in Chapter 3 (the laundering study), the chest region may be more highly contaminated due to a lack of barrier protection between it and the skin, with the garment being in direct contact with the skin. Additionally, this region is close to the mouth and may become contaminated by bacteria from the oral cavity, which have previously been found on the skin (Dewhirst *et al.* 2010). Interestingly, as previously discussed, an unpublished study by Tayside Pharmaceuticals Quality Assurance Department (2007), testing the bacterial bioburden of clean room clothing by swabbing, also recovered higher numbers of bacteria from the front neck area of clean room suits (which correlates to the chest area in this current study) donned by operators, compared to other sites tested. However, these were very low levels of growth (1 - 2 cfu / swab), compared to the levels of bacteria recovered on contact plates during this part of our study. However, these lower numbers could be due to the decreased bacteria recovery efficiency obtained from antistatic carbon filament polyester fabric using swabbing compared to the direct agar contact method, as found in Chapter 2 of this study.

Also during this part of our study, a higher percentage of contact plates used to test the oral cavity region of hoods donned by operators dressing wearing either no gloves, non – sterile gloves or sterile clean room gloves displayed high levels of growth compared to the other sites tested. Aside from plates used to test the left carpus of suits donned with non – sterile gloves, a statistically higher percentage of plates used to test the oral cavity of suits donned under the same condition displayed high levels of growth (* $p < 0.05$) (Figure 4 – 12 (b)). These high levels are not thought to be due to hand borne bacterial transmission, as the hood is never touched over the oral cavity area during the donning process, but

this may be due to transmission of bacteria from the oral cavity, as previously discussed, dispersed through operators talking and breathing during the dressing process, and which can permeate the polyester hood, as shown in Chapter 2. Although this issue draws no relevance to glove use whilst donning the garment, this study highlights the high level of contamination in this area and suggest that bacterial levels could be potentially reduced by limiting operators' speech, however, in many cases this may prove impractical. Also in this study, a high percentage of plates used to test the left carpus suits donned using non - sterile gloves were found to be contaminated with low levels of growth. This may be due to two out of the three operators taking part in the study being left handed. However, the same pattern was not observed in contact plates used to test the same operators' garments donned with no gloves or sterile clean room gloves. In fact, only 11.1 % [1 / 9] of plates used to test the left carpus of suits donned with no gloves displayed low levels of growth compared to 66.7 % [6 / 9] of those used to test the same region of garments donned with non – sterile gloves (Figure 4 – 12 (b)).

It could be argued that the recovery of bacteria from the surface of garments during this stage of the study was not a direct result of hand borne transfer during the donning process but the result of bacteria being present on the surface of the garments following the laundering process and prior to the donning process, as was discussed in Chapter 3 of this study. However, a considerably higher percentage of plates used to test the surface of garments following donning displayed growth when compared to those used to test garments laundered under the same conditions, during the laundering comparison study in chapter 3 (no gloves - 64 %, non - sterile gloves - 57 % and sterile clean room gloves - 59 %) (Figure 4 – 7) vs. 6.1 % (Figure 3 – 6). Therefore, although a small percentage of bacteria on the suits may be the direct result of an inefficient laundering process, the results of our study show that contamination of clean room garments occurs via the hand borne route during donning process. Therefore, to help ensure the sterility of clean room garments during the donning process it is imperative that clean room operators adhere a standardised protocol to ensure that garments are handled and donned in a systematic manner which does not increase the bacterial bioburden of the garment (European Commission 2008). However, the validation of clean room operator's clothing after every change is impossible, due to the

unique operations undertaken whilst donning the garments and the range of variables associated with its use, nevertheless, the bacterial bioburden of the surface of clean room garments following donning should be monitored regularly (Rhodes 2006). As previously discussed in Chapter 3, monitoring of clean room garments following the donning process should be undertaken as part of staff training. Testing should be carried out in the changing area of the clean room facility following dressing by the operator, as was undertaken in this study. Both the right and left hand side of the front hood, collar cuff, wrist, leg and boots should be monitored using contact plates or wetted swabs and investigative and corrective action taken if any sample achieves levels greater than 5 cfu / plate (Rhodes 2006). However, the levels of contamination recovered on contact plates in this current study were in some cases considerably higher than the recommended 5 cfu / plate action level. However, it is important to remember that this study was undertaken by novice clean room operators. Although these operators were fully supervised during the gowning and sampling processes, it is suggested that clean room operators should undertake at least 30 hours of initial training on donning clean room garments before they can work in the clean room, with continuous weekly training thereafter (Larkin 2009). Ideally, to ensure reproducibility of the results obtained during this part of our study, further investigation of the bacterial bioburden of clean room garments following their donning should be undertaken. This should include the sampling of garments donned by a larger pool of operators. Unfortunately, this could not be undertaken during our study due to a limited number of operators available to commit their time to the research. Furthermore, ideally, a greater number of sites would be tested on each operator's garment. Preferably these sites would be the same as those garments sites tested during the laundering comparison study (Chapter 3), to include sites below the waist. This would allow for a total comparison of the bacterial bioburden between laundered suits and those tested following the donning process. This was not undertaken in our current study due to time limitations and the need to sample operators in a short time frame following their working duration in the clean room.

Finally, as previously discussed in Chapter 2 of our study, the percentage recovery of bacteria from pre - inoculated antistatic carbon filament polyester squares using the direct contact method may have been significantly underestimated due to the

time in which contact plates were incubated (24 hours), as well as the composition of agar used to fill these plates. The issue of agar composition was addressed in Chapter 3, where there was found to be no significant difference between the percentage of contact plates displaying growth used to test the surface of clean room suits laundering with and without gamma sterilisation, whether these were filled with nutrient or tryptone soya agar. The issue of incubation time was addressed in this current chapter. Although not shown to be significant, the results of the study showed an increase in the percentage of plates displaying each level of growth following a 48 hour agar incubation period as opposed to a 24 hour agar incubation period (Figure 4 – 7). These results confirm that contact plates used for the recovery of bacteria from clean room garments require a 48 hours incubation period to allow the viable cells sufficient time to grow. A 24 hour incubation period as undertaken in previous contact plate studies (Tebbutt 1991; Foschino *et al.* 2003; Obee *et al.* 2006; Pinto *et al.* 2008) may underestimate the level of contamination on a surface. It also suggests the poor recovery efficiency observed in Chapter 2 of this study may be due to inadequate incubation time and that all further results in this study should only be considered following a 48 hour incubation period.

4.6 Conclusion

In this chapter the bacterial bioburden of the surface of clean room garments donned by operators dressing wearing either no gloves, non – sterile gloves or sterile clean room gloves was compared using the direct agar contact method. Bacteria were recovered from the surface of garments under each condition, however, there was found to be no significant difference between the total percentage of contact plates displaying growth and their subsequent levels of growth and the variable, dressing wearing no gloves, non – sterile gloves or sterile clean room gloves. Additionally, there was shown to be no significant difference between the bacterial bioburden of the 7 garments sites tested and glove type, thus suggesting that sterile clean room gloves can be substituted for their non – sterile equivalent or no gloves during the clean room garment donning process without subsequently increasing the surface bacterial bioburden of the clean room garment. However, on examination of the bacterial bioburden of garment sites within glove types, in each case, a higher percentage of plates used to test the chest region displayed low levels of growth, and a higher percentage of those used

to test the oral cavity region displayed high levels of growth, compared to the levels of growth observed on plates used to test the other sites examined. Finally in this chapter, an increase in the percentage of contact plates displaying growth, as well as an increase in the levels of growth displayed, following a 48 hour incubation period compared to a 24 hour incubation period was observed.

Chapter 5:

The Impact of Gender upon the
Surface Bacterial Bioburden of Antistatic Carbon Filament
Polyester Clean Room Garments Worn by Operators Working
In a Clean Room Environment

5.1 Introduction

The bacterial bioburden of the surface of clean room operators' garments should remain low during their working period within the clean room environment (Rhodes 2006). However, it has shown that despite specialist clean room garments reducing the airborne dispersion of particles and their associated microorganisms from operators, these will not retain all sources of operator related detriment (Ramstorp *et al.* 2005; Whyte and Hejab 2007). Furthermore, evading microorganisms can contaminate the outer surface of clean room operators' garments whilst they work (Grangè *et al.* 2010). As a result, these specialist garments can become a vehicle for the transmission of microorganisms, having a potentially detrimental impact upon the sterility and integrity of a clean room environment and its products, as previously discussed. The number of bacteria and skin scales dispersed from individuals has been shown to significantly differ from person to person (Hall *et al.* 1986). In fact, the total number of particles and / or microorganisms shed by individuals over time has been found to be dependent upon a number of factors including clothing type worn (Whyte *et al.* 1976; Mitchell *et al.* 1978; Reinmüller and Ljungqvist 2003, Ramstorp *et al.* 2005; Bischoff *et al.* 2007; Whyte and Hejab 2007) and activity being undertaken (Ramstorp *et al.* 2005), as well as skin pathology (Noble and Davies 1965), skin care regimes (Hall *et al.* 1986) and gender (Noble and Davies 1965; Noble *et al.* 1976; Mitchell *et al.* 1978; Hall *et al.* 1986; Bischoff *et al.* 2007; Whyte and Hejab 2007). Therefore, in this part of our study the bacterial bioburden of the surface of reusable antistatic carbon filament clean room garments worn by male and female operators having worked in a clean room environment for a fixed period of time was assessed and compared using the direct agar contact method developed in Chapter 2. To the author's knowledge, there is only one such previous study (Grangè et al. 2010), however, this earlier research examines the bacterial bioburden of the surface of clean room operators' garments with respect to time spend working in the clean room rather than gender as such.

5.1.1 Microbial & Particulate Dispersion between Genders

Previous research studies have assessed particulate and / or bacterial dispersion rates from individuals by quantifying the number of particles and / or bacteria dispersed over a period of time, most commonly using a modified dispersal chamber (Noble and Davies 1965; Noble *et al.* 1976; Whyte *et al.* 1976; Mitchell

et al. 1978; Hall *et al.* 1986; Reinmüller and Ljungqvist 2003, Ramstorp *et al.* 2005; Bischoff *et al.* 2007; Whyte and Hejab 2007). However, only three of these previous studies focus upon the dispersal of particles with respect to clean room operators and their garments (Reinmüller and Ljungqvist 2003; Ramstorp *et al.* 2005; Whyte and Hejab 2007). Furthermore, these previous clean room garment studies do not directly assess the bacterial bioburden of the surface of clean room garments and therefore the results of this part of our study cannot be compared directly to these previous studies. Despite an absence of published literature assessing the bacterial bioburden of the surface of clean room operators' garments, those particulate / microbial dispersion studies which do exist have shown that gender plays an important role in the dispersion of skin related microorganisms into the air, with males shedding significantly more particles and / or microorganisms than their female counterparts (Noble and Davies 1965; Noble *et al.* 1976; Mitchell *et al.* 1978; Whyte and Hejab 2007). In fact, Noble *et al.* (1976) showed that males dispersed between 2.5 – 5 times more MCPs than their female equivalents. Furthermore, males have been shown to disperse twice as much *S. aureus* into the air as females. However, the reason for this remains unclear (Bischoff *et al.* 2007). Previous research has also shown that males may disperse approximately 1.45 times more skin squames into the environment than their female counterparts of the same skin surface area (Noble *et al*. 1976). Therefore, it is important to consider that in this respect the degree of bacterial dispersion may be proportionate and directly related to skin surface area rather than gender as such.

Gender has also been shown to effect the microbial diversity of the human skin (Reichel *et al.* 2011; Zeeuwen *et al.* 2012), with males having been shown to harbour more microorganisms at all body sites when compared to their female counterparts (Reichel *et al.* 2011). For example, Fierar *et al.* (2008) found that the microbial diversity on the surface of the hand varies significantly between genders. The reason for this remained unclear, however, it is important to consider that any differences may be due to hand washing practices rather than gender as such (Fierar *et al.* 2008). The exact mechanism behind which factors play a role in the microbial diversity and dispersion between genders is not well documented. Furthermore, there are very few published studies which assess the physiological

skin differences between genders and those studies which do exist are contradictory (Jacobi *et al.* 2005). The problem is that the skin is a complex organ, host to a number of gender specific physical and chemical features within its structure. For example, differences between the skin of males and females can include their levels of sebum, sweat and collagen, as well as differences in skin pH, thickness and tone (Giacomoni *et al.* 2009). Such physiological and morphological differences between the skin of males and females may be due to the varying levels and types of the sex hormones estrogen, progesterone and testosterone (Giacomoni *et al.* 2009). These can affect the thickness of the skin, immunity to disease, as well as its pH (Dao and Kazin 2007), all of which can affect the skin's microbial community. For example, the androgen group of sex hormones, which encompasses testosterone (levels of which are 10 fold in males) is responsible for coarser body hair, increased muscle development and a denser network of collagen fibres (Maxwell 2016).

5.1.2 Employing Clean Room Operators

In a bid to control particulate and microbial numbers within the clean room environment there should be strict standards for employing clean room operators. Within this, individual characteristics including size, allergies and environmental and chemical sensitivity should be considered (Eudy 2003). In addition, operators should report any medical conditions which cause the abnormal shedding of the skin including eczema and psoriasis (MHRA 2017). However, gender is a characteristic which cannot be discriminated against, despite male operators potentially shedding more microorganisms into the environment than their female counterparts, possibly to the detriment of the product and ultimately patient. The high prevalence of females working in hospital pharmacies has resulted in the majority of clean room workers in this industry being female (Jackson and Wilson 2006). However, males must still be considered for clean room operator positions. Therefore, it would be advantageous to the clean room industry to determine the quantity and diversity of bacteria shed through clean room operators' clothing with respect to gender and body site. Such research may provide the clean room industry with a better understanding of the role gender plays in regard to contamination levels within clean room facilities, with a view to implementing

additional dressing measures which could reduce any significant difference in microbial dispersion between male and female operators.

5.2 Aims & Objectives

The aim of this aspect of the project was to assess the bacterial bioburden of the surface of clean room garments, following their wear in the clean room environment, with respect to gender. In order to achieve this, a couple of specific objectives were developed:

- To use the direct agar contact method, developed in Chapter 2, to recover, enumerate and compare the levels of bacteria on the surface of clean room garments worn by male and female operators having worked in a clean room environment for a fixed period of time.
- With respect to gender, male and female operators, use the direct agar contact method to compare the surface bacterial bioburden of clean room garments at various body sites following their wear.

5.3 Materials & Methodology

In order to achieve the specific objectives set out in our current study the following materials and methods were used.

5.3.1 Clean Room Facility & Maintenance

This current investigation was carried out within the clean room facility based within the Technical Building at Robert Gordon University in Schoolhill Aberdeen, as discussed Chapter 4 - Section 4.3.1 (the layout of which can be seen in Figure 4 – 5). Maintenance of the clean room was undertaken as discussed in Chapter 4 - Section 4.3.2.

5.3.2 Ethical Approval & Consent

As previously discussed in Chapter 4 - Section 4.3.3, a research ethics self-assessment (RESA) form was submitted and the application was approved by the ethical review panel of the School of Pharmacy and Life Sciences at the Robert Gordon University. For this part of the project 3rd year undergraduate Master of Pharmacy students were invited to voluntarily participate in the study. Prior to their sampling a consent form giving a written explanation of the procedure was

presented to all volunteers. Each volunteer signed the consent form prior to taking part in the study. The form also allowed the individual the opportunity to discreetly opt out of the study. Furthermore, beyond gender, no private, confidential or personal information was recorded.

5.3.3 Clean Room Clothing & Laundering

Clean room attire and gloves used in this part of the current study were the same as those discussed in Chapter 4 - Section 4.3.4. Clean room garments were laundered by Fishers Laundry Group (Aberfeldy, UK) as per the process described in Chapter 4 - Section 4.3.4.

5.3.4 Preparation of Contact Plates

Fifty five mm nutrient agar contact plates were prepared and stored as per the method discussed in Chapter 2 - Section 2.3.4. Agar plates were removed from the fridge and left at room temperature for two hours prior to use to eliminate condensation on the surface of the agar.

5.3.5 Clean Room Garment Donning

Operators entered the clean room changing area as described in Chapter 4 - Section 4.3.5. Following hand washing operators donned appropriately sized non – sterile gloves. Operators working in the Grade C clean room donned a sterile cleanroom suit, as described in Chapter 4 - Section 4.3.5. Operators working in the Grade A / B clean room donned a sterile hood as described in Chapter 4 - Section 4.3.6 and sterile clean room suit (Chapter 4 - Section 4.3.7). In addition, operators working in the Grade A / B clean room donned sterile over boots in the following manner. The operator tore open the bag containing the boot and touching only the insides of the fabric it was removed from the packaging. This was placed over the foot (with shoes already removed and with a plastic overshoe on) and tied securely at the knee and ankle. This was repeated with the other foot.

5.3.6 Direct Agar Contact Method of Bacterial Recovery

Operators spent either ~ 30 minutes working in a Grade A / B clean room or ~ 60 minutes working in a Grade C clean room. Immediately following working operators exited the room and entered into the grey area of the changing room (Figure 4 – 5). Operators having worked in the Grade C clean room undertook the

direct contact method as described in Chapter 4 - Section 4.3.9 on six areas of their clean room suit (Figure 4 – 6). In addition, operators having worked in the Grade A / B area tested the same 6 areas as well as their hood, covering their oral cavity. Following testing contact plates were inverted and incubated at 37 ^{o}C. These were examined for growth after 24 and 48 hours. All results are reported as those at 48 hours due to an increase in the percentage of contact plates displaying low, moderate and high levels of growth following a further 24 hour incubation period at 37 ^{o}C (taking the total incubation period to 48 hours) observed during Chapter 4 of our study. The areas of the garments which were tested were then wiped with a 70 % ethanol impregnated wipe and these were laundered as per the process described in Chapter 4 - Section 4.3.4.

5.3.7 Statistical Analysis of Results

The results of this study were analysed using GraphPad Prism 4.0 (GraphPad Software Inc., La Jolla, CA). Two – way Analysis of Variance (ANOVA) at a 95 % confidence level was used to statistically analyse any differences between the variable of gender

5.4 Results

A total of 497 contact plates were used to test the exterior surface of clean room garments having been worn by a subject group of 77 novice operators (26 males and 51 females) following their working period in a clean room environment, either ~ 30 minutes working in a Grade A / B clean room or ~ 60 minutes working in a Grade C clean room. Fifty six contact plates (representing 100 %) were used to test the exterior surface of garments worn by 8 males operators working in a Grade A / B clean room and 189 plates (representing 100 %) used to test the surface of garments worn by 27 female operators working under the same condition. In addition, 108 contact plates (representing 100 %) were used to test the surface of garments worn by 18 male operators working in a Grade C clean room and 144 contact plates (representing 100 %) used to test the surface of garments worn by 24 female operators working under the same condition. A comparison of gender against the total percentage of contact plates displaying growth following a 48 hour incubation period, used to test the surface of clean room garments worn by male and female operators having worked for ~ 30 minutes in a Grade A / B clean room and ~ 60 minutes in a Grade C clean room, is shown in Figure 5 – 1.

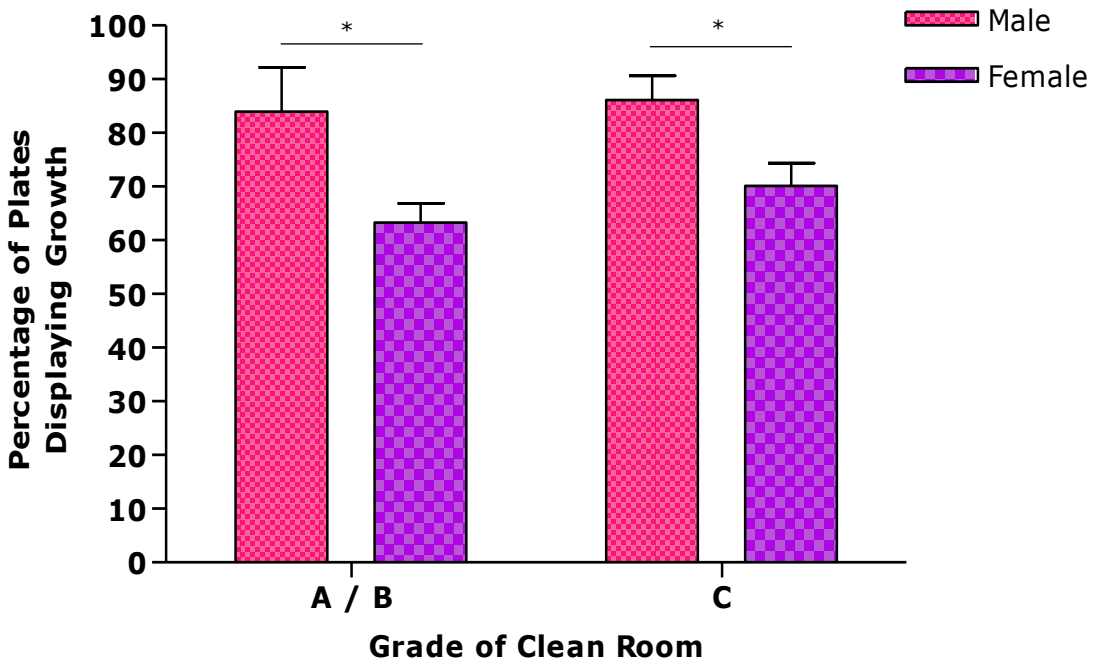


Figure 5 – 1: A comparison of gender against the total percentage of contact plates displaying growth following a 48 hour incubation period at 37 ^{o}C, used to test the surface of clean room garments worn by operators working in either a Grade A / B clean room for ~ 30 minutes or a Grade C clean room for ~ 60 minutes (* $p < 0.05$).

As shown in Figure 5 – 1, statistically, there was shown to be a significantly higher percentage of plates used to test the surface of garments worn by male operators displaying growth, under both working conditions, when compared to the percentage displaying growth used to test the surface of garments worn by female operators working under the same circumstance. As shown, 83.9 % [47 / 56] of contact plates used to test the exterior surface of clean room garments worn by male operators having worked in a Grade A / B clean room for ~ 30 minutes displayed growth. In comparison, 63.3 % [120 / 189] of plates used to test the surface of garments worn by female operators working under the same conditions displayed growth. Of contact plates used to test the surface of garments worn by operators having worked in a Grade C clean room for ~ 60 minutes, 86.1 % [93 / 108] of those used to test garments worn by males and 70.1 % [101 / 144] of those used to test garments worn by females displayed growth.

To further assess the bacterial bioburden of clean room garments following wear with respect to gender, a comparison was also drawn between operator gender and the levels of growth displayed on these contact plates following their incubation, under each working condition. A comparison of operator gender against the percentage of contact plates displaying either 0 (no growth), 1 - 9 (low level growth), 10 - 20 (moderate level growth) or > 20 (high level growth) cfu / plate, used to test the surface of garments worn by operators working in either a Grade A / B clean room for ~ 30 minutes or a Grade C clean room for ~ 60 minutes is shown in Figure 5 – 2.

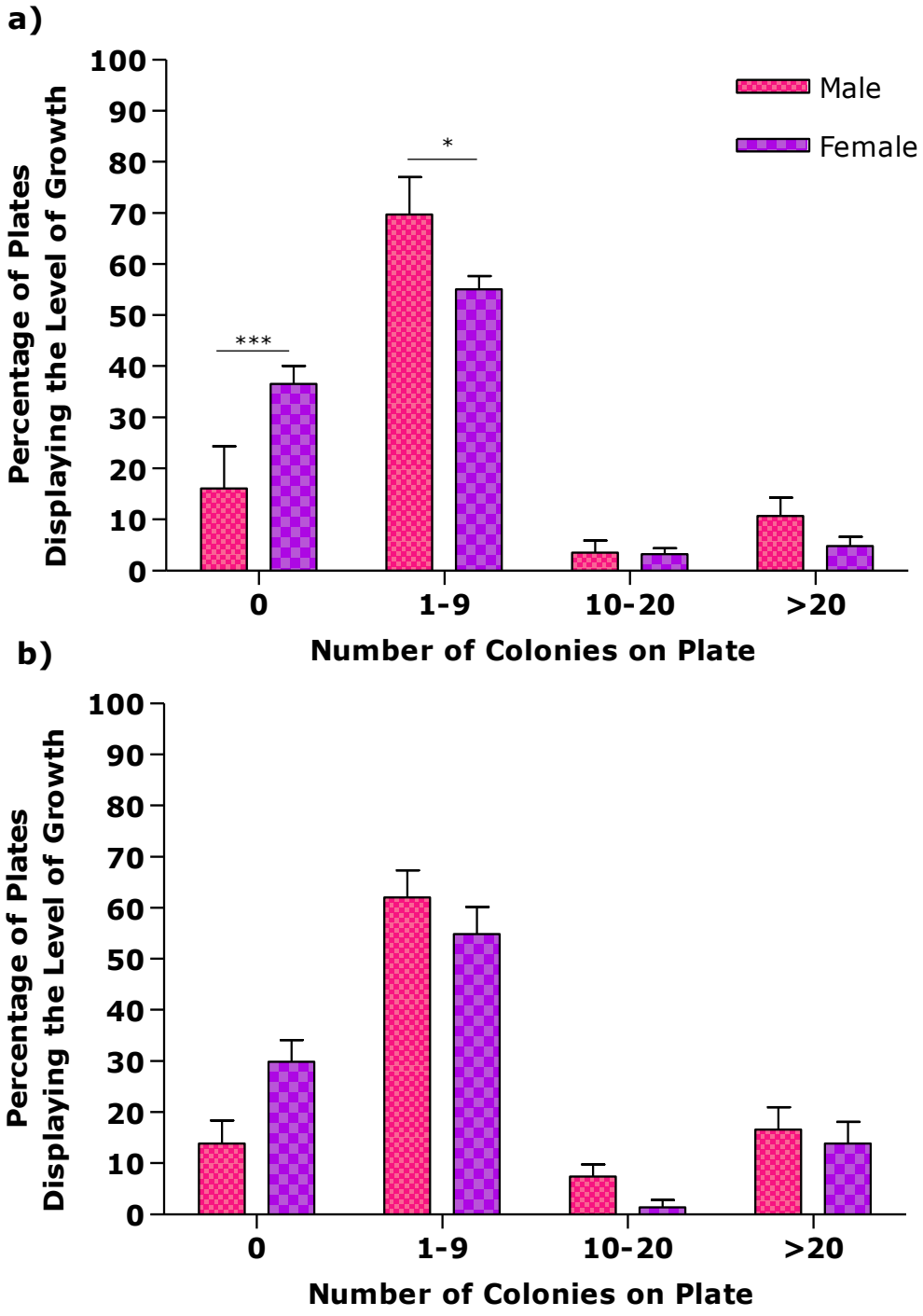


Figure 5 – 2: A comparison of gender against the total percentage of contact plates displaying either 0 (no growth), 1 – 9 (low level growth), 10 – 20 (moderate level growth) or >20 (high level growth) cfu / plate following a 48 hour incubation period at 37 °C, used to test the surface of clean room garments worn by operators working in either (a) a Grade A / B clean room for ~ 30 minutes or (b) a Grade C clean room for ~ 60 minutes (*** $p < 0.001$; * $p < 0.05$).

As shown in Figure 5 – 2, under both working conditions, a higher percentage of plates used to test the surface of male operators' garments displayed low, moderate and high levels of growth compared to those contact plates used to test the surface of garments worn by female operators working under the same condition. However, this was only found to be statistically significant between the percentage of plates displaying low levels of growth (* $p < 0.05$) and gender, in plates used to test the garments of operators working in a Grade A / B clean room for ~ 30 minutes (Figure 5 – 2 (a)). As also shown in Figure 5 – 2 (a), 16.1 % [9 / 56] of plates used to test the surface of garment worn by male operators having worked in a Grade A / B clean room displayed no growth. This was shown to be statistically less than the 36.7 % [69 / 189] of contact plates with displayed no growth used to test female operators working under the same condition (*** $p < 0.001$). Of the 83.9 % [47 / 56] of contact plates which displayed growth when used to test the surface of garments worn by the male operators (Figure 5 – 1), 69.6 % [39 / 56] of these displayed low levels of growth, 3.6 % [2 / 56] moderate levels of growth and 10.7 % [6 / 56] high level growth (Figure 5 – 2 (a)). Of the 63.3 % [120 / 189] of contact plates displaying growth which were used to test the surface of garments worn by female operators having worked under the same conditions (Figure 5 – 1), 54.5 % [103 / 189] of these showed low level growth, 3.2 % [6 / 189] moderate level growth and 5.8 % [11 / 189] high level growth.

As shown in Figure 5 – 2 (b), 13.9 % [15 / 108] of contact plates used to test the surface of garments worn by male operators having working in a Grade C clean room displayed no growth, this was shown to be less than the 29.9 % [43 / 144] of contact plates with displayed no growth used to test the surface of garments worn by female operators having worked under the same conditions. Of the 86.1 % [93 / 108] of contact plates displaying growth which were used to sample the surface of garments worn by male operators working in a Grade C clean room (Figure 5 – 1), 61.1 % [66 / 108] of these displayed low levels of growth, 7.4 % [8 / 108] moderate levels of growth and 17.6 % [19 / 108] high levels of growth. Additionally, of the 70.1 % [101 / 144] of contact plates displaying growth which were used to test the surface of garments worn by female operators having worked under the same condition (Figure 5 – 1), 54.9 % [79 / 144] showed low level growth, 1.4 % [2 / 144] moderate level growth and 13.8 % [20 / 144] high level growth.

In order to fully address the issue of gender with respect to the bacterial bioburden of clean room garments following their wear in the clean room environment a further comparison was drawn between gender and the total percentage of contact plates displaying growth used to test each garment site. A comparison of gender against the total percentage of plates displaying growth, used to test the chest, umbilical, posterior cervicis, lumbus, left and right carpus of male and female operators having worked in a Grade A / B clean room for ~ 30 minutes or a Grade C clean room for ~ 60 minutes. As well as the oral cavity of hoods worn by the same operators working in the Grade A / B clean room is shown in Figure 5 – 3.

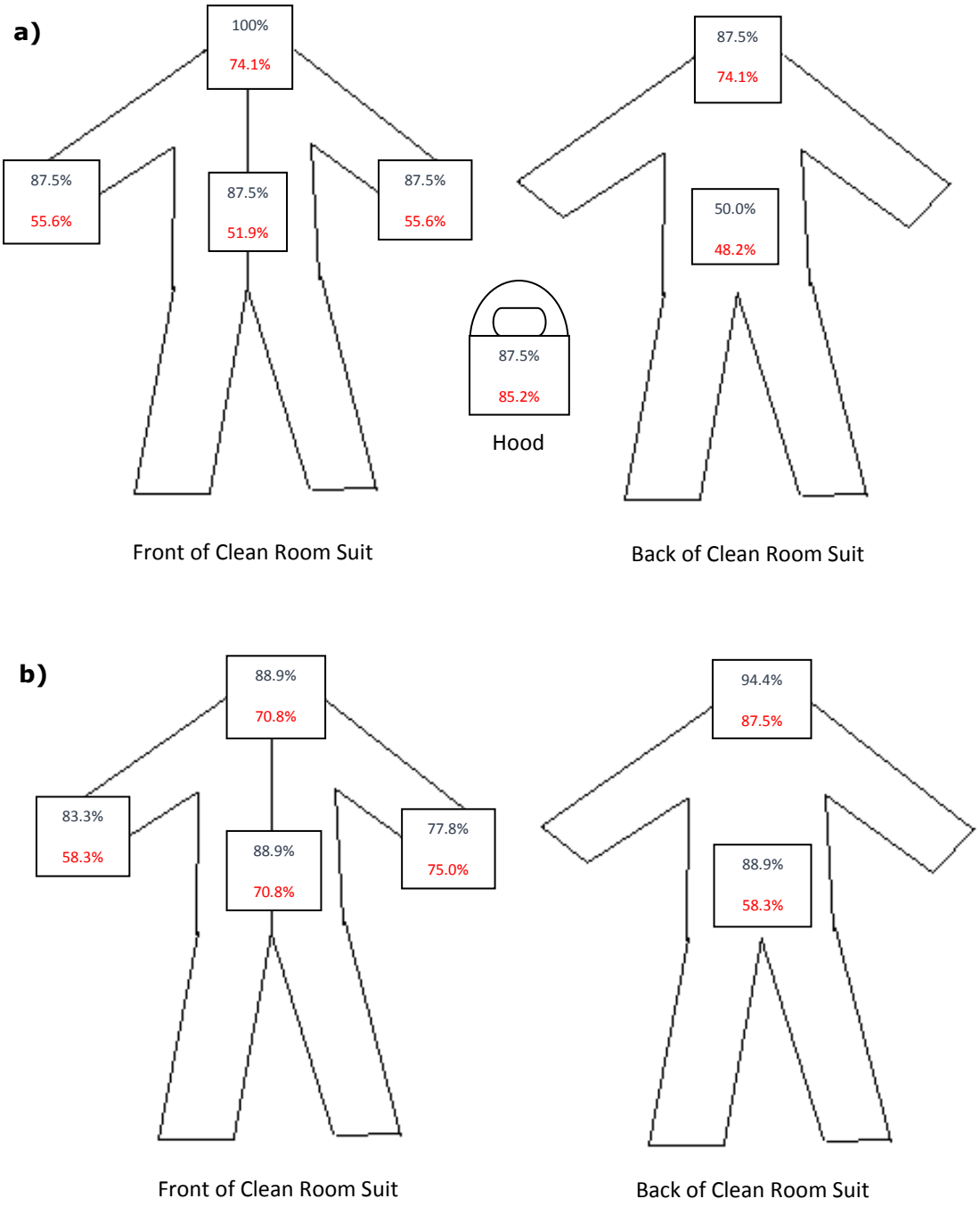


Figure 5 – 3: A comparison of gender against the total percentage of contact plates displaying growth at each site tested (chest, umbilicus, posterior cervicis, lumbus, left carpus, right carpus and oral cavity (Figure 4 – 6)) of garments worn by male (blue) and female (red) operators working in either (a) a Grade A / B clean room for ~ 30 minutes and (b) a Grade C clean room for ~ 60 minutes.

As shown in Figure 5 – 3 (a), although not found to be statistically significant, a higher percentage of contact plates used to test all 7 sites of garments worn by male operators having worked in a Grade A / B clean room for ~ 30 minutes displayed growth compared to the percentage of plates displaying growth which were used to test the same sites of garments worn by female operators working under the same condition (chest – 100 % [8 / 8] vs. 74.1 % [20 / 27]; posterior cervicis - 87.5 % [7 / 8] vs. 74.1 % [20 / 27]; umbilicus – 87.5 % [7 / 8] vs. 51.9 % [14 / 27]; lumbus – 50.0 % [4 / 8] vs. 48.2 % [13 /27], left and right carpus – both 87.5 % [7 / 8] vs. 55.6 % [15 / 27]; oral cavity 87.5 % [7 / 8] vs. 85.2 % [23 / 27]. Furthermore, as shown in Figure 5 – 3 (b), although not shown to be statistically significant, a higher percentage of contact plates used to test all 6 sites of garments worn by male operators having worked in a Grade C clean room for ~ 60 minutes displayed growth compared to the percentage of plates displaying growth which were used to test the same sites of garments worn by female operators working under the same conditions was also observed (chest and umbilicus - both 88.9 % [16 / 18] vs. 70.8 % [17 / 24]; posterior cervicis - 94.4 % [17 / 18] vs. 87.5 % [21 / 24]; lumbus – 88.9 % [16 / 18] vs. 58.3 % [14 / 24]; left carpus – 77.8 % [14 / 18] vs. 75.0 % [18 / 24]; right carpus – 83.3 % [15 / 18] vs. 58.3 % [14 / 24]).

In order to fully investigate the matter, a further comparison was drawn between each gender and the levels of growth detected on these plates for each of the garment sites tested. A comparison of gender against the percentage of contact plates displaying either 0 (no growth), 1 - 9 (low level growth), 10 - 20 (moderate level growth) or > 20 (high level growth) cfu / plate, used to test the chest, umbilical, posterior cervicis, lumbus, left and right carpus suits worn by operators working in Grade C clean room for 60 minutes is shown in Figure 5 – 4 (b), and the same 6 sites, as well as the oral cavity of hoods, worn by operators working in a Grade A / B clean room for ~ 30 minutes, is shown in Figure 5 – 4 (a).

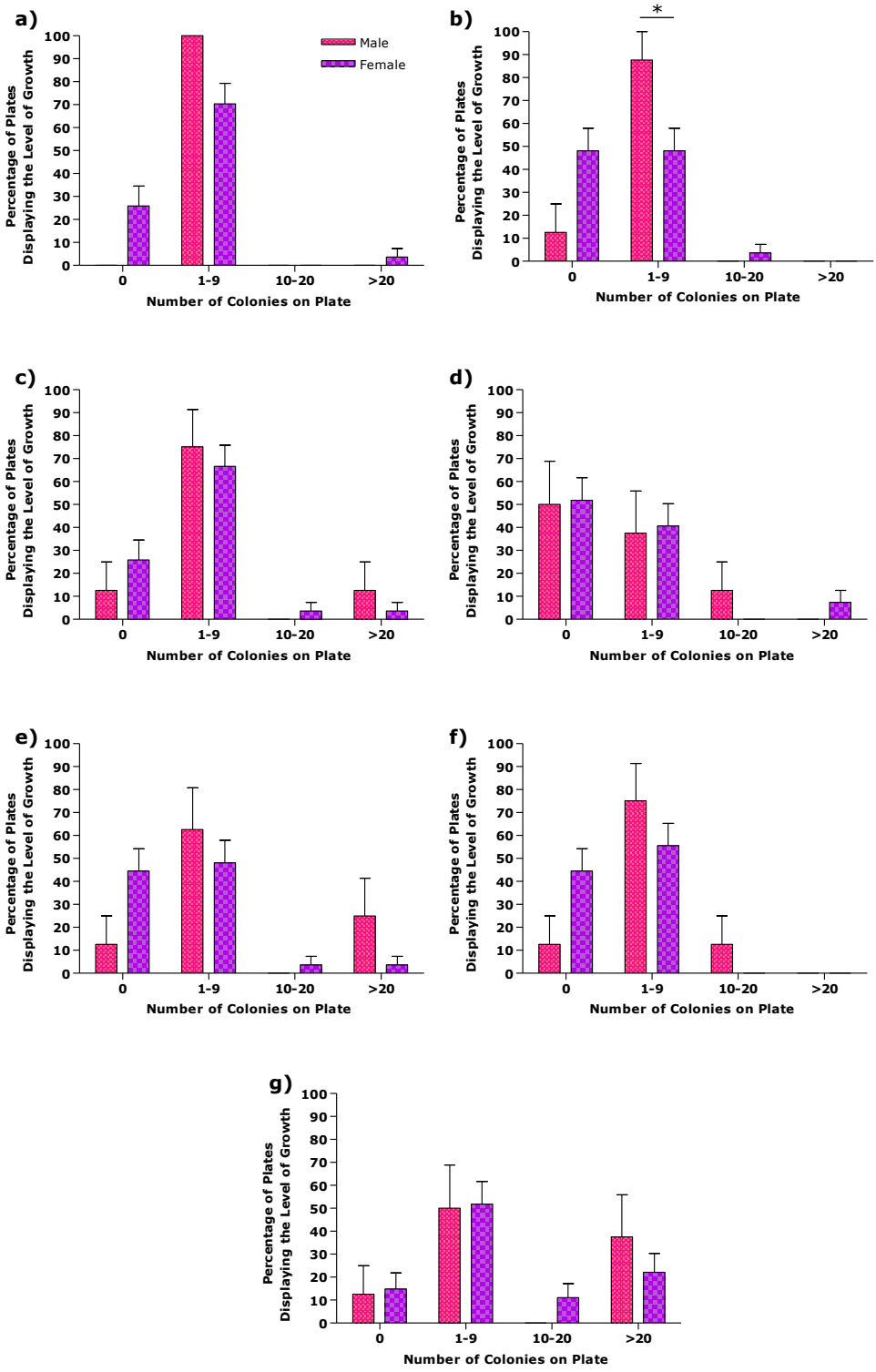


Figure 5 – 4a: A comparison of gender against the total percentage of contact plates displaying either 0 (no growth), 1 – 9 (low level growth), 10 – 20 (moderate level growth) or >20 (high level growth) cfu / plate, used to test the (a) chest, (b) umbilicus, (c) posterior cervicis, (d) lumbus, (e) left carpus, (f) right carpus and (g) oral cavity region of clean room garments worn by operators working in a Grade A / B clean room for ~ 30 minutes (* $p < 0.05$).

As shown in Figure 5 – 4a (a), 100 % [8 / 8] of contact plates used to test the chest of male operators working in a Grade A / B clean room for ~ 30 minutes all displayed low level growth. In comparison, of the 100 % [27 / 27] of contact plates used to test the same region of garments worn by female operators working under the same conditions 25.9 % [7 / 27] displayed no growth, 70.4 % [19 / 27] % low level growth and 3.7 % [1 / 27] % high levels growth. A shown in Figure 5 – 4a (b), 12.5 % [1 / 8] of those plates used to test the umbilicus of male operators' garments displayed no growth and 87.5 % [7 / 8] % low level growth. Of those used to test the same site of female operators' garments 48.15 % [13 / 27] displayed no growth, 48.15 % [13 / 27] low level growth and 3.7 % [1 / 27] moderate level growth. Statistically, significantly more plates used to this region of male operators suits displayed low level growth compared to those used to test the same site of suits worn by female operators working under the same conditions (* $p < 0.05$). Of those contact plates used to test the posterior cervicis regions of male operators suits (Figure 5 – 4a (c)) only 12.5 % [1 / 8] displayed no growth, 75 % [6 / 8] low level and 12.5 % [1 / 8] high level growth. Plates used to test the same sites on female operators garments displayed as 25.9 % [7 / 27], 66.7 % [18 / 27], 3.7 % [1 / 27] and 3.7 % [1 / 27] respectively. As shown in Figure 5 – 4a (d), 50 % [4 / 8] of plates used to test the lumbus region of suits donned by male operators displayed no growth, 37.5 % [3 / 8] displayed low levels growth and 12.5 % [1 / 8] displayed moderate level growth. In comparison, 51.9 % [14 / 27] of plates used to test the lumbus region of female operators' suits displayed no growth, 40.7 % [11 / 27] displayed low level and 7.4 % [2 /27] high levels of growth. Plates used to test the left carpus of male operators (Figure 5 – 4a (e)) displayed as 12.5 % [1 / 8] no growth, 62.5 % [5 / 8] low level and 25.0 % [2 / 8] high level growth. Those used to test the same site in females displayed as 44.4 % [12 / 27] no growth, 48.2 % [13 / 27] low level growth and 3.7 % [1 / 27] of both moderate and high level growth. Plates used to test the right carpus of male operators' suits displayed as 12.5 % no growth [1 / 8], 75 % [6 / 8] low level and 12.5 % [1 / 8] moderate level growth. In those used to test female operators garments at the same region this was found to be 44. 4 % [12 / 27] no growth and 55.6 % [15 / 27] low level growth only. Lastly, plates used to test the oral cavity of hoods donned by male operators displayed 12.50 % [1 / 8] no growth, 50 % [4 / 8] low level and 37.5 % [3 / 8] high level growth. In those used to test the oral cavity of female operators' hoods levels of no growth, low, moderate and

high growth were 14.8 % [4 / 27], 51.9 % [14 / 27], 11.1 % [3 / 27] and 22.2 % [6 / 27] respectively (Figure 5 – 4a (a)). Overall, at all sites tested a lower percentage of plates used to test the surface of garments worn by male operators displayed no growth compared to those used to test the same sites in garments worn by female operators working under the same conditions. Aside from a statistically higher percentage of plates used to test the chest of male operators displaying low levels of growth compared to the percentage used to test the same site of female operators having worked under the same conditions, there was shown to be no significant difference between gender and the levels of growth displayed on contact plates at each of the sites tested.

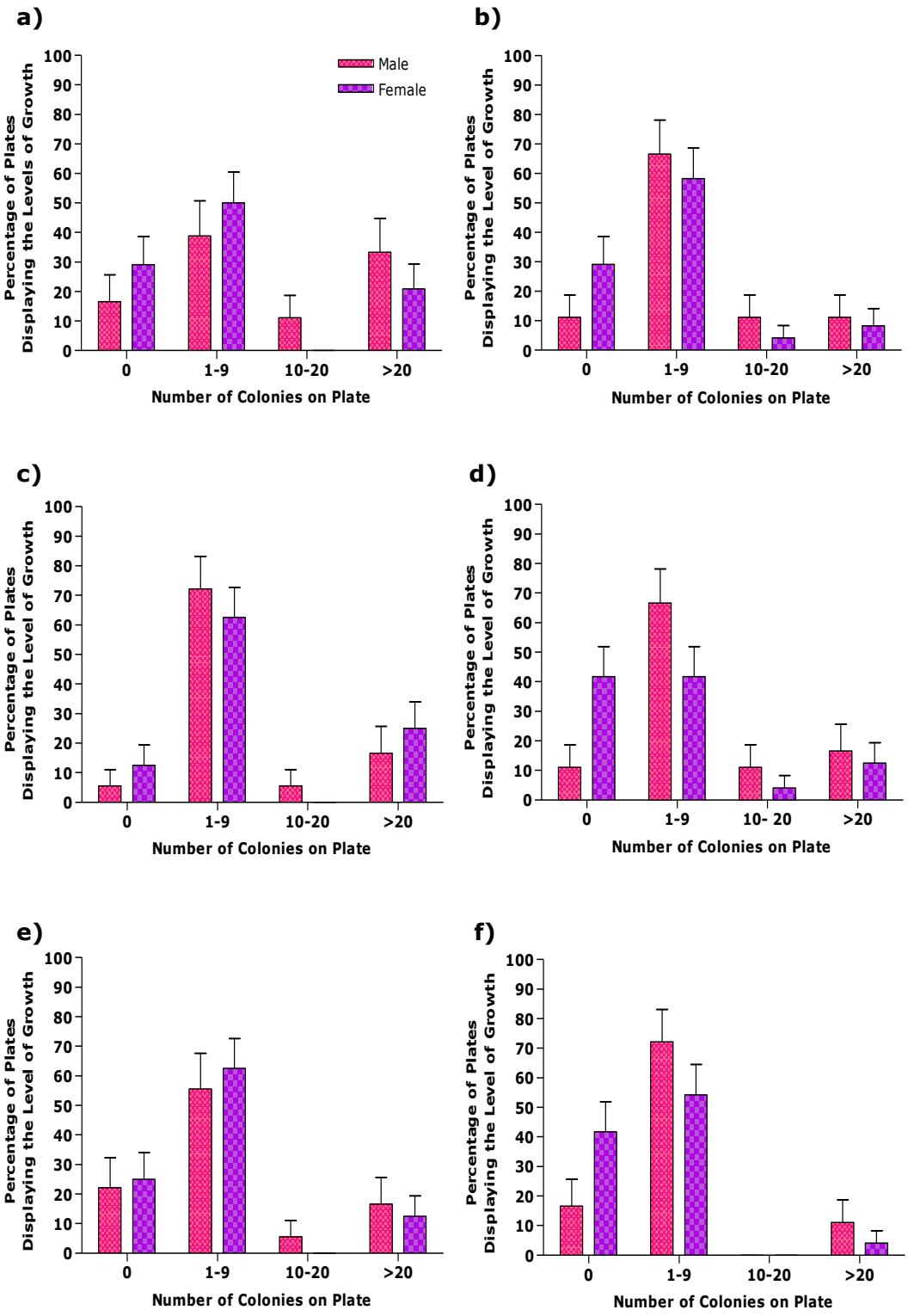


Figure 5 – 4b: A comparison of gender against the total percentage of contact plates displaying either 0 (no growth), 1 – 9 (low level growth), 10 – 20 (moderate level growth) or >20 (high level growth) cfu / plate, used to test the (a) chest, (b) umbilicus, (c) posterior cervicis, (d) lumbus, (e) left carpus and (f) right carpus region of clean room garments worn by operators working in a Grade C clean room for ~ 60 minutes.

As shown in Figure 5 – 4b (a), of the 100 % [18 / 18] contact plates used to test the chest region of garments worn by male operators having worked in a grade C clean room for ~ 60 minutes 16.7 % [3 / 18] displayed no growth, 38.9 % [7 / 18] low level growth, 11.1 % [2 / 18] moderate level growth and 33.3 % [6 / 18] high level growth. In comparison, of the 100 % [24 / 24] of contact plates used to test the chest region of female operators working under the same conditions 29.2 % [7 / 24] displayed no growth, 50 % [12 / 24] low and 20.8 % [5 / 24] high level growth. Those plates used to test the umbilicus region of male operators' garment equated to 11.1 % [2 / 18] no growth, 66.7 % [12 / 18] low level and both 11.1 % [2 / 18] moderate and high level growth and those used to test the same region of female operators garments as 29.2 % [7 / 24] no growth, 58.3 % [14 / 24] low level growth, 4.2 % [1 /24] moderate level and 8.3 % [2 / 24] high level growth (Figure 5 – 6b (b)). Those used to test the posterior cervicis region of male operators displayed as 5.6% [2 / 18] no growth, 72.2 % [13 / 18] low level growth, 5.6 % [1 / 18] moderate level growth and 16.7 % [3 /18] high level growth. Contact plate used to test the same site of female operators' garments displayed as 12.5 % [3 / 24], 62.5 % [15 / 24], 0 % [0 / 24] and 25.0 % [6 / 24] respectively (Figure 5 – 4a (c)). As shown in Figure 5 – 6b (d), contact plates used to test the lumbus of male operators garments displayed 11.1 % [2 /18] no growth, 66.7 % [12 / 18] low level, 11.1 % [2 / 18] moderate level and 16.7 % [3 / 18] high level growth. In comparison, those use the test the same region of female operators displayed as 41.7 % [11 / 24], 41.7 % [11 / 24], 4.2 % [1 / 24] and 12.5 % [3 / 24] respectively. Of plates used to test the left carpus of male operators 22.2 % [4 / 18] displayed no growth, 55.6 % [10 / 18] low level, 5.6 % [1 / 18] moderate level and 16.7 % [3 / 18] high level growth and those used to test the left carpus of female operators as 25.0 % [6 / 24], 65.5 % [16 / 24], 0 % [0 / 24] and 12.5 % [3 / 24] respectively. Lastly, the plates used to test the right carpus of male operators' garments displayed as 16.7 % [3 / 18], 72.2 % [13 / 18], 0 % [0 / 18] and 11.1 % [2 / 18] and female operators' garments as 41.7 % [10 / 24], 54.2 % [13 / 24], 0 % [0 / 24] and 4.2 % [1 / 24], all respectively. Overall, as found with contact plates used to test garments worn by operators having working in a Grade A / B clean room (Figure 5 – 4a) at all sites a lower percentage of plates used to test the surface of garments worn by male operators displayed no growth compared to those used to test the same sites in garments worn by female operators working under the same conditions. However, there was shown to be no

significant differences observed between gender and the percentage of contact plates displaying either low, moderate or high levels of growth at each of the sites tested.

To further investigate the bacterial bioburden of clean room operators' garments with respect to gender an additional comparison was drawn between suit site and the levels of growth (discussed above) displayed on contact plates for each of the genders, under each working condition – ~ 30 minutes in a Grade A / B clean room or ~ 60 minutes in a Grade C clean room. A comparison of garment site against the percentage of contact plates displaying either 0 (no growth), 1 - 9 (low level growth), 10 - 20 (moderate level growth) or > 20 (high level growth) cfu / plate for garments worn by male operators having worked in a Grade A / B clean room for ~ 30 minutes is shown in Figure 5 – 5a (a) and in a Grade C clean room for ~ 60 minutes is shown in Figure 5 – 5a (b) and for female operators having worked in a Grade A / B clean room for ~ 30 minutes is shown in Figure 5 – 5b (a) and a Grade C clean room for ~ 60 minutes is shown in Figure 5 – 5b (b).

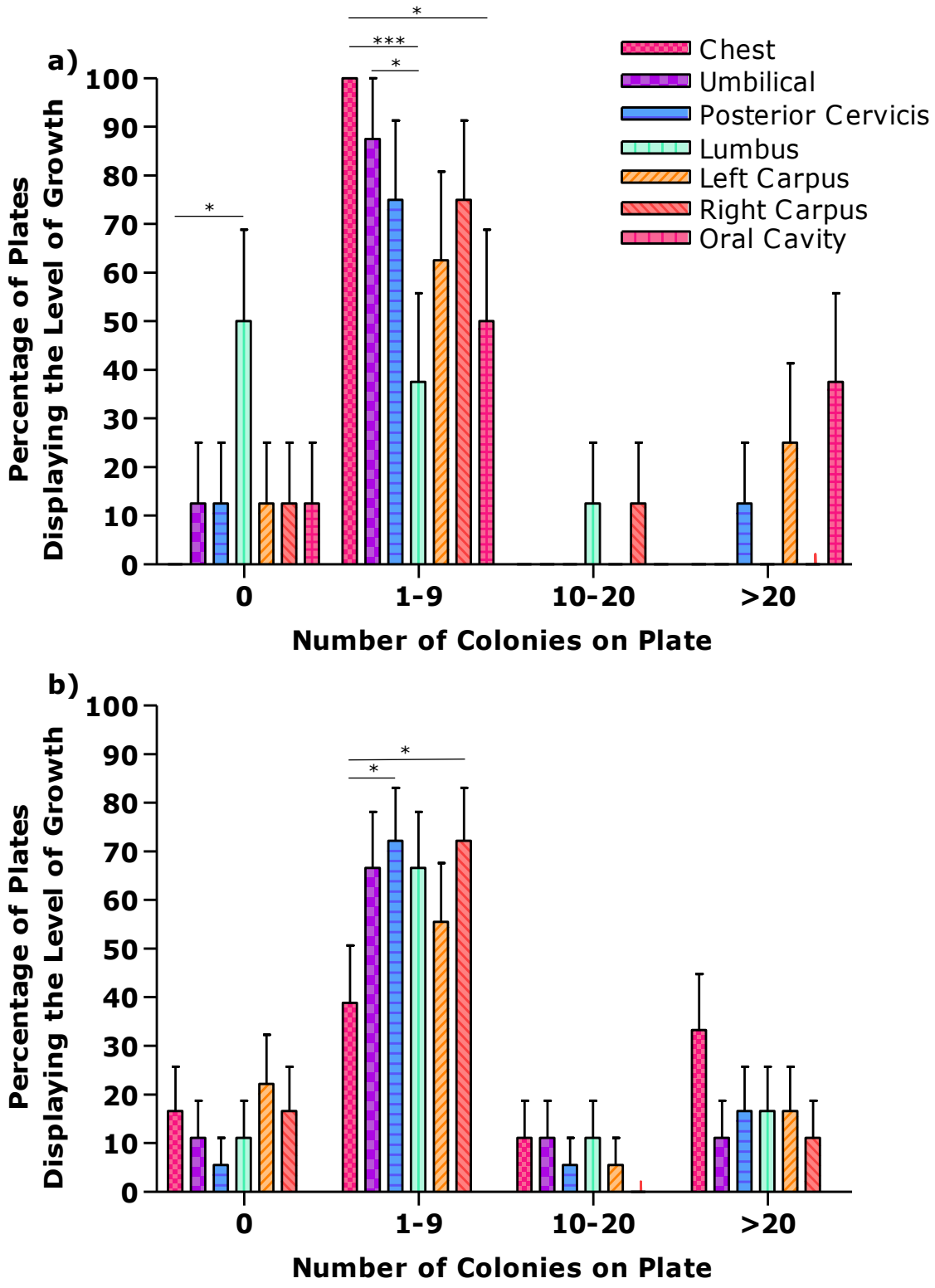


Figure 5 – 5a: A comparison of suit site against the percentage of contact plates displaying either 0 (no growth), 1 - 9 (low level growth), 10 - 20 (moderate level growth) or > 20 (high level growth) cfu / plate, used to test the surface of garments worn by male operators having worked in either (a) a Grade A / B clean room for ~ 30 minutes or (b) a Grade C clean room for ~ 60 minutes (* $p < 0.05$; *** $p < 0.001$).

As shown in Figure 5 – 5a (a), a higher percentage of plates used to test the lumbus region of suits worn by male operators working in a Grade A / B clean room displayed no growth (50 % [4 / 8]) compared to the percentage of those displaying growth, used to test all other sites. This was found to be significantly higher than those used to test the chest (0 % [0 / 8]) (* $p < 0.05$). Additionally, a higher percentage plates used to test the chest region displayed low level growth than the other sites tested, this was found to be significantly more than those used to test the oral cavity (100 % [8 / 8] vs. 50 % [4 / 8]) (* $p < 0.05$) and the lumbus (100 % [8 / 8]) vs. 37.5 % [3 / 8]) (*** $p < 0.001$). A significantly higher percentage of plates used to test the umbilicus region also displayed low level growth than those used to test the lumbus (87.5 % [7 / 8] vs. 37.5 % [3 / 8]) (* $p < 0.05$). A higher percentage of plates used to test the oral cavity over the hood of male operators working in a Grade A / B clean room also displayed high levels of growth compared to the other sites tested however, this was not found to be significant.

There was found to be no statistical significance shown between suit site and the percentage of contact plates displaying either no growth, moderate level growth or high level growth, used to test the surface of suits worn by male operators working in a Grade C clean room (Figure 5 – 5a (b)). However, a higher percentage of plates used to test the chest region of suits displayed high levels of growth compared to the percentage displaying this level of growth used to test the other sites. Additionally, a higher percentage of contact plates used to test right carpus and posterior cervicis displaying low level growth compared to the percentage displaying this level of growth those used to test the chest (72.2 % [13 / 18] vs. 38.7 % [7 / 18] (* $p < 0.05$).

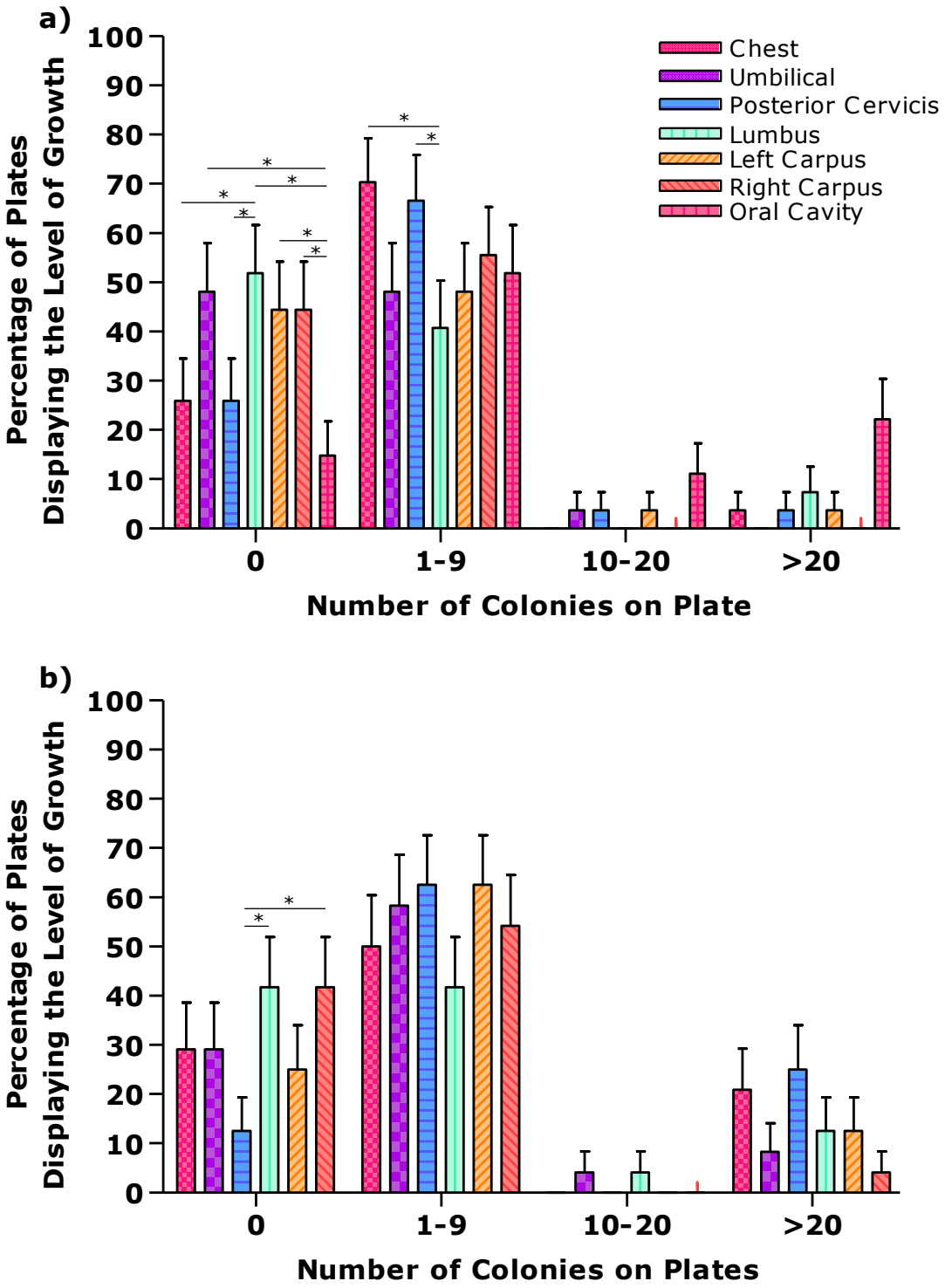


Figure 5 – 5b: A comparison of suit site the percentage of contact plates displaying either 0 (no growth), 1 - 9 (low level growth), 10 - 20 (moderate level growth) or > 20 (high level growth) cfu / plate, used to test the surface of garments worn by female operators having worked in either (a) a Grade A / B clean room for ~ 30 minutes or (b) a Grade C clean room for ~ 60 minutes (* $p < 0.05$).

As shown in Figure 5 – 5b (a), a significantly lower percentage of plates used to test the oral cavity of hoods worn by female operators working in a Grade A / B clean room displayed no growth compared to those used to test the right and left carpus (both 14.8 % [4 / 27] vs. 44.4 % [12 / 27] (both * $p < 0.05$), umbilical (14.8 % [4 / 27] vs. 48.15 [13 / 27]) and lumbus (14.8 % [4 / 27] vs. 51.9 % [14 / 27]) (both ** $p < 0.01$). Furthermore, a significantly higher percentage of plates used to test the lumbus region displayed no growth compared to those used to test the chest and posterior cervicis (both 51.9 % [14 / 27] vs. 25.9 % [7 / 27]) (both * $p < 0.05$). In addition, a significantly higher percentage of contact plates used to test the chest (70.4 % [19 / 27]) and posterior cervicis (66. 7 % [18 / 27]) displayed low level growth compared to the other sites tested, this was significantly different between plates used to test these sites and those used to test the lumbus (40.7 % [11 / 27]) (both * $p < 0.05$). A higher percentage of plates used to test the oral cavity over the hood worn by female operators working in a Grade A / B clean room displayed growth compared to the other sites tested however, this was not found to be statistically significant.

As shown in Figure 5 – 5b (b), a lower percentage of plates used to test the posterior cervicis region (12.5 % [3 / 24]) of suits worn by female operators working in a Grade C clean room for ~ 60 minutes displayed growth compared to those used to test the other sites. This was found to be significantly lower than the percentage used to test the lumbus and right carpus region of suits (both 41.2 % [10 / 24]) (both * $p < 0.05$). This is due to a higher percentage of plates used to test the posterior cervicis displaying low and high level growth compared to those used to test the other sites, however, this was not found to be statistically significant. There was also found to be no significant difference between suit site and the percentage of plates displaying either low, moderate or high levels of growth.

5.5 Discussion

In order to protect the clean room environment and ultimately product from human associated microbial contamination, the bacterial bioburden of the surface of clean room operators' garments should remain low or at zero during their working period within the clean room environment (Rhodes 2006). However, as found during Chapter 2 of our study, the surface of clean room garments may be contaminated

with bacteria following laundering, and as shown during Chapter 3 of our study, the levels of such bacterial contamination can increase as a result of hand borne transfer during the donning process. In this part of our study, using the direct agar contact method developed in Chapter 2, bacteria were recovered from the surface of clean room garments worn by both male and female operators following their working in either a Grade A / B clean room for approximately ~ 30 minutes or a Grade C clean room for ~ 60 minutes (Figure 5 – 1). This finding suggests that microorganisms attached to particles will permeate every day and specialist clean room clothing, contaminating the outer surface of clean room garments whilst operators work. This theory is also supported by Grangè *et al.* (2010), who also recovered skin commensal bacteria from the surface of clean room operators' garments following their wear within the clean room environment, using a direct agar contact method similar to that employed during our study. This particulate and microbial migration through the garments is thought to be due to the abrasiveness of the clothing against the skin, as well as operator movement - detaching squames from the skin and aiding their transportation from the garment into the airflow (Clark and de Calcina - Goff 2009). The results of our study help reiterate the suggestion that although specialist clean room garments will reduce the number of airborne particles and their associated microorganisms entering into the clean room environment, these garments will not retain all source of human detriment, as found by Whyte and Hejab (2007) and Ramstorp *et al.* (2005). Whyte and Hejab (2007), assessing the dispersion of MCPs into the air through both every day and clean room garments, found that the addition of clean room garments reduced the dispersion of MCPs from approximately 2400 per minute to 177 particles per minute (Whyte and Hejab 2007). However, Ramstorp *et al.* (2005) found that such a reduction in airborne particles by wearing cleanroom garments was dependent upon operator activity, with the efficiency of such garments improving only as the operator's movement increased. However, it is important to note that only one operator was investigated during this previous study (Ramstorp *et al.* 2005). The ability of microorganisms to permeate clean room clothing fabric was also found in Chapter 2 of this current study. During this earlier stage of our study we showed that *S. aureus* NCTC 6571, inoculated onto the topside of antistatic carbon filament polyester fabric, permeated the material and was recovered from its underside. However, it is important to remember that during Chapter 2 of our study the microorganism (*S. aureus* NCTC 6571) was in

its unicellular form (~ 0.8 - 1 μm), and therefore bacterial migration during this earlier chapter does not consider the bacteria's transportation mode - skin squames - and their size, which may reduce the rate of bacterial migration through the fabric's pores. However, the movement of microorganisms through clean room garments as found in this part of our current study is supported by various previous studies each of which have identified predominant clean room isolates as species of *Staphylococcus* and *Micrococcus* (Sandle 2011b; Martín *et al.* 2012; Park *et al.* 2013; Moissi – Eichinger *et al.* 2015), implying operators are the primary source of cleanroom contamination. This is despite operators wearing full clean room attire (Sandle 2011b). This is further supported by the identification of skin commensal bacteria on the surface of cleanroom garments worn by operators working in the clean room environment (Grangè *et al.* 2010). The findings of our study, in conjunction with those of Grangè *et al.* (2010), should be of concern to the clean room industry because as earlier discussed these textiles may act as a transmission mode for microorganisms following their acquisition. Furthermore, such skin host organisms which contaminate the outer surface of clean room operators' garments as they work may be to the detriment of the clean room environment and its product sterility. In a bid to establish the identification and therefore source of isolates recovered from the surface of clean room garments during this stage of our study a representative selection of these isolates underwent genotypic 16S rRNA gene sequencing. These results will be presented and discussed in Chapter 6.

The main aim of this part of our study was to assess the role that operator gender plays upon the resultant bacterial bioburden of garments which have been worn for a working duration in a clean room environment – an area which severely lacks in published literature. The results of our study show that a significantly higher percentage of contact plates used to test the surface of garments worn by male operators working in either a Grade A / B or Grade C clean room displayed growth compared to the percentage of those displaying growth, used to test female operators working under the same condition (* $p < 0.05$) (Figures 5 – 1). In addition, on evaluation of the levels of growth displayed on these contact plates, there was found to be a higher percentage of plates used to test male operators' garments displaying low, moderate and high levels of growth following incubation

compared to the percentage of plates displaying the same levels of growth used to test female operators' garments worn under the same conditions (Figure 5 – 2). In particular, a significantly higher percentage of plates used to test the garments of male operators working in a Grade A / B clean room displayed low levels growth compared to the percentage of plates displaying the same level of growth used to test female operators working under the same condition (* $p < 0.05$) (Figure 5 – 2 (a)). In addition, a higher percentage of plates used to test clean room garments worn by males operators displayed growth at all body sites tested when compared to those used to test the same sites of garments worn by female operators under the same conditions (Figure 5 – 3). This was not found to be significant, however, this lack of significance is thought to be due to the high percentage of plates displaying growth at each of the sites tested, for each gender. An additional comparison was drawn between the levels of growth displayed on these plates and gender. A significantly higher percentage of plates used to test the umbilicus region of garments worn by males operators having worked in a Grade A / B clean room displayed low levels growth compared to those used to test the same region of garments worn by female operators having worked under the same conditions (* $p = 0.05$) (Figure 5 – 4a (b)). However, the percentage of plates displaying either low, moderate or high levels of growth following incubation presented no correlation between levels of growth and gender for each site tested under each working condition (Figures 5 – 4a and 5 – 4b). This could however be due to the small pool number of male operators assessed compared to the number of female operators tested - one of the biggest limitations of this part of our study. However, this is a factor which could not be controlled due to the overall pool of volunteers being female heavy. Ideally, this study would be replicated with a higher number of volunteers which are equal in gender numbers. Furthermore, it is important to note, as previously discussed, this study was undertaken with, although supervised, novice operators, which may account for the high percentage of plates displaying growth at each of the sites tested.

Despite this, the results of our study show that gender plays a significant role in the quantity of bacteria dispersed by clean room operators through and onto their garments and subsequently into the clean room environment. Our findings suggest that male clean room operators will disperse more microorganisms than their female counterparts under the same working conditions, as well as substantiating

that the outer surface of garments worn by male operators are more highly contaminated than those worn by their female colleagues. As previously discussed, differences in the microbial and / or particulate dispersion between genders is an issue which has previously been investigated (Mitchell *et al.* 1978; Noble and Davies 1965; Noble *et al.* 1976; Whyte and Hejab 2007). These previous studies support the findings of our current study by all showing males disperse more microorganisms into the environment than females. However, these previous studies investigated the dispersion of skin particles and / or microorganisms from individuals into the air and not onto clean room garments. Therefore, these cannot be directly compared to our study. Furthermore, the results of our study cannot be directly compared to any previous studies as these do not assess the bacterial bioburden of clean room garments with respect to gender. Overall, on review of the literature the exact explanation for any differences between the particulate and bacterial dispersion numbers between genders remains uncertain. Increased bacterial dispersion by males may be due to gender linked physiological skin differences as previously discussed in Section 5.1.1. In addition, Noble *et al.* (1976) suggests that greater microbial dispersion by males may simply be due to the increased concentration of bacteria on male skin compared to numbers on the female skin. Greater dispersion may be due to males having slightly smaller skin scales than females (Noble *et al.* 1976) and therefore more of these scales would be able to evade the pores of clean room garments. However, Whyte and Hejab (2007) could not find a difference in the size of skin cells between genders. Greater dispersion by males may simply be due to males dispersing a higher quantity of skin squames than their female counterparts (Noble *et al.* 1976) or due to an increase in the surface area of their skin, as previously discussed. Males are generally larger than females and have a larger proportionate surface area of skin. So equal shedding rate would logically result in males releasing more squames, with proportionally more microorganisms as a result, however, Whyte and Hejab (2007) also failed to find a relationship between weight and skin area and particle dispersion rates.

The results of our study suggest that consideration should be given to the arrangement of clothing worn between the sexes in a bid to reduce any microbial and particle dispersion rates between male and female operators. In our study it may be important to consider that the nature of everyday clothing worn between

the sexes may account for the greater dispersal of microorganisms from the skin of males. It was suggested by Noble *et al.* (1976), that the difference between microbial dispersion of males and females may be due to the types of clothing worn varying between genders. However, this previous study was carried out over 40 years ago, when greater differentiation existed between garments worn by each of the genders. Furthermore, it is important to note that the arrangement and the number of layers of everyday clothing worn under the clean room clothing in this current study was not a constant factor and may have skewed the results obtained. Unfortunately, this is a limitation of the current study could not be controlled due to the study being undertaken by student volunteers, working in the clean room under specific circumstances. However, in general, to reduce desquamation from operators, due to abrasion of everyday clothing, this could be substituted for either specialist clean room underwear or non – linting polyester surgical scrubs. In fact, surgical scrubs have been shown to reduce microbial dispersion of individuals by 75 %, with the addition of specialist clean room garments increasing this to 80 % (Ljungqvist and Reinmüller 2003). In fact, there are a number of ways in which the bacterial dispersion of operators may be reduced, not only by male operators but by female operators. Movement of individuals has been shown to cause billowing or pumping of clothing (Clark and de Calcina - Goff 2009), as a result particles will travel up and down the garments in the direction of both the head and feet (Eudy 2003), exiting the garment within the airstream (Clark and de Calcina - Goff 2009). Contamination of clean room garments caused by this pump effect may be reduced by operators wearing clean room suits which are elasticated or tight at the wrist, neck and ankles. In addition, operators could don sterile protective arm sleeves. Mitchell *et al.* (1978) found that the addition of a rubber band over loose arm sleeves reduced bacterial dispersion by 35 %, whilst Whyte *et al.* (1976) found wearing a suit with tight ankle fittings significantly reduced the dispersion of bacteria into the environment. As found by Noble *et al.* (1976), the overall release of microorganisms from both males and females is greater below the waist. In fact, 80 % of bacterial particles have been found to be released from this lower area (Whyte *et al.* 1976). This suggests a limitation of the current study, as garments worn by clean room operators were only tested above the waist. However, future research could be undertaken to investigate the bacterial bioburden of the surface of clean room garments worn by operators below the waist. Furthermore, this would also allow us to draw comparison between the

bacterial bioburden of clean room garments with respect to the upper and lower regions of the body. It has previously been demonstrated that garments constructed of a tighter woven, more occlusive fabric, will retain larger particles, reducing the dispersion of particles and / or microorganisms into the air (Whyte *et al.* 1976; Whyte and Bailey 1985; Whyte and Bailey 1989). In fact, bacterial retention could be improved overall by both genders wearing clean room clothing which is constructed of impermeable fabric. Studies have shown that donning complete suits or gowns constructed of such non - woven fabric, instead of conventional loose weave clothing, can reduce bacterial dispersion of males by approximately 60 % (Mitchell *et al.* 1978). However, although fewer microorganisms will penetrate such impermeable fabric, or those with an additional layer, air permeability between the skin and garment may be significantly reduced (Laing 2008). Over time wearing a suit that is not well ventilated significantly increases the body temperature and stress of the operator (Hao *et al.* 2004). This may make the garment uncomfortable to wear. One previous study found that wearing an additional non - woven layer could reduce the permeability of air between the skin and environment by approximately 50 %. Additional layers were found to reduce air permeability further (Militky and Havrdova 2001). Bacterial dispersion from operators may also be reduced by operators wearing garments treated with an antimicrobial finish. As discussed in Chapter 3, such finishes have been shown to reduce the number of microorganisms on fabrics (Bajpai *et al.* 2011) and therefore may reduce their release into the environment (Hobson 2007). However, it is also important to note that such a fabric finish may create a problem as a result of bacterial mutations (McIlvaine and Tessien 2006).

In this part of our study a comparison was also drawn between the levels of growth displayed on contact plates and each garment site tested in garments worn by each gender, under each working condition (Figure 5 – 5a and Figure 5 - 5b). A higher percentage of plates used to test the chest region of suits worn by male and female operators working in a Grade A / B clean room displayed low level growth compared to the other sites tested, as well as a higher percentage of plates used to test the umbilicus of male operators and the posterior cervicis of female operators working under the same condition displaying low levels of growth. Additionally, a higher percentage of plates used to test the oral cavity of hoods working in a Grade A / B clean room displayed high levels of growth compared to

the percentage of plates used to test the other sites (Figure 5 – 5a (a) and Figure 5 – 5b (a)). These findings are similar to those found in Chapter 3 and 4 of our study – with a higher percentage of plates used to test the chest and umbilicus of suits during the laundering comparison study displaying growth (Chapter 3) and a higher percentage of plates used to test the chest and oral cavity regions of suits, donned with no gloves, non – sterile gloves or sterile clean room gloves, displaying growth (Chapter 4), compared to the other sites tested. As previously discussed, the high percentage of plates displaying growth used to test the oral cavity region of the hood are thought to be due to the sheer volume of bacteria originating from the oral cavity (Dewhirst *et al.* 2010) and the higher percentage used to test the chest displaying growth being the result of a combination of hand – borne bacterial transfer during the donning process, the area being in direct contact with the skin, as well as from contamination arising from the oral cavity. Interestingly, as shown in Figures 5 – 5a (b) and 5 – 5b (b), the omission of a hood (operators working in a Grade C clean room) saw a reduction in the percentage of plates used to test the chest region of suits worn by both male and female operators displaying low levels of growth but an increase in the percentage of plates displaying high level of growth. This was compared to the percentage of plates used to test the same region of operators working in a Grade A / B clean room (wearing a hood) (Figures 5 – 5a (a) and 5 – 5b (a)). Therefore, the results of our study suggest that the addition of a hood could reduce contamination not only entering the environment from the oral cavity but the presence of a hood provides an additional layer of fabric between the chest region and clean room suit. The hood is also thought to reduce the dispersion of squames from the neck of the clean room suit via the pump effect. Although face masks have been shown to reduce the dispersion of airborne bacteria (Bischoff *et al.* 2007), the effectiveness of clean room head coverings is an area which lacks in published literature. This finding may be monumental for the clean room industry. Therefore, we suggest that a hood donned in addition to a clean room suit can reduce the bacterial bioburden of clean room garments. In addition, a higher percentage of plates used to test the posterior cervicis region of suits worn by both male and female operators working in a grade C clean room (without a hood) displayed high levels of growth compared to the percentage displaying the same level of growth, used to test the same region of garments worn by operators working in a Grade A / B clean room (with a hood) (Figures 5 – 5 (a) and 5 – 5 (b)). This increase in the percentage of plates

displaying growth is thought to be due to contamination of the area by the hair. Therefore, a hood is thought to help retain bacterial from the scalp, reported to be host to a million microorganisms per cubic centimetre, the forehead, host to 100 – 1000 microorganisms over the same area and saliva and nasal fluids containing 10 million microorganisms per gram (Sandle 2014). However, it is also important to remember that this study was carried out using two different variables – ~ 30 minutes working in a Grade A / B clean room or ~ 60 minutes working in a Grade C clean room. Therefore, the increase in levels of growth may be due to an increase time in wearing the garments rather than the absence of a hood. As shown by Grangè *et al.* (2010), the levels of bacteria on the surface of operators garments can increase over time spent working in the clean room environment. Employing two different time points was found to be one of the limitations of this study – a factor which unfortunately could not be controlled due to the timetabled working sessions of the volunteers taking part. Ideally, in order to determine whether the addition of a hood or the time factor ultimately effected the levels of bacteria recovered from garments this part of the research should be repeated with the operator working in the same grade of clean room, for the same period of time, both with and without wearing a hood. To the author's knowledge there are only two previous studies which examine the dispersal of microorganisms from the body with respect to body site (Noble *et al.* 1976; Whyte *et al.* 1976). Furthermore, only one of these studies also considered this topic with respect to gender (Noble *et al.* 1976). Noble *et al.* (1976) found that microbial dispersion from individuals at various body sites into the air varies according to gender, with males releasing more microorganisms from the thighs and abdomen and females releasing more from the shin area, however, as previously discussed this study is over 40 years old and also discusses bacterial release with regard to clothing type, a factor which is thought to be less apparent nowadays.

The majority of contact plates used in this current study displayed growth well in excess of 10 cfu per contact plate action limit recommended by the NHS Pharmaceutical Quality Assurance Committee (Rhodes 2006). It is also important to consider that as earlier discussed and previously shown in Chapter 2, contact plates were only found to less than 3 % of bacteria inoculated into antistatic carbon filament polyester fabric squares. Therefore, bacterial numbers on garments tested in that part of the current study may be significantly underestimated. The

levels of growth recovered on plates in this current study are also considerably higher than those of an unpublished study by Tayside Pharmaceuticals (2010). However, it is important to consider that this current study was undertaken by inexperienced clean room operators (although under personal supervision of a trained operator), lacking awareness and skill of not only the donning process but correct clean room working discipline. There were also a group of between 7 – 9 operators present in the clean room at one given time. Each of which may have contributed to increased levels of bacteria on garments. In addition, it is also important to consider that microorganisms on clean room garments may be a direct result of microorganisms being present on the reusable clean room suits following laundering, as found in Chapter 3, or as a direct results of contaminating of garments via the hand borne route during dressing, as found in Chapter 4. Ideally, this part of our study would be replicated using experienced clean room operators, using both an equal number of male and female subjects, working for equal time periods under both working conditions.

5.6 Conclusion

In this chapter the bacterial bioburden of the surface of male and female clean room operators' garments following a working period in a clean room environment was assessed using the direct agar contact method developed in Chapter 2. The results of our study show that operator associated bacteria will penetrate every day and specialist clean room clothing, contaminating the exterior surface of clean room garments whilst operators work. A higher percentage of contact plates used to test the surface of garments worn by male operators displayed low, moderate and high levels of growth at all sites tested compared to those used to test the same regions of clean room garments worn by female operators working under the same conditions. The donning of a clean room hood was shown to reduce the levels of bacteria at both the chest and posterior cervicis regions of suits worn by both male and female operators. However, further investigation should be undertake to determine whether this was a direct result of time spent working in the clean room.

Chapter 6:

Qualitative Identification of a Representative Selection of Bacterial Isolates Recovered from the Surface of Reusable Antistatic Carbon Filament Polyester Clean Room Garments during the Laundering and Gender Comparison Studies

6.1 Introduction

Contamination of a pharmaceutical clean room or product by a microbial contaminant can lead to a facility being temporarily shut down, having an impact not only upon its output but also its expenditure (Champagne 2008). Furthermore, as previously discussed, in a worst case scenario, microbial contamination of a pharmaceutical product can lead to patient disease and ultimately death (MDPH 2012; Pharmacy Practice News 2013; Public Health England 2014; Sprinks 2014; Torjesen 2014). The number and diversity of microorganisms which are present in a clean room environment can be studied in order to establish effective microbial control (Sandle 2011b), allowing a facility to address cases of contamination before they occur (Sheraba *et al.* 2010). This knowledge can prove invaluable to a facility, helping establish the normal micro flora within the room, as well as identifying any predominant isolates and their sources (Akers 1997). The origin of a microbial clean room contaminant can be determined by classifying the microorganism using various phenotypic or genotypic methods of identification (Sandle 2011b). Examples of clean room contaminates and their sources include Gram - positive cocci which are associated with the human skin, Gram - positive rods which inhabit the environment and may be transferred into the clean room via materials and Gram - negative rods which are found in water (Sandle 2011b; Sandle 2017). In this part of the study quantitative microbial identification was undertaken on a representative sample of the bacterial isolates recovered from the surface of reusable clean room garments during the laundering (Chapter 4) and gender comparison (Chapter 5) studies, in a bid to determine their source.

6.1.1 Microbial Identification of Unknown Isolates

Microbial identification tests are undertaken to assign unknown bacteria, parasites and fungi to their genus and sometimes species level under their Latin or Greek names (Janda and Abbott 2002). Early 20th Century identification methods included physiological and biochemical testing, as well as bacterial diversity assessments; permitting the classification of bacterial groups using a systematic approach. In 1957 numerical taxonomy methods using computers were first introduced, which later led to the first comprehensive phenotypic methods of identification (Janda and Abbott 2002). Phenotypic means of identification are based upon a microorganism's morphological and physiological behaviours

(Sheraba *et al.* 2010). This method of identification is well recognised (Champagne 2008) and is reported to be the most commonly employed method of microbial identification in pharmaceutical associated laboratories (Sandle 2011a). In contrast genetic techniques classify isolates using the organism's nucleotides and genes (Sandle 2011b). Such methods of identification are considered more attractive (Boye *et al.* 1999), objective (Petti *et al.* 2005) and accurate (Champagne 2008; Woo *et al.* 2008) than traditional phenotypic identification methods. Furthermore, such genotypic methods tend to be quicker than phenotypic analysis (Champagne 2008) and allow for a greater degree of strain distinction (Emerson *et al.* 2008).

6.1.1.1 Phenotypic Identification

Traditional phenotypic methods involve identifying microorganisms based upon their phenotypic observable characteristics, derived using a number of assays (Champagne 2008). Some examples of phenotypic tests used to identify bacteria and based on colony morphology, growth and their biochemical reactions are shown in Table 6 – 1.

Table 6 – 1: Some examples of microbial characteristics and their associated phenotypic identification tests.

Characteristic		
Colony Morphology	**Colony Growth**	**Biochemical Reactions**
Form, Elevation, Margin	Aerobic and Anaerobic Growth	Acid Fasting
Gram – Stain	Antibiotic Resistance	Oxidation
Flagella Arrangement	Incubation Temperature	Catalase
Cell Shape and Size	Nutritional Requirements	Oxidation / Fermentation

The first stage in phenotypic isolate identification usually begins with determining the Gram – stain of the isolate, furthermore, the morphological characteristics of the colony should also be noted (Sandle 2017). Further tests can be undertaken which observe growth characteristics and requirements, as well as enzymatic and metabolic activities (Sandle 2017). The numbers of phenotypic tests which are undertaken during identification are organisation and laboratory specific. For example, the Centers for Disease Control and Prevention (CDC) identify some Gram – negative bacteria using just less than 50 tests, whereas some laboratories rely on less criteria by using commercially available identification kits (Baron 1996). An example are analytical profile index (API®) strips - with the API® 20E identification product containing 20 tests (Biomérieux 2017). However, despite their popularity, phenotypic identification assays are subject to a number of limitations. The primary problem is that identification relies heavily upon the expression of cell metabolic activities, as well as the morphological features of the isolate (Sheraba *et al.* 2010). Furthermore, phenotypic tests are time consuming (Boye *et al.* 1999; Champagne 2008) and it can also prove difficult to interpret their subjective results (Stager and Davies 1992). Identification is also limited to the size of the phenotypic database, which is also predisposed towards microorganisms recovered in the clinical field rather than those in the pharmaceutical industry. However, it is important to note that the same problem may be encountered when using genotypic identification methods (Sandle 2011b). There are a number of advanced phenotypic techniques which have developed in recent years including mass spectroscopy (Sandle 2017). One of these techniques is MALDI - TOF MS (Matrix – Assisted Laser Desorption / Ionisation Mass Spectroscopy), a rapid, economical and sensitive method which is based upon identifying organisms from a database upon their generated peptide mass fingerprint (Singhal 2015). Another fast emerging method, hailed as the future of microbial identification, is Raman Spectroscopy. This method generates a spectroscopic fingerprint of an unknown microorganism which can quantitatively and qualitatively be used to determine its identification (Ashton *et al.* 2011).

6.1.1.2 Genotypic Identification

There are two genotypic identification methods available either fingerprint or sequence based analysis (Emerson *et al.* 2008). Fingerprint techniques are used to produce a number of DNA fragments of the microorganism's genome, separated

by size (Emerson *et al.* 2008). Alternatively, sequence based identification methods rely upon sequencing the whole genome (Donkor 2013), or a specific gene within the isolate (Emerson *et al.* 2008). In each case the fingerprint or sequence is then compared against a database of known organisms (Emerson *et al.* 2008).

6.1.1.2.1 16S Ribosomal RNA Gene Sequencing

16S rRNA gene sequencing is the most commonly applied genotypic method of identifying isolates (Janda and Abbott 2002; Wang *et al.* 2015). The 16S rRNA gene, present in all bacteria, is responsible for coding for a bacterial ribosome's small subunit (Wang *et al.* 2015). This highly conserved gene sequence consists of variable regions which allow discrimination between the genus and species of isolates (Harris and Hartley 2003). 16S rRNA gene sequencing has been widely used in a number of previous research studies to study the diversity of the molecular bacteria communities in spacecraft assembly clean rooms (Moissl *et al.* 2007; La Duc *et al.* 2009, Vaishampayan 2010), as well as to study the viability of organisms in such extreme environments (La Duc *et al.* 2007). A systematic diagram representing the process of identifying bacteria recovered from the skin using 16S ribosomal RNA gene sequencing is shown in Figure 6 – 1.

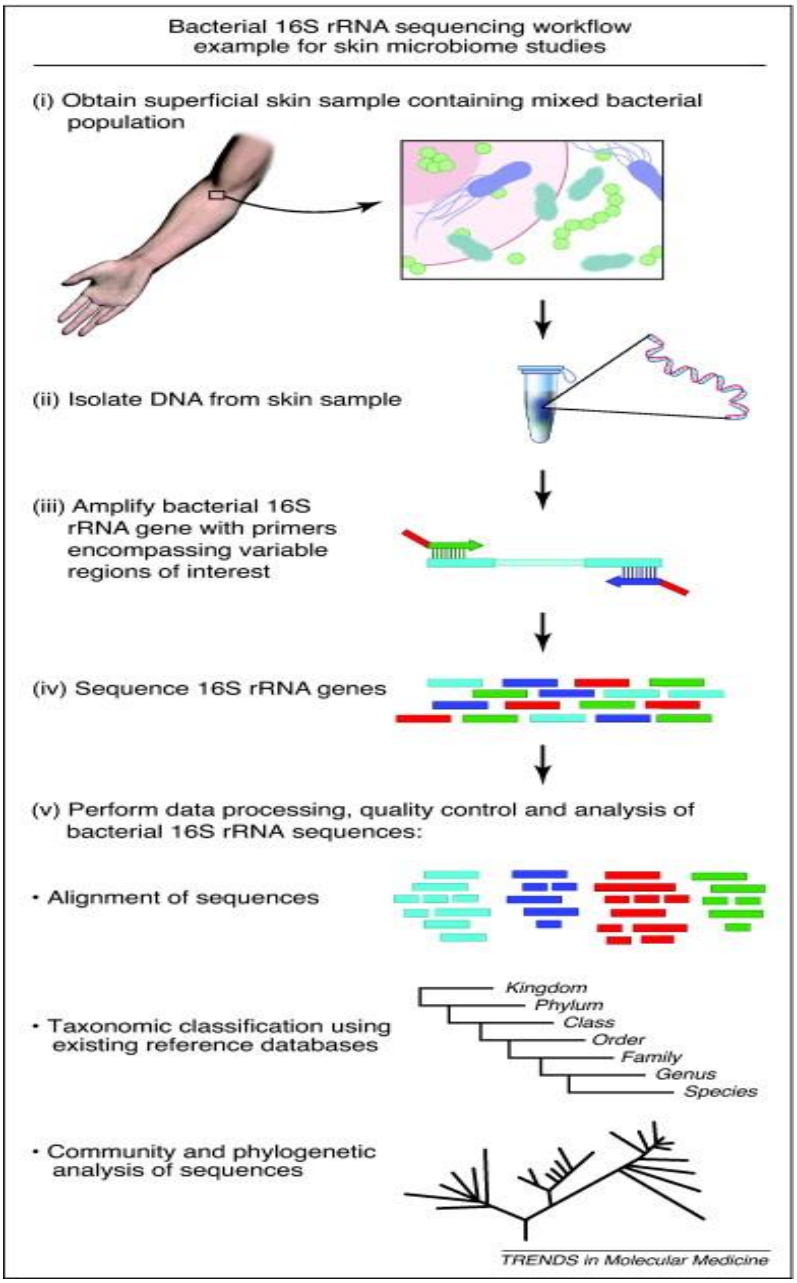


Figure 6 – 1: Schematic view of 16S ribosomal RNA gene-based bacterial sequencing workflow. The 16S ribosomal RNA gene is universal among prokaryotes and can be used for phylotyping of bacterial sequences. This diagram shows the process from collecting a skin sample to DNA sequencing and analysis used in many skin microbiome studies (Reprinted from: Trends in Molecular Medicine, 17 (6), Kong, H, Skin Microbiome: Genomics – based insights into the diversity and role of skin microbes, pp. 320 – 328, Copyright (2011), with permission from Elsevier).

6.1.2 Microbial Proportions within the Clean Room Environment

To date there are a limited number of published studies which assess the number and diversity of microorganisms which exist in the clean room environment. However, Martín *et al.* (2012) and Park *et al.* (2013), studying the microbial

diversity in pharmaceutical clean rooms, as well as Moissi – Eichinger *et al.* (2015) studying the same in a spacecraft associated clean room, found the predominant genera to be species of *Staphylococcus* and *Micrococcus* species. In fact, Martín *et al*. (2012), found the most frequently observed species to be *S. epidermidis*, *S. hominis* and *M. luteus*, all skin commensal bacteria, suggesting operators are the primary source of clean room contamination, as previously discussed. Furthermore, a nine year review of studies assessing the diversity of clean room isolates found that over 50 % of these were species of *Micrococcus* and *Staphylococcus* (49 % of isolates recovered from Grade A / B clean room and 51 % of those recovered from Grade C / D clean rooms). In addition, 10 - 13 % of isolates were identified as environmental *Bacillus* species (Sandle 2011b). A low percentage of Gram – negative bacteria were also identified, thought to have been transferred into the clean room environment via poor operator hygiene (Sandle 2011b) (Table 6 – 2). To the author's knowledge there is only one study which has identified unknown isolates recovered from the surface of clean room operators garments following wear (Grangè *et al.* 2010), however, this previous study does not state by which method the isolates were identified. Despite this, the predominant specie on the garments were identified as skin commensal *S. epidermidis*, as well as *Micrococcus* species (Grangè *et al.* 2010), thus confirming, as previously thought, that MCPs will permeate clean room garments contaminating their outer surface as operators work.

6.2 Aims & Objectives

The aim of this stage of the project was to seek the identification of a representative selection of bacterial isolates recovered from the surface of clean room garments during the laundering and gender comparison studies. The specific objectives were:

- To compare the identification efficiencies of traditional phenotypic identification tests and genotypic 16S rRNA gene sequencing.
- To seek qualitative identification of a representative selection of isolates recovered from the surface of clean room garments during the laundering and gender comparison studies using phenotypic and genotypic methods, and use this knowledge to help identify their source.

6.3 Materials & Methodology

In order to achieve the specified objectives the following materials and methods were used.

6.3.1 Phenotypic Testing

Phenotypic identification tests were undertaken on a representative selection of isolates recovered from the surface of clean room garments during the laundering comparison study. Twenty three colonies were selected for identification, these were all selected based on their morphology and represented the range of the colonies recovered during the study. Of these, 16 were recovered from the surface of suits laundered without sterilisation and 7 were recovered from the surface of suits laundered with additional gamma sterilisation. Phenotypic tests were selected from Cowan and Steel's Manual for the Identification of Medical Bacteria (Barrow and Feltham 1993), in order to achieve isolate identification to the genus level. During all assays relevant control organisms were simultaneously analysed to give a known positive or negative response.

6.3.1.1 Preparation of Agar Plates

Agar plates were prepared and stored as described previously in Chapter 2 - Section 2.3.3. Master and working plates of each isolate were prepared as described in Chapter 2 - Section 2.3.5. A fresh working plate was prepared weekly during the identification study.

6.3.1.2 Preparation of OF Agar Test Tubes

Oxidation fermentation (OF) test nutrient agar was prepared according to the manufacturer's instructions by dissolving 9.88 g of OF Test Nutrient Agar powder (Oxoid Ltd, Basingstoke, UK) in 1 L of water. This was autoclaved at 121 ^{o}C for 15 minutes (Astell Scientific Ltd, Sidcup, UK) then left to cool to 50 ^{o}C. Once cooled, 100 mL of sterile filtered 10 % D - (+) glucose (Sigma Aldrich, Missouri, USA) was added to the agar. Within a Laminar airflow cabinet (Hepaire Products Corporation, Kanata, Canada) 5 mL of sterile molten agar was dispensed into sterile test tubes and left to set. Test tubes were covered to ensure sterility and then stored at 4 ^{o}C prior to use.

6.3.1.3 Gram Staining Test

A glass microscope slide (Fisher Scientific Ltd, Loughborough, UK) was dipped into ethanol (Fisher Scientific Ltd, Loughborough, UK), drained and passed through a Bunsen flame to burn off any excess alcohol before being left to cool. A 0.5 cm diameter circle was marked onto both the top and corresponding underside of the slide. Using a loop, a drop of Ringer's solution (Sigma Aldrich, Missouri, USA) was transferred into the centre of the circle on the upper surface of the slide. Using a flamed, cooled loop, a small amount of colony from the working plate was obtained by touching its edge followed by transfer to the drop of Ringer's solution. The loop was gently rubbed into the liquid and smeared out over the circle. Slides were left to air dry. To heat fix the film the slide was briefly passed horizontally through the hottest part of the Bunsen flame, smear facing upward, using sterile forceps 3 times. The film was flooded with 1 % aqueous crystal violet (Sigma Aldrich, Missouri, USA) and left for 1 minute. The film was then covered in Gram's iodine (Sigma Aldrich, Missouri, USA) and left for a further 1 minute, before being rinsed off with water. Holding the slide at an angle it was flooded with ethanol for 10 seconds, then water. The film was then covered with Gram's Safranin Solution (Sigma Aldrich, Missouri, USA) and left for 1 minute. The slide was rinsed with water and left to air dry. Slides were observed using bright field microscopy (Baden – Wurttemberg, Germany) at x 1000 magnification with oil immersion (Pro – Lab Diagnostics, Wirral, UK). An observation and record was made of Gram stain reaction, cell shape, size and arrangement.

6.3.1.4 Oxidase Test

Using sterile forceps an oxidase disc (Sigma Aldrich, Missouri, USA) was transferred into a sterile empty petri dish and the lid replaced. The lid was removed and using a sterile inoculating loop and small amount of organism from a well isolated colony was spread onto the oxidase disc. The lid was replaced and the reaction left for 2 minutes. A positive reaction was observed if the disc turned a deep blue colour. No colour change indicated a negative reaction.

6.3.1.5 Catalase Test

A sterile microscope slide (Fisher Scientific, Loughborough, UK) was placed inside a sterile Petri dish, the lid was removed and using a sterile inoculating loop and small amount of organism from a well isolated colony was transferred onto the

microscope slide. Using a dropper, one drop of 3 % Hydrogen Peroxide (Fisher Scientific, Loughborough, UK) was transferred onto the microorganism, on the slide. The lid of the Petri dish was replaced and an observation was made for an immediate bubble formation. An immediate bubble formation indicated a positive results and the absence of a bubble formation indicated a negative result.

6.3.1.6 Aerobic & Anaerobic Growth Tests

A well isolated colony was streaked into the surface of a nutrient agar plate. The plate was inverted and incubated aerobically at 37 °C. For anaerobic growth plates were inverted and transferred into an anaerobic jar (Fisher Scientific, Loughborough, UK) along with a 2.5 L AnaeroGen™ sachet (Fisher Scientific, Loughborough, UK), this was then sealed and incubated under anaerobic conditions at 37 °C. An observation of growth was made at both 24 and 48 hours. Growth indicated a positive result whereas no growth indicated a negative result.

6.3.1.7 Glucose Oxidation / Fermentation Test

Two test tubes containing OF agar were inoculated with a pure colony of the isolate using a sterile loop. One of the tubes was overlaid with 1 cm of sterile mineral oil (Sigma Aldrich, Missouri, USA). The tubes were incubated at 37 °C and an inspection for colour change was made after 24 and 48 hours. A fermentative result was indicated by a yellow colour change in both tubes. An oxidative result was indicated by a yellow colour change in the open tube and a green colour change in the covered tube. A negative result is observed by a green colour change in both tubes.

6.3.2 Genotypic Testing

Genotypic testing was undertaken on 47 of the isolates recovered from the surface of clean room garments during the gender comparison study, as well as the same 23 isolates having previously undergone phenotypic testing, recovered from the surface of clean room suits during the laundering comparison study. All colonies were selected based on their morphology and represented the range of the colonies recovered from the suits following each of the laundering processes or worn by operators within the clean room.

6.3.2.1 Preparation of 0.1 x TE Buffer

One litre of 1 M Tris EDTA (TE) solution was prepared by dissolving 121.14 g of Tris base (Fisher Scientific, Loughborough, UK) in 1 L of deionised water. A 0.5 M EDTA solution was prepared by dissolving 18.6 g of EDTA (Fisher Scientific, Loughborough, UK) in 100 mL of deionised water. Both solutions were adjusted to a pH of 8.0. One litre of 1 x TE buffer was prepared by adding 10 mL of 1 M Tris solution with 2 mL of 0.5 M EDTA solution and making this up to 1 L with deionised water. One litre of 0.1 x TE buffer was prepared by diluting 100 mL of 1 x TE buffer in 900 mL of deionised water.

6.3.2.2 Preparation of 1 x TBE Buffer

One litre of 10 x Tris Borate EDTA (TBE) buffer was prepared by dissolving 242 g of Tris base, 123.4 g of boric acid (Fisher Scientific, Loughborough, UK) and 12.88 g of EDTA in deionised water. One litre of 1 x TBE buffer was prepared but diluting 100 mL of 10 x TBE buffer in 900 mL of deionised water.

6.3.2.3 Preparation of 10 mg / ml Lysozyme Buffer

A 10 mg / mL lysozyme solution was prepared by dissolving 10 mg of lysozyme powder (≥ 40,000 units protein / mg) (Sigma Aldrich, Missouri, USA) in 1 mL of 0.1 M TE Buffer.

6.3.2.4 Preparation of 10 % SDS Solution

A solution containing 10 % SDS was prepared by dissolving 1 g of SDS powder (Fisher Scientific, Loughborough, UK) in 10 mL of deionised water.

6.3.2.5 Preparation of Sterile Nutrient Broth

Sterile nutrient broth was prepared and sterilised as described in Chapter 2 Section 2.3.2.

6.3.2.6 Preparation of Overnight Culture

Overnight suspensions of each isolate were prepared as previously discussed in Chapter 2 - Section 2.3.6.

6.3.2.7 Bacterial Genomic DNA Extraction of Isolates

One millilitre of overnight culture was transferred into a sterile 1.5 mL micro centrifuge tube (Fisher Scientific, Loughborough, UK) and then centrifuged at 14,263 x g for 5 minutes in a Technico Maxi centrifuge (Fisher Ltd, Loughborough, UK). The supernatant was removed from the tube and the pellet re - suspended in 100 µL of 10 mg / mL lysozyme solution for 45 minutes at 37 ^{o}C. Following incubation, 1 µL of 20 mg / mL Proteinase K (Sigma Aldrich, Missouri, USA) and 1 µL of 10 % SDS solution were added to the tube and incubation continued for a further 30 minutes. At this stage 100 µL of 30 % (w / v) BT Chelex® 100 resin (BioRad, California, USA) was added to the tube and mixed using a vortex mixer (Fisher Scientific, Loughborough, UK). The sample was heated in a heating block set at 56 ^{0}C (Fisher Scientific, Loughborough, UK) for 30 minutes, before being incubated for a further 10 minutes in a boiling water bath (Grant Instruments, Cambridge, UK). The tube was then centrifuged for 5 minutes at 14,263 x g. The supernatant (~ 100 – 200 µL) was transferred into a new sterile 1.5 mL micro centrifuge tube – this contained the bacterial DNA. Following extraction, agarose gel electrophoresis using 10 µL of supernatant on a 1.5 % (w/v) agarose gel was performed to determine if DNA extraction was successful. The remainder of the sample was frozen at - 20 ^{o}C for future analysis.

6.3.2.8 Preparation of 1.5 % Agarose Gel

A 1.5 % agarose gel was prepared by adding 3 g of agarose powder (Bioline, London, UK) into 200 mL of 1 x TBE buffer in a conical flask; this was heated in the microwave at medium power for approximately 2 minutes, whilst mixing occasionally. This was left to cool and once touch warm 20 µL of GelRed™ (nucleic acid dye) (Sigma Aldrich, Missouri, USA) was added to the flask. The gel solution was poured into a sealed gel tray containing a 16 well comb. The gel was left to set for approximately 30 minutes and the seals removed. The reservoir was then filled with 1 x TBE buffer to the fill mark and the comb removed.

6.3.2.9 Agarose Gel Electrophoresis

Into a sterile 1.5 mL micro centrifuge tube, 2 µL of loading dye (Promega, Wisconsin, USA) was added to 10 µL of extracted bacterial DNA sample and mixed. This 12 µL of sample was loaded into a well (a separate well per sample), along with 5 µL of Hyperladder™ 1 kb (Promega, Wisconsin, USA) in another well. The

tank was set to run for 90 minutes at 110 volts. Following electrophoresis the gel was visualised and photographed under UV light using the Bench – top UV trans – illuminator and Photo DocIt™ imaging system (Ultra – Violet Products Limited, California, USA).

6.3.2.10 Preparation of Samples for PCR

16s rRNA forward and reverse primers (synthesised by Sigma Aldrich, Missouri, USA) were used to amplify a 320 bp hypervariable region at the 5‵ - end of the 16S rRNA gene in each of the isolates selected. These primer sequences have previously been shown to be successful in amplification of the 16S rRNA gene in over 100 bacterial isolates (Harris and Hartley 2003). Primers were stored at - 20 ^{o}C and thawed at room temperature prior to dilution. Primers were diluted to obtain a working molar concentration of 10 mM. Typically 10 µL of each primer was diluted with 90 µL of sterile Milli – Q water. The sequence and melting temperatures of the primers to achieve an anticipated PCR amplicon size of 320 bp can be seen in Table 6 – 2.

Table 6 – 2: 16S rRNA forward and reverse primer sequence and melting temperatures to achieve an anticipated PCR amplification size of 320 bp (Harris *et al.* 2002; Harris and Hartly 2003; Rogina *et al.* 2014).

Primer	Sequence	Melting Temperature Tm^{o} (^{o}C)
Forward 16S F	5′ – GCTCAGATTGAACGCTGG – 3′	62.3
Reverse 16S R	5′ – TACTGCTGCCTCCCGTA – 3′	60.6

For each sample a 50 µL volume of PCR mastermix was prepared by addition of 10 µL of 5 x buffer, 3 µL of 25 mM $MgCl_2$, 1 µL of 10 mM dNTPs (all Bioline, London, UK), 1 µL of each forward and reverse primer and 34 µL of Milli – Q water in to a sterile micro centrifuge tube. A PCR tube (Fisher Scientific, Loughborough, UK)

was then prepared which contained the 50 µL of PCR mastermix, 5 µL of extracted bacterial DNA and 1 µL of Taq polymerase (5 units / µL) (Bioline, London. UK).

6.3.2.11 PCR of Bacterial DNA

PCR was undertaken in a Jencons – PLS Techne TC – 312 thermo cycler (Jencons Scientific Ltd, Bedfordshire, UK). Initially, samples were denatured at 94 °C for 5 minutes. Samples were further denatured at 94 °C for 1 minute, this stage was then repeated along with annealing and elongation 30 times. Primer annealing was undertaken at 63 °C for 1 minute and elongation at 72 °C for 2 minutes. A final elongation stage of 10 minutes at 72 °C was undertaken. Following PCR the samples (10 µL) underwent gel electrophoresis on a 1.5 % (w/v) agarose gel to determine if amplification was successful, as described in Section 6.4.2.9.

6.3.2.12 PCR Purification

PCR samples were purified using the Wizard® SV Gel and PCR Clean Up System (Promega, Wisconsin, USA). This system can be used to purify DNA fragments from PCR amplifications and has been shown to efficiently purify 95 % of DNA fragments at 320 bp (Betz and Strader 2002). Into a sterile micro centrifuge tube 40 µL of PCR sample was added to 40 µL of membrane binding solution (4.5 M guanidine isothiocyanate and 0.5 M potassium acetate (pH 5.0)) and mixed. An SV mini column was inserted into a collection tube and the prepared PCR product and membrane binding solution mix transferred into the mini column. This was incubated for 1 minute at room temperature, before being centrifuged for a further 1 minute at 14,263 x g. The mini column was removed from the collection tube and the flow - through discarded. The mini column was re – inserted into the collection tube and washed using 700 µL of membrane wash solution (75 mL of 95 % ethanol added) (after ethanol addition – 10 mM potassium acetate (pH 5.0), 80 % ethanol and 16.7 µM EDTA (pH 8.0)) and centrifuging at 14,263 x g for 1 minute. The flow through was once again discarded and the mini column re – inserted back into the collection tube. This washing process was repeated with 500 µL of membrane binding solution and centrifuged for 5 minutes, the collection tube was emptied and the column re - centrifuged to allow evaporation of any residual ethanol. To elute the PCR product from the mini column this was placed into a sterile micro centrifuge tube and 50 µL of Nuclease Free Water added. This was incubated at room temperature for 1 minute and centrifuged at 14,263 x g for a

further minute. The cleaned up DNA product was stored at – 20 ^{o}C and the mini column was discarded.

6.3.2.13 Preparation of Samples for Sequencing

Following clean up, each PCR sample underwent gel electrophoresis on a 1.5 % (w/v) agarose gel (as described in 6.4.2.9) to determine the concentration of DNA within the sample. Each sample (containing 5 µL of PCR product and 2 µL of loading dye) was run against 5 µl and 10 µl samples of 1 kb Hyperladder™. The resultant gel was visualised under UV light and the concentration of DNA in the sample was estimated by comparing the intensity of the band against that of the Hyperladder 1 kb scale (Bioline 2016). The DNA concentration of each sample was adjusted to give between 6 - 20 ng in 30 µL of Milli – Q water (0.2 – 0.67 ng / µl). DNA sequencing was performed by DNA Sequencing & Services (MRC I PPU, School of Life Sciences, University of Dundee, Scotland, www.dnaseq.co.uk) using Applied Biosystems Big-Dye Ver 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer.

6.3.2.14 Basic Local Alignment Selection Tool (Blast) Analysis of DNA Sequences

DNA sequences obtained were examined to determine if there was adequate differentiation between the background and sample signals. Sequences were converted to the FASTA (test – based) format and entered into the BLAST software (http://blast.ncbi.nlm.nih.gov/). This compares the sequences with those stored in the 16S rRNA sequences database for bacteria and archaea. Identification to the species level was considered sufficient when a similarity of > 97 % was achieved to a sequence in the database (Rogina *et al.* 2014). Sequences which did not met this criteria were identified to the genus level if a > 95 % similarity was observed (Harris and Hartley 2004).

6.4 Results

To validate the first – stage phenotypic methods of identification, to be undertaken on unknown bacterial isolates recovered from the surface of clean room garments, these assays were firstly undertaken on 4 known control species – *Staphylococcus aureus* NCTC 6571, *Staphylococcus epidermidis* NCTC 8558, *Micrococcus luteus* NCTC 9278 and *Enterococcus faecalis* NCTC 775. In each case observation of the

colonies' morphological characteristics following their growth on agar were made including shape, elevation, form, size and colour. Next, each control specie underwent 6 different phenotypic assays – Gram staining, aerobic and anaerobic growth, catalase, oxidase and oxidation / fermentation testing, as well as observations made to cell shape, size and arrangement. The results of these assays and any observations made were compared against the first - stage table for identifying Gram – positive bacteria (Barrow and Feltham 1993). The results of each test, any observation made and the resultant identification of each of the control species to the genus level is shown in Table 6 – 3.

In order to compare the identification efficiency of traditional phenotypic testing to that of genotypic 16S rRNA gene sequencing, first - stage phenotypic assays were undertaken on 23 of the isolates recovered from the surface of garments during the laundering comparison study in Chapter 3 (16 of the isolates recovered from the surface of clean room suits laundered without sterilisation and 7 of the isolates recovered from the surface of clean room suits laundered with terminal gamma sterilisation). The results of each phenotypic test undertaken, any observations made and resultant identification of 16 of the isolates recovered from the surface of clean room suits laundered without gamma sterilisation is shown in Table 6 – 4 (a) and of 7 of the isolates recovered from the surfaces of suits laundered with gamma sterilisation in Table 6 – 4 (b).

Table 6 – 3: First – stage phenotypic identification of control species to the genus level.

Control Species	Colony shape, elevation, form & size (mm)	Colony colour	Gram Stain & Shape	Cell Arrangement & Size (µm)	Aerobic Growth	Anaerobic Growth	Catalase Test	Oxidase Test	Oxidation / Fermentation Test	Species Identification (Genus level)
Staphylococcus aureus **NCTC 6571**	Circular, convex, entire, 2.0	Cream	+ve Cocci	Cluster 1.0	+	+	+	-	F	*Staphylococcus*
Staphylococcus epidermidis **NCTC 8558**	Circular, flat, entire, 1.0	White	+ve Cocci	Cluster 1.0 – 2.0	+	+	+	-	F	*Staphylococcus*
Micrococcus luteus **NCTC 9278**	Circular, raised, entire, 2.0	Yellow	+ve Cocci	Cluster 2.0	+	-	+	+	0 / -	*Micrococcus*
Enterococcus faecalis **NCTC 775**	Circular, raised, entire, 1.5	Cream / white	+ve Cocci	Short chains & Pairs 1.0 – 2.0	+	+	-	-	F	*Enterococcus*

(+) indicates a positive result / (-) indicates a negative result. OF – Glucose Oxidation / Fermentation Test (F) indicates a fermentative result and (O) an oxidative result (o/-) indicates oxidation in one tube and no change in the other.

Table 6 – 4 (a): First - stage phenotypic identification of 16 of the isolates recovered from the surface of clean room suits laundered without sterilisation during the laundering comparison study based on their first stage phenotypic characteristics.

Isolate Identifier	Site of Recovery	Colony shape, elevation, form & size (mm)	Colony colour	Gram Stain & Shape	Cell Arrangement & Size (µm)	Aerobic Growth	Anaerobic Growth	Catalase Test	Oxidase Test	Oxidation / Fermentation Test	Species Identification (Genus level)
LSA	Chest	Circular, flat, entire, 1.4	Yellow	+ve Cocci	Cluster 0.8	+	-	+	-	F	Unknown
LSO	Chest	Circular, raised, entire, 2.0	Cream / yellow	+ve Cocci	Cluster 1.0	+	+	+	-	F	*Staphylococcus*
LSV	Posterior Cervicis	Circular, flat, entire, >10 (large cluster)	white	- ve Rod	Linked Rods 3.0	+	+	+	+	F	Unknown
LSC	Chest	Circular, convex, entire, 1.3	Yellow	- ve Cocci	Cuboid Pack 2.0	+	-	+	-	F	Unknown
LSQ	Left crus	Circular, convex, entire, 1.3	Yellow	- ve Cocci	Cluster 2.0	+	-	+	-	-	Unknown
LSM	Left crus	Irregular, convex, undulated, 1.0	Cream / white	- ve Cocci	Cluster 2.0	+	+	+	-	-	Unknown
LSW	Umbilical	Circular, flat, entire, 2.65	Cream / yellow	+ ve Cocci	Cluster 2.0	+	+	+	-	F	*Staphylococcus*
LSU	Posterior Cervicis	Circular, flat, entire, 2.0	Yellow	- ve Cocci	Rods 2.0	+	-	+	-	-	Unknown
LSH	Umbilical	Irregular, raised, undulated, 10.3	Cream / white	- ve Rod	Straight Rods 2.0 – 3.0	+	+	+	-	F	Unknown
LSK	Left Crus	Circular, raised, entire, 0.7	Cream / white	- ve Cocci	Cluster 2.0	+	+	+	-	-	Unknown
LSI	Umbilical	Circular, flat, entire, 1.4	Yellow	- ve Cocci	Cluster 2.0	+	+	-	-	-	Unknown
LSF	Chest	Circular, flat, entire, 1.4	White	- ve Rod	Straight Rods 2.0 – 3.0	+	-	-	-	-	Unknown
LSN	Chest	Circular, flat, entire, 2.7	Cream / white	- ve Cocci	Cluster 2.0	+	-	+	-	-	Unknown
LSX	Chest	Circular, flat, entire, 0.83	Cream / white	+ve Cocci	Cluster 1.0 – 2.0	+	+	+	-	F	*Staphylococcus*
LSS	Umbilical	Circular, flat, entire, 2.6	Cream / white	+ve Cocci	Singular 1.0	+	+	+	-	F	*Staphylococcus*
LSL	Chest	Circular, convex, entire, 1.7	Yellow	+ve Rod	Rods 2.0 – 3.0	+	-	+	-	-	*Kurthia*

(+) indicates a positive result / (-) indicates a negative result. OF – Glucose Oxidation / Fermentation Test (F) indicates a fermentative result and (O) an oxidative result (o/-) indicates oxidation in one tube and no change in the other.

Table 6 – 4 (b): First - stage phenotypic identification of 7 of the isolates recovered from the surface of clean room suits laundered with gamma sterilisation during the laundering comparison study based on their first stage phenotypic characteristics.

Isolate Identifier	Site of Recovery	Colony shape, elevation, form & size (mm)	Colony colour	Gram Stain & Shape	Cell Arrangement & Size (µm)	Aerobic Growth	Anaerobic Growth	Catalase Test	Oxidase Test	Oxidation / Fermentation Test	Species Identification (Genus level)
LST	Right Sura	Circular, convex, entire, 2.0	Cream / yellow	+ve Cocci	Clusters 1.0	+	-	+	-	-	Unknown
LSP	Left Axilla	Circular, convex, entire, 0.7	White	+ve Cocci	Cluster 2.0	+	-	+	-	-	Unknown
LSG	Posterior Cervicis	Circular, convex, entire, 1.4	Cream / yellow	+ve Cocci	Cluster 0.8	+	+	+	-	F	*Staphylococcus*
LSZ	Posterior Cervicis	Circular, convex, entire, 1.0	Cream	+ve Cocci	Cluster 1.0	+	+	+	-	F	*Staphylococcus*
LSE	Left Axilla	Circular, convex, entire, 2.1	Cream / yellow	+ve Cocci	Cluster 1.0 – 2.0	+	+	+	-	-	Unknown
LSJ	Right Axilla	Circular, convex, entire, 1.3	Cream / white	+ve Cocci	Cluster 1.0	+	+	-	-	O	Unknown
LSB	Posterior Cervicis	Circular, convex, entire, 1.6	Cream / white	+ve Cocci	Cluster 0.8	+	+	+	-	F	*Staphylococcus*

(+) indicates a positive result / (-) indicates a negative result. OF – Glucose Oxidation / Fermentation Test (F) indicates a fermentative result and (O) an oxidative result (o/-) indicates oxidation in one tube and no change in the other.

As shown in Table 6 - 3, all 4 control species were identified to their corresponding genus level using first – stage phenotypic tests. However, identification to the species and strain level could not be achieved. As shown in Table 6 – 4 (a), phenotypic identification of 16 of the isolates recovered from the surface of clean room garments laundered without sterilisation was achieved to the species level with only 5 out of the 16 isolates investigated (31.3 % identification efficiency). Of these, 4 isolates, recovered from the umbilical and chest regions of suits, were identified as species of *Staphylococcus,* and the other isolate, recovered from the chest of a suit, as a *Kurthia* species. The results of the phenotypic tests undertaken, as well as any observations made on the remaining 11 isolates did not conform to the first – stage table for phenotypic identification and therefore could not be classified. As shown in Table 6 – 4 (b), first – stage phenotypic testing of 7 of the isolates recovered from the surface of garments laundered with gamma sterilisation resulted in only 3 isolates being identified to the genus level (42.9 % identification efficiency). These isolates were recovered from the posterior cervicis region of suits and were identified as species of *Staphylococci.* Overall, only 8 out of the 23 isolates investigated using phenotypic means were identified to the genus level (34.8 % identification efficiency). The results of the phenotypic tests undertaken on the further 15 isolates did not conform to the first - stage table for identifying bacteria and therefore could not be classified.

Due to the poor identification efficiencies obtained using phenotypic methods of classification during the first stage of this study and in a bid to compare the efficiency of both phenotypic and genotypic methods of bacterial identification, 16S rRNA gene sequencing was undertaken on the same 23 isolates investigated above. During this procedure DNA was extracted from each of the isolates. The presence of DNA in each sample was confirmed using gel electrophoresis, an example of which is shown in Figure 6 – 2 (a). As shown, DNA was visually observed by the presence of a high molecular weight band in Figure 6 – 2 (a). Next, a hypervariable region of the genomic DNA which codes for the 16S rRNA gene, present in each sample, was amplified using specific PCR primers (Table 6 – 2). Gel electrophoresis was again undertaken to confirm the presence of the PCR amplification product at ~ 320 bp in each of the samples, as shown in Figure 6 – 2 (b) (please note the absence of PCR product for sample LSU – this sample was

subsequently run until a product was observed at ~ 320 bp). Following confirmation each PCR product was purified. To ensure the purity of the PCR product following purification gel electrophoresis was subsequently undertaken and an observation of a purified PCR product at ~ 320 bp made, as shown in Figure 6 – 2 (c). The remaining isolates, as well as those recovered during the gender comparison study gave similar results (data not shown).

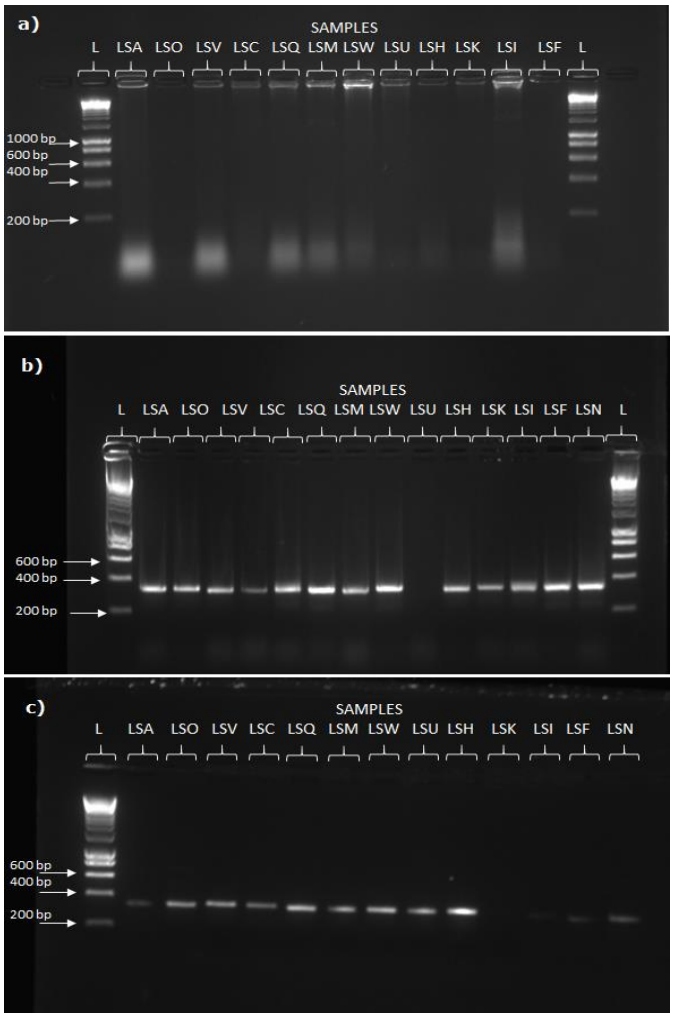


Figure 6 – 2: (a) an image of agarose gel electrophoresis confirming the presence of the DNA of 12 of the isolates recovered from the surface of clean room garments during the laundering comparison study, visualised under UV light using GelRed™, (b) confirmation of a ~ 320 bp PCR amplification product from the same 12 isolates plus an additional isolate and (c) confirmation of their purified PCR product at ~ 320 bp. (L) represents 5 µL of 1 kb HyperLadder. 200, 400, 600 and 1000 bp band sizes are indicated where necessary.

The Purified PCR amplification products were then diluted and subsequently sequenced by Dundee DNA Sequencing Service. Sequences were converted to the FASTA format and entered into the BLAST software, which compares these to sequences held in the BLAST 16S rRNA database for bacteria and archaea. An example of the resultant FASTA formatted sequence and the closest matched sequence from the BLAST 16S rRNA bacteria and archaea database for isolate LSA can be seen in Figure 6 – 3. Another 21 isolates from the laundering comparison

study and 46 isolates from the gender comparison study gave similar results (data not shown). However, for two isolates (one from each comparison study) there were no significant matches found within the database.

a) LSA:

GGCCGCAATCGACGATGAGCCCAGCTTGCTGGGTGGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCTTAACTCTGGGAT
AAGCCTGGGAAACTGGGTCTAATACCGGATAGGAGCGCCCACCGCATGGTGGGTGTTGGAAAGATTTATCGGTTTTGGATGGACTCGCG
GCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGACTGAAA
CACGCCCCAAACTCCTACGGGAGGCAGCAGTA

b) **Micrococcus luteus strain NCTC 2665** 16S ribosomal RNA gene, partial sequence
Sequence ID: ref|NR_075062.2|Length: 1525Number of Matches: 1

Related Information

Range 1: 56 to 353GenBankGraphics Next Match Previous Match First Match

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand	Frame
508 bits(275)	5e-144()	291/298(98%)	3/298(1%)	Plus/Plus	

Features:

```
Query  5    GCAA-TCG-ACGATG-AGCCCAGCTTGCTGGGTGGATTAGTGGCGAACGGGTGAGTAACA  61
            |||| ||| |||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  56   GCAAGTCGAACGATGAAGCCCAGCTTGCTGGGTGGATTAGTGGCGAACGGGTGAGTAACA  115

Query  62   CGTGAGTAACCTGCCCTTAACTCTGGGATAAGCCTGGGAAACTGGGTCTAATACCGGATA  121
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  116  CGTGAGTAACCTGCCCTTAACTCTGGGATAAGCCTGGGAAACTGGGTCTAATACCGGATA  175

Query  122  GGAGCGCCCACCGCATGGTGGGTGTTGGAAAGATTTATCGGTTTTGGATGGACTCGCGGC  181
            |||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  176  GGAGCGTCCACCGCATGGTGGGTGTTGGAAAGATTTATCGGTTTTGGATGGACTCGCGGC  235

Query  182  CTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGA  241
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  236  CTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGA  295

Query  242  GGGTGACCGGCCACACTGGGACTGAAACACGCCCCAAACTCCTACGGGAGGCAGCAGT  299
            |||||||||||||||||||||||| ||||| |||| ||||||||||||||||||||
Sbjct  296  GGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGT  353
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Figure 6 – 3: (a) an example of a FASTA formatted sequence and (b) the closest matched sequence from the BLAST 16S rRNA bacteria and archaea database.

In order to prove the accuracy and reliability of 16S rRNA gene sequencing, this technique was firstly undertaken on 4 known control species – Pseudomonas *aeruginosa* NCTC 6570*, Escherichia coli* LY180*, Staphylococcus aureus* NCTC 6571 and *Micrococcus luteus* NCTC 9278. The molecular identification of each specie, using the BLAST 16S rRNA database for bacteria and archaea, can be seen in Table 6 – 5.

Table 6 - 5: Molecular identification of control species using 16S rRNA gene sequencing.

Control Species	Genus Identified	Species Identified	Strain Identified	No of Matching Nucleotides [% Match]
***Pseudomonas aeruginosa* NCTC 6570**	*Pseudomonas*	*aeruginosa*	NCTC 6570	284 / 284 [100]
***Escherichia coli* LY180**	*Escherichia*	*coli*	LY180	288 / 289 [99]
***Staphylococcus aureus* NCTC 6571**	*Staphylococcus*	*aureus*	Z172	295 / 297 [99]
***Micrococcus luteus* NCTC 9278**	*Micrococcus*	*luteus*	NCTC 2665	289 / 295 [98]

As shown in Table 6 – 5, 16S rRNA gene sequencing successfully identified each of the 4 control species to the species level (> 97 % similarity match). In two cases, *Pseudomonas aeruginosa* NCTC 6570 and *Escherichia coli* LY180, these control species were identified to the correct strain level. However, in the remaining 2 cases (*Staphylococcus aureus* NCTC 6571 and *Micrococcus luteus* NCTC 9278) accurate identification was not achieved at this level. Therefore, for the remainder of this study identification of unknown isolates using 16S rRNA gene sequence was only deemed accurate and therefore recorded to the genus, and where achieved, species level.

Due to the poor identification rate observed during phenotypic testing of the isolates recovered during the laundering study (34.8 % [8 / 23 isolates]). 16S rRNA gene sequencing was undertaken on the same 23 isolates. The resultant

identifications of the 16 isolates, recovered from the surfaces of suits laundered without sterilisation, are shown in Table 6 – 6 (a).

Table 6 – 6 (a): Molecular identification of 16 of the isolates, recovered from the surface of suits laundered without gamma sterilisation during the laundering comparison study, using 16S rRNA gene sequencing.

Isolate Identifier	Site of Recovery	Genus Identified	Species Identified	Number of Matching Nucleotides [% match]
LSA	Chest	*Micrococcus*	*luteus*	291 / 298 [98]
LSO	Chest	*Micrococcus*	*luteus*	296 / 300 [99]
LSQ	Left crus	*Micrococcus*	*luteus*	286 / 295 **[97]**
LSW	Umbilical	*Micrococcus*	*luteus*	291 / 295 [99]
LSK	Left Crus	*Micrococcus*	*luteus*	255 / 261 [98]
LSI	Umbilical	*Micrococcus*	*luteus*	284 / 289 [98]
LSX	Chest	*Micrococcus*	*luteus*	290 / 300 **[97]**
LSS	Umbilical	*Staphylococcus*	*epidermidis*	292 / 295 [99]
LSN	Chest	*Staphylococcus*	*epidermidis*	297 / 298 [99]
LSM	Left crus	*Staphylococcus*	*epidermidis*	266 / 289 [**92**]
LSL	Chest	*Bacillus*	*infantis*	291 / 305 **[95]**
LSU	Posterior Cervicis	*Bacillus*	*infantis*	298 / 302 [99]
LSV	Posterior Cervicis	*Bacillus*	*licheniformis*	309 / 312 [98]
LSF	Chest	*Bacillus*	*oceanisediminis*	250 / 256 [98]
LSH	Umbilical	*Bacillus*	*Subtilis subsp. spizizenii*	265 / 267 [99]
LSC	Chest	No significant similarities found		

As shown in Table 6 – 6 (a), 13 of the isolates recovered from the surface of clean room garments laundered without gamma sterilisation were successfully identified to the genus level (> 95 % match to a sequence in the BLAST bacteria and archaea database) (81.3 % identification efficiency to the genus level), in addition, 11 of these isolates were identified to the species level (> 97 % match to a sequence in the BLAST bacteria and archaea database) (68.8 % identification efficiency to the species level). Two isolates (LSM and LSL) were discredited due to being less than

a ≤ 95 % similarity match to their corresponding sequences. One organism (LSC) could not be identified as there were found to be no significant similarities within the database.

Five of the isolates, recovered from the chest, left crus and umbilical region of suits laundered without sterilisation, were identified as *Micrococcus luteus,* with a further two isolates recovered from the left crus and chest of suits also being identified as species of *Micrococcus*. Two isolates recovered from the chest and umbilical region of suits laundered under the same condition were identified as *Staphylococcus epidermidis* and an isolate recovered from the posterior cervicis identified as *Bacillus Infantis.* The remaining isolates were identified as *Bacillus lichenformis,* recovered from the posterior cervicis region, *Bacillus subtilis subsp. spizizenii,* recovered from the umbilical region and *Bacillus oceanisediminis,* recovered on a contact plate used to test the chest region of a suit. The resultant identifications of 7 of the isolates recovered from the surfaces of clean room suits laundered without sterilisation using 16S rRNA gene sequencing is shown in Table 6 – 6 (b).

Table 6 – 6 (b): Molecular identification of 7 of the isolates, recovered from the surface of suits laundered with gamma sterilisation during the laundering comparison study, using 16S rRNA gene sequencing.

Isolate Identifier	Site on Suit	Genus Identified	Species Identified	Number of Matching Nucleotides [% match]
LSE	Left Axilla	*Micrococcus*	*luteus*	282 / 289 [98]
LSJ	Right Axilla	*Micrococcus*	*luteus*	283 / 292 **[97]**
LSB	Posterior Cervicis	*Micrococcus*	*luteus*	296 / 300 [99]
LST	Right Sura	*Micrococcus*	*luteus*	295 / 300 [98]
LSP	Left Axilla	*Staphylococcus*	*warneri*	302 / 308 [98]
LSG	Posterior Cervicis	*Kocuria*	*rhizophila*	297 / 301 [99]
LSZ	Posterior Cervicis	*Kocuria*	*rhizophila*	302 / 307 [98]

As shown in Table 6 – 6 (b), 6 of the 7 isolates selected from those recovered from the surface of clean room suits laundered with gamma sterilisation were identified

to the species level (85.7 % identification efficiency to the species level). A remaining isolate (LSJ) was identified to the genus level (100 % identification efficiency to the genus level). Three isolates recovered from the right sura, left axilla and posterior cervicis region of suits were identified as *Micrococcus luteus*, with a further isolate recovered from the right axilla being identified to the same genus. A further 2 isolates recovered from the posterior cervicis of suits were identified as *Kocuria rhizophila* and the remaining isolate, recovered from the left axilla of a suit, as *Staphylococcus warneri*.

Overall, using 16S rRNA gene sequencing, identification of 20 of 23 of the isolates investigated, recovered from the surface of clean room suits during the laundering comparison study, was achieved to the genus level (> 95 % match to a sequence on the BLAST bacteria and archaea database) (87.0 % identification efficiency to the genus level). Furthermore, 17 of these isolates were considered successfully identified to the specie level (> 97 % match to a sequence on the BLAST bacteria and archaea database) (73.9 % identification efficiency to the species level). In order to fully compare the identification efficiency of each of the methods undertaken in this part of the study an overall comparison was drawn between the identification of isolates recovered from the surface of clean room garments during the laundering comparison study and identification method – phenotypic assays or 16S rRNA genotypic testing (Table 6 – 7)

Table 6 – 7: Comparison of the identification of 23 of the isolates recovered from the surface of clean room suits during the laundering comparison study, using 1st stage phenotypic identification methods and 16s rRNA gene sequencing. (-) indicates an unidentified isolate.

Isolate	Isolate Identification (Genus Level)		
	Gram Stain & Shape	**Phenotypic Testing (1st Stage)**	**Genotypic Testing (16S rRNA gene Sequencing)**
LSA	+ve cocci	-	*Micrococcus*
LSO	+ve cocci	*Staphylococcus*	*Micrococcus*
LSV	- ve rods	-	*Bacillus*
LSC	- ve cocci	-	-
LSQ	- ve cocci	-	*Micrococcus*
LSM	+ ve cocci	-	*Staphylococcus*
LSW	+ ve cocci	*Staphylococcus*	*Micrococcus*
LSU	- ve rods	-	*Bacillus*
LSH	- ve cocci	-	*Bacillus*
LSK	- ve cocci	-	*Micrococcus*
LSI	- ve cocci	-	*Micrococcus*
LSF	- ve rods	-	*Bacillus*
LSN	- ve cocci	-	*Staphylococcus*
LSX	+ ve cocci	*Staphylococcus*	*Micrococcus*
LSS	+ ve cocci	***Staphylococcus***	***Staphylococcus***
LSL	+ ve rods	*Kurthia*	*Bacillus*
LST	+ ve cocci	-	*Micrococcus*
LSP	+ ve cocci	-	*Staphylococcus*
LSG	+ ve cocci	*Staphylococcus*	*Kocuria*
LSZ	+ ve cocci	*Staphylococcus*	*Kocuria*
LSE	+ ve cocci	-	*Micrococcus*
LSJ	+ ve cocci	-	*Micrococcus*
LSB	+ ve cocci	*Staphylococcus*	*Micrococcus*

Assuming the genotypic identification of each isolate to the genus level is accurate, it can be seen from Table 6 - 7 that phenotypic identification misidentified 6 out of 7 isolates identified using phenotypic assays (85.7 % misidentification). Only one isolate (LSS) was identified to the same bacterial genus using both methods. Furthermore, in 8 cases (LSV, LSU, LSH, LSQ, LSK, LSI, LSN and LSF) the results of the genotypic testing did not conform to the Gram - stain results observed during phenotypic identification of the unknown isolates. Furthermore, in 1 case (LSH) the shape of the organisms observed during phenotypic testing did not conform to these expected given the results of the genotypic testing.

Due to the poor levels of identification efficiency observed with unknown isolates using phenotypic identification methods, as well as the misidentification of isolates (Table 6 – 7), 47 of the unknown isolates recovered from the surface of clean room garments during the gender comparison study underwent 16S rRNA gene sequencing only. The resultant identifications of 36 of the isolates, recovered from the surface of garments worn by female operators working in a clean room environment, is shown in Table 6 – 8 (a). In addition, the resultant identifications of 11 of the isolates recovered from the surface of clean room garments worn by males operators, having worked under the same conditions, is shown in Table 6 – 8 (b).

Table 6 – 8 (a): Molecular identification of 36 of the isolates, recovered from the surface of clean room garments worn by female operators during the gender comparison study, using 16S rRNA gene sequencing.

Isolate Identifier	Garment Site	Genus	Species	No of Matching Nucleotides [% match]
LS13	Oral Cavity	*Micrococcus*	*luteus*	275 / 279 [99]
LS3	Lumbus	*Micrococcus*	*luteus*	273 / 273 [100]
LS4	Umbilicus	*Micrococcus*	*luteus*	293 / 295 [99]
LS21	Posterior Cervicis	*Micrococcus*	*luteus*	291 / 299 **[97]**
LS40	Left Carpus	*Micrococcus*	*luteus*	253 / 255 [99]
LS42	Chest	*Micrococcus*	*luteus*	294 / 300 [98]
LS58	Left Carpus	*Micrococcus*	*luteus*	293 / 297 [99]
LS69	Right Carpus	*Micrococcus*	*luteus*	292 / 295 [99]
LS1	Posterior Cervicis	*Staphylococcus*	*equorum*	248 / 250 [99]
LS2	Chest	*Staphylococcus*	*equorum*	298 / 302 [99]
LS71	Umbilicus	*Staphylococcus*	*equorum*	305 / 312 **[97]**
LS51	Oral Cavity	*Staphylococcus*	*succinus*	304 / 307 [99]
LS52	Lumbus	*Staphylococcus*	*succinus*	305 / 311 [98]
LS74	Umbilicus	*Staphylococcus*	*succinus*	300 / 307 [98]
LS29	Left Carpus	*Staphylococcus*	*cohnii subsp. urealyticus*	302 / 307 [98]
LS30	Left Carpus	*Staphylococcus*	*cohnii subsp. urealyticus*	298 / 298 [100]
LS31	Chest	*Staphylococcus*	*cohnii subsp. urealyticus*	307 / 310 [99]
LS42B	Chest	*Staphylococcus*	*cohnii subsp. urealyticus*	249 / 293 [**85**]

LS26	Posterior Cervicis	*Staphylococcus*	*capitis*	306 / 310 [99]
LS43	Umbilicus	*Staphylococcus*	*capitis*	293 / 296 [99]
LS39	Umbilicus	*Staphylococcus*	*hominis*	296 / 301 [98]
LS41	Umbilical	*Staphylococcus*	*saprophyticus*	307 / 313 [98]
LS87	Oral Cavity	*Bacillus*	*anthracis*	308 / 315 [98]
LS89	Oral Cavity	*Bacillus*	*anthracis*	308 / 313 [98]
LS73	Right Carpus	*Bacillus*	*anthracis*	301 / 310 **[97]**
LS19	Chest	*Bacillus*	*aerius*	287 / 295 **[97]**
LS61	Posterior Cervicis	*Bacillus*	*aerius*	298 / 306 **[97]**
LS57	Left Carpus	*Bacillus*	*safensis*	299 / 301 [99]
LS60	Umbilicus	*Bacillus*	*pumilus*	287 / 289 [99]
LS48	Left Carpus	*Arthrobacter*	*koreensis*	213 / 250 **[85]**
LS34	Oral Cavity	*Brachybacterium*	*conglomeratum*	296 / 302 [98]
LS12	Umbilicus	*Dermacoccus*	*nishinomiyaensis*	295 / 304 **[97]**
LS5	Umbilicus	*Kocuria*	*gwangalliensis*	299 / 304 [98]
LS68	Lumbus	*Paenibacillus*	*amylolyticus*	302 / 312 **[97]**
LS22	Lumbus	*Psychrobacter*	*pulmonis*	268 / 272 [99]
LS23	Posterior Cervicis	*No significant similarities found*		

As shown in Table 6 – 8 (a), using 16S rRNA gene sequencing, 33 of 36 of the unknown isolates investigated, recovered from the surface of clean room garments worn by female operators during the gender comparison study, were identified to the genus level (> 95 % match to their corresponding species on the database) (91.7 % identification efficiency to the genus level). Twenty six of these were successfully identified to the species level (> 97 % match to their corresponding species on the database) (72.2 % identification efficiency to the species level). The resultant identification of two of the isolates (LS42b and LS48) were considered inconclusive due to these only being a 85 % match to their corresponding species on the database and one isolate (LS23) showed no significant similarities to the species of bacteria on the database.

Of the 33 isolates successfully identified, recovered from the surface of garments worn by female operators, 8 of these were classified as *Micrococcus* species with 7 of these identified as *Micrococcus luteus*, having been displayed on contact plates used to test the chest, lumbus, umbilicus, oral cavity, and left and right carpus

regions of clean room garments. In addition, 13 isolates were identified as species of *Staphylococcus* including *S. equorium* [2 / 33], recovered from the posterior cervicis and chest regions, *S. succinus* [3 / 33], recovered from the umbilical, oral cavity and lumbus regions, *S. cohnii subsp. urealyticus* [3 / 33], recovered from the left carpus and chest regions, *S. capitis* [2 / 33], recovered from the posterior cervicis and umbilical and *S. hominis* [1 / 33] and *S. saprophyticus* [1 / 33], both recovered from the umbilical region. A further seven isolates were identified as species of *Bacillus* species including *B. anthracis* [2 / 33] displayed on plates used to test the oral cavity region of hoods, *B. safensis* [1 / 33] recovered from the left carpus of a suit and *B. pumilus* [1 / 33] from the umbilical region. In addition, an isolate recovered from the umbilical region of a suit was identified as *Kocuria gwangalliensis.* Another recovered from the oral cavity was identified as *Brachybacterium conglomeratum,* and two isolates recovered from the lumbus region were identified as *Psychrobacter pulmonis* and a species of *Paenibacillus.* Lastly, another isolate, recovered from the umbilicus region was also identified as a species of *Dermacoccus*.

Table 6 – 8 (b): Molecular identification of 11 of the isolates, recovered from the surface of clean room garments worn by male operators during the gender comparison study, using 16S rRNA gene sequencing.

Isolate Identifier	Garment Site	Genus	Species	No of Matching Nucleotides [% match]
LS49	Right Carpus	*Micrococcus*	*luteus*	292 / 296 [99]
LS90	Umbilicus	*Staphylococcus*	*succinus*	304 / 308 [99]
LS75	Umbilicus	*Staphylococcus*	*succinus*	306 / 311 [98]
LS32	Umbilicus	*Staphylococcus*	*cohnii subsp. urealyticus*	303 / 307 [99]
LS67	Chest	*Staphylococcus*	*saprophyticus*	264 / 266 [99]
LS82	Chest	*Bacillus*	*anthracis*	304 / 311 [98]
LS83	Umbilicus	*Bacillus*	*anthracis*	289 / 290 [99]
LS59	Posterior Cervicis	*Bacillus*	*safensis*	305 / 310 [98]
LS62	Posterior Cervicis	*Microbacterium*	*maritypicum*	290 / 306 **[97]**
LS63	Right Carpus	*Microbacterium*	*maritypicum*	246 / 249 [99]
LS64	Chest	*Microbacterium*	*maritypicum*	296 / 301 [98]

As shown in Table 6 – 8 (b), 100 % [11 / 11] of the unknown isolates selected from contact plates used to test the surface of garments worn by male operators during the gender comparison study were successfully identified to the genus level using 16 S rRNA gene sequencing. In addition, 90.9 % [10 / 11] of these were identified to the species levels. Of these, an isolate recovered from the right carpus region of a suit was identified as *Micrococcus luteus* and 4 isolates recovered from the umbilical and chest region of suits were identified as *Staphylococcus* species including *S. succinus* [2 / 11], *S. saprophyticus* [1 / 11] and *S. cohnii subsp. urealyticus* [1 / 11]. In addition, 2 isolates recovered from the chest and umbilicus regions were identified as *Bacillus anthracis* and another isolate from the posterior cervicis as *Bacillus safensis*. The 3 remaining isolates, recovered from the posterior cervicis, right carpus and chest regions were identified as *Microbacterium species including M. maritypicum* [2 / 11].

6.5 Discussion

During this aspect of our research study genotypic 16S rRNA gene sequencing was found to be considerably more reliable at identifying unknown isolates, recovered from the surface of clean room garments during the laundering and gender comparison studies, than more traditional phenotypic identification assays. The genotypic approach successfully identified 87.0 % [20 / 23] of the self – selected pool of isolates recovered during the laundering study to the genus levels, of which 73.9 % [17 / 23] were also identified to the species level (Tables 6 – 6 (a) and (b)). In comparison, only 34.8 % [8 / 23] identification efficiency was observed with traditional phenotypic methods, undertaken on the same isolates (Table 6 – 4 (a) and (b)). Furthermore, when these resultant phenotypic identifications were compared to that of their genotypic sequencing match the identity of only 1 isolate (LSS) was found to correspond (Table 6 – 7), suggesting an 85.7 % incidence of misidentification using the phenotypic approach. Furthermore, a high identification efficiency was also observed with 16S rRNA gene sequencing during the classification of 47 of the isolates recovered from the surface of clean room garments during the gender comparison study. Overall, 93.6 % [44 / 47] of these isolates were identified to the genus level, with 76.6 % [36 / 47] of these also identified to the species level (Table 6 – 8 (a) and (b)). The results of our research supports previous literature, each of which report genotypic identification methods to be preferable to traditional phenotypic methods (Tang *et al.* 1998; Petti *et al.* 2005, Champagne 2008; Emerson *et al.* 2008; Sheraba *et al.* 2010; Amaral *et al.* 2014). The identification of microorganisms using traditional phenotypic testing is challenging to perform, as well as being time - consuming (Tang *et al.* 1998), as was also found during our study. The results can also be difficult to reproduce (Amaral *et al.* 2014). The problem is that the accuracy and reliability of phenotypic assays depend entirely upon the microorganism's behaviour and morphology under certain conditions (Sheraba *et al.* 2010). However, these observable characteristics will change over time (Petti *et al.* 2005). As a result, and as found during our phenotypic study, isolates are misidentified or remain unidentified (Sheraba *et al.* 2010). Therefore, in our study, the isolates recovered during the laundering comparison study, and evaluated using phenotypic methods, were further assessed using 16S rRNA gene sequencing to achieve accurate identification, as recommended by Janda and Abbott (2002), Bosshard *et al.* (2004) and Petti *et al*. (2005).

Genotypic methods of identification are not hindered by a cell's ever changing characteristics and behaviours (Champagne 2008). Mignard and Flandrois (2006) found that 16S rRNA gene sequencing provided identification to the species level in > 83 % of isolates, with only 1 % of samples remaining unidentified. Additionally, Drancourt *et al.* (2000) observed similar results, with > 89 % of samples being > 97 % similar to a sequence on their database. As previously discussed during our genotypic study, identification was achieved to the genus level in between 87.0 % - 93.6 % of the isolates investigated and to the species level in between 73.9 % – 76.6 % of these. During our study identification at the strain level was not considered accurate. As shown during the 16S rRNA gene identification of control species, 2 out of 4 of the organisms were misidentified at the strain level (Table 6 – 5) and therefore isolates recovered during the laundering and gender comparison studies were only considered accurately identified to the genus and where appropriate species level. Misidentification at the strain level is thought to be due to closely related bacterial strains exhibiting sequence similarities within their 16S rRNA gene. An example of this being *Bacillus* species *globisporus* and *psychrophilus* each of which share a > 99.5 % gene sequence similarity (Fox *et al.* 1992). During our study, initial phenotypic identification was only achieved (inaccurately in most cases) to the genus level. Identification of isolates to the species level using phenotypic assays may be possible, however, this would require second stage testing, which during our study would have proven expensive, time consuming and labour intensive. Furthermore, identification of isolates to the strain level using phenotypic tests is also difficult because different bacteria may exhibit the same phenotypes (Amaral *et al.* 2014).

Assuming the results of the 16S rRNA gene sequencing undertaken in our current study were correct to the genus level, the cumulative results of the phenotypic assays misidentified 7 of the 8 isolates originally identified (87.5 %) (Table 6 – 7). The frequency of the misidentification of isolates using phenotypic assays is a factor which remains unknown (Petti *et al.* 2005) and therefore is a subject which should be studied further. In our study isolate misidentification may have been the result of the misinterpretation of the subjective phenotypic results (Stager and Davies 1992). For example, of the 23 of the isolates investigated, recovered from the surface of clean room clothing during the laundering comparison study, the Gram - stain observations made of 8 of these isolates (LSV, LSU, LSH, LSQ, LSK,

LSI, LSN and LSF) did not conform to that expected given their identification using 16S rRNA gene sequencing and the morphology of another isolate (LSH) did not obey that expected (Table 6 – 7). This suggests a problem in analysing the results of the phenotypic tests. During our study various problems existed, for example, during the Gram - staining assay it was difficult to differentiate between the resultant colours of the cells and therefore challenging trying to determine whether the isolate was a Gram – positive or a Gram – negative bacteria. This problem was also encountered when determining the resultant colours obtained in the tubes during the oxidation / fermentation test, making it difficult to differentiate between an oxidative or fermentative result. Furthermore, the results of phenotypic tests are dependent upon a number of other variables including incubation time and culture / growth media age (Sandle 2011b), each of which may have impacted upon the inability to correctly identify an isolate in our current study. As shown in Table 6 – 3, four control species were identified to the correct genus level using the results of the first - stage phenotypic tests. However, this is thought to be due to the results of the tests undertaken on the isolate being pre – known and therefore easier to interpret. Culture age may also have played a role in the misinterpretation and reliability of the results obtained during the current study. Isolates recovered during the laundering study were stored on master plates in the fridge for a number of months before undergoing the identification stage of the study. This may have caused changes in the characteristics of the isolate. However, this factor was unavoidable due to time constraints. Overall, time plays a major contributing factor to the inherent limitation of phenotypic identification, with methods being time - consuming and labour intensive. Identification can take in excess of 48 hours after isolation. However, in some cases full identification of some organisms can take up to a few weeks (Tang *et al.* 1998), as was found in this in this current study. In comparison, identification of isolates using 16S rRNA gene sequencing can be achieved in less than 48 hours (Champagne 2008).

However, despite the apparent advantages and success of 16S rRNA gene sequencing, no bacterial identification method is 100 % accurate (Janda and Abbott 2002) and therefore relying on a single method may lead to an isolate being misidentified. As shown in this current study, 16S rRNA gene sequencing did not achieve 100 % identification. Two isolates (LSC – an isolate from the laundering comparison study and LS23 – an isolate from the gender comparison study) could

not be identified due to there being no significantly similar sequence found in the 16S rRNA bacterial and archaea database. Furthermore LSM and LSL (both isolates recovered from the surface of suits laundered without sterilisation during the laundering comparison study), LS48 (recovered from the left carpus of a male operator's suit during the gender comparison study) and LS42B (recovered from the chest region of a female operator's suit during the gender comparison study) were considered inconclusively identified due to a less than 97 % similarity match to a sequence within the database. This could be due to novel bacterial species not yet on the sequencing database. Another major problem associated with 16S rRNA gene sequencing is the potential contamination of the sample with exogenous bacterial DNA. However, during this study this was minimised by identifying the risks and contamination hazards and ensuring careful management of the working environment and personnel.

The presence of bacteria on clean room garments following laundering with / without sterilisation is of concern to the clean room industry because the specialist garments must not add to microbial numbers being introduced into the environment. If such organisms were to enter into the clean room and product this could result in patient disease and potentially death (MDPH 2012; Pharmacy Practice News 2013; Public Health England 2014; Sprinks 2014; Torjesen 2014). One of the aims of this part of the study was to identify a representative pool of isolates recovered from these suits and determine their source. Using 16S rRNA gene sequencing, bacteria isolated from the surface of clean room garments during the laundering comparison study were predominantly identified as Gram - positive species of *Micrococcus, Staphylococcus and Bacillus* (Tables 6 – 6 (a) and (b)). There were no incidences of Gram - negative species identified during our study. Sixty five % [13 / 20] of the isolates classified were successfully identified as species of skin commensal bacteria including *Micrococcus luteus* (40 % [8 / 20], *Staphylococcus epidermidis* (10 % [2 / 20] and *warneri* (5 % [1 / 20], and *Kocuria rhizophila* (10 % [2 / 20]). This finding is of concern because although these bacteria are predominantly non – pathogenic each of these species has the ability to cause disease (Dürst 1991; Kamath *et al.* 1992; Otto 2012; Moissenet *et al.* 2012), which should be considered as patients receiving sterile pharmaceutical products are usually immunocompromised. Furthermore, the identification of skin commensal bacteria on garments having been laundered with / without

sterilisation suggests that such processes are inadequate at removing human sources of microbial contamination following their wear. However, it is important to consider that the presence of skin commensal bacteria on garments post laundering / sterilisation may be due to the re - contamination of garments during the laundering processing phase, post washing / drying. It could also be argued that the identification of skin commensal bacteria on garments post laundering / sterilisation may be the result of sampling technique, with the personnel being the contamination source during the physical sampling. Therefore, it could be argued that the findings in this study are not a true reflection of the garment's bacterial bioburden. However, as previously discussed in Chapter 3, contamination risk prior to sampling was minimised by ensuring that the individual took every precaution available to avoid cross – contamination. Contamination risk by personnel was reduced by the operator dressing in specialist clean room garments including donning a sterile face mask and by wearing sterile rubber gloves prior to sampling. Contamination risk (from the operator, materials and environment) was also minimised prior to sampling by ensuring the outer packaging of each suit was sterilised prior to its opening and that the operators did not touch the exterior surface of the suit. Furthermore, the sampling surface was repeatedly decontaminated with 70 % ethanol throughout the testing period.

The remaining 20 % [4 / 20] of isolates successfully identified to the species level during this part of the study, recovered from suits having undergone laundering without sterilisation only (Table 6 – 6 (a)), were classified as environmental *Bacillus* species, including water inhabitant *infantis* [1 / 20], *oceanisediminis* [1 / 20], and soil inhabitants' *licheniformis* [1 / 20] and *subtilis subsp. spizizenii* [1 /20]. The identification of bacteria which inhabit water could suggest that suits have become contaminated during the washing process, whereas the presence of soil inhabitants suggests inadequate decontamination. The presence of *Bacillus* species on suits having undergone laundering but not on suits having undergone additional gamma - irradiation would suggest that gamma sterilisation is successful at eliminating environmental species of contamination. However, it is very important to remember that isolates having undergone identification in this part of the study are self – selected 'representative' colonies from a much larger pool of colonies and this will impact upon the results. To the author's knowledge, there are no previous published studies which have identified isolates recovered

from the surface of clean room garments following laundering with or without terminal sterilisation, with this being the first such study. Therefore, the results of our research cannot be compared directly to any previous studies. The results of this study suggest that contaminated reusable clean room garments could pose a hazard to the clean room environment and in turn have a detrimental impact upon the sterility of the product manufactured within the room. The presence of potentially pathogenic microbial contamination on the surface of reusable clean room garments is of great concern and reiterates the worries by clean room operators who have questioned the lack of sterility of garments returning from outsourced laundering facilities in the past (Larkin 2009). As previously discussed this could be mitigated by using reusable garments with an antimicrobial finish, which potentially reduces the microbial bioburden of the garment overall (McIlvaine and Tessien 2006), or by operators wearing single use disposable garments (Larkin 2009; Larkin 2012). Such disposable garments assure a predictable and consistent performance, eliminating variability in barrier filtration efficiency associated with reusable garments (Larkin 2012).

Overall, the majority of the isolates which were selected from contact plates used to recover bacteria from the surface of garments worn by operators having worked in a clean room environment were identified as skin commensal in origin including species of *Staphylococcus*, *Micrococcus* and *Dermacoccus* (38.6 % [17 / 44]). Of these, *Micrococcus luteus* was the sole *Micrococcus* specie identified (18.2 % [8 / 44]). However, a number of skin commensal *Staphylococcus* species were identified including *S. cohnii subsp urealyticus* (9.1 % [4 / 44]), *S. captis* (4.5 % [2 / 44])*, S. saprophyticus* (4.5 % [2 / 44]) *and S. hominis* (2.3 % [1 / 44]) (Tables 6 – 8 (a) and (b)). With over 35 % of isolates studied being identified as human in origin this finding helps support the argument raised in Chapter 5, that microorganisms attached to skin squames will penetrate every day and specialist clean room clothing, contaminating the outer surface of clean room garments whilst operators work. This is also supported by the findings of Grangè *et al.* (2010). This data also supports previous theories, which state that operators are the primary source of clean room contamination (Champagne 2008; Casser 2011a; Strauss *et al.* 2011; Chen *et al.* 2013; Sandle 2014). As previously discussed, a review by Sandle (2011b) identified over 50 % of clean room isolates as species of *Micrococcus* and *Staphylococcus.* Additionally, Martín *et al.* (2012) identified the

main clean room isolates as species of *Staphylococcus*, *Micrococcus* and *Kocuria* and concluded these were a result of operators working in the clean room. As suggested in Chapter 5 – Section 5.5, such contamination could be reduced by operators substituting normal everyday clothing for either specialist clean room underwear (Ljungqvist and Reinmüller 2005) or polyester suits (Eudy 2014), wearing garments treated with an antimicrobial finish (McIlvaine and Tessien 2006) or wearing garments of an improved barrier efficiency (Mitchell *et al.* 1978; Hao *et al.* 2004; Chen *et al.* 2013).

In addition to a high percentage of the isolates recovered from the surface of garments worn by operators in the clean room being identified as skin commensal bacteria, a further 13 isolates (29.5 %) were classified as the environmental genus' *Bacillus* [10 / 44] and *Microbacterium* [3 / 44], as well as another isolate as a species of *Paenibacillus*, also environmental in origin (Table 6 – 8 (a) and (b)). Those species identified include *B. safensis* (4.5 % [2 / 44]) and *B. pumilus* (2.2 % [1 / 44]) which are frequent contaminants of pharmaceutical clean rooms (Branquinho *et al.* 2014). In addition, four of the isolates recovered were identified as *B. anthracis,* an environmental microorganism which has the potential to cause anthrax (Spencer 2003) and which has previously been identified in soil samples (Pepper *et al.* 2002). The identification of this organism was considered correct in relation with aligning our data with that of the comparable sequence obtained from the BLAST database. However, the sequence within the database may be incorrect in relation to the sequence for *B. anthracis,* potentially due to the organism being uncommonly isolated and identified. Furthermore, as previously discussed, misidentification at the strain level is common and thought to be due to closely related bacterial strains exhibiting sequence similarities within their 16S rRNA gene (Fox *et al.* 1992). However, if such an organism was to be identified within a clinical diagnostic laboratory the appropriate authority should be notified (Scottish Government 2012). Environmental microbial species may be transferred into the clean room via dust and personnel (Sandle 2017). The presence of predominantly soil associated bacteria identified in isolates recovered from clean room operators garments during the gender comparison study suggests that outdoor shoes may be acting as a vehicle for the transmission of such bacteria into clean room changing area, which is then picked up on the garments and transferred into the clean room environment. Moissi – Enichinger *et al.* (2015) found the changing area

of a clean room facility to be host to highest colony numbers, due to high level activity, as well as being a primary source of contaminants entering the clean room. Unfortunately, the introduction of contamination into the changing area and clean room cannot be avoided, but it could be minimised. In this study clean room operators entered the changing area wearing their outdoor shoes, stepping onto an adhesive mat at the changing room entrance; shoes were then removed and stored in the black area of the changing room (Chapter 4 - Section 4.6.6). The results of this part of our study suggest that shoes should be removed prior to entering the changing area or an additional adhesive mat could be stationed at the external side of the changing room door to reduce the introduction of such contamination into the changing area.

Whilst there were similar proportions of skin commensal *Staphylococcus* species and environmental species of *Bacillus* identified in the isolates recovered from garment worn by both male and female operators between genders (*Staphylococcus:* Male - 36.4 % [4 / 11] vs. Female – 39.4 % [13 / 33]; Bacillus: Males - 27.3 % [3 / 11] vs. Females – 21.2 % [7 / 33]), a higher percentage of those isolates recovered from the surface of garments worn by female operators were identified as species of *Micrococcus* (Female – 24.4 % [8 / 33] vs. male - 9.1 % [1 / 11]). Additionally, 3 isolates (27.3 %) recovered from the surface of garments worn by male operators were identified as environmental *Microbacterium* species. This specie was not identified in isolates recovered from garments worn by female operators. Furthermore, isolates recovered from the surface of female operators' garments were also identified as seawater inhabitant *Kocuria gwangalliensis* [1 / 33] (Seo *et al.* 2009) and foodborne species *Brachybacterium conglomeratum* [1 / 33] (Wenning *et al.* 2005), *Staphylococcus equorum* [2] and *Psychrobacter pulmonis* [1 / 33]. The results of the gender comparison study (Chapter 5) suggest that the surface of garments worn by male operators were host to more microorganisms than the surface of those worn by female operators. However, the results of this part of the current study suggest that the surface of clean room garments worn by females are host to a more diverse bacterial community than the surface of those worn by their male counterparts (Tables 6 – 8 (a) and (b)). However, it is very important to remember that this study was undertaken on a self – selected 'representative' selection of colonies recovered from the surface of garments worn by operators and does not represent the overall

bacteria community on the garments. Furthermore, although a representative and proportionate number of isolates were selected from each of the pools with respect to the percentage of plates displaying growth and number of garments tested in Chapters 3 and 5 (suits laundered without gamma sterilisation [16], suits laundered with gamma sterilisation [7] and garments worn by male [11] and female operators [36]) these were not in equal numbers. Therefore, the greater diversity observed in isolates used to test female operators may likely be due to the number of isolates identified.

Overall, throughout this entire study, emphasis has been placed upon bacterial contamination at specific garment sites (Chapters 3, 4 and 5). However, in this part of the study there seems to be no correlation between garment site and the presence of any particular specie at that site. However, once again this may be due to the number of representative isolates within each pool and is something that could be investigated with 16S rRNA gene sequencing of the remaining isolates recovered during the study. This would also allow for a definitive answer to whether gamma sterilisation can successfully eliminate the presence of environmental bacteria on suits having undergone laundering only and whether the surface of females operators' garments are more diversely populated than those of their male counterparts. The identification of all the isolates (or a greater number of isolates) recovered during the laundering and gender comparison studies was not possible during our study due to time and financial constraints.

6.7 Conclusion

In this section of the research project the efficiency of two microbial identification techniques were compared. Phenotypic assays and genotypic 16S rRNA gene sequencing were used to identify a representative pool of isolates recovered from the surface of clean room suits during the laundering comparison study. 16S rRNA gene sequencing was found to be more reliable and accurate at identifying bacteria recovered from the surface of clean room garments to the species level than traditional phenotypic methods of identification. 16S rRNA sequencing successfully identified 87.0 % of the isolates investigated, recovered during the laundering comparison study, to the genus level and 73.9 % of same isolates to the species level. In comparison, first – stage phenotypic identification methods were initially found to identify 34.8 % of the same isolates to the genus level, of which, when

using 16S rRNA gene sequencing, over 85 % were subsequently proven to be misidentified. In addition, 16S rRNA gene sequencing successfully identified 93.6 % of isolates investigated, recovered during the gender comparison study, to the genus level and 76.6 % of these to the species level.

In addition, using 16S rRNA gene sequencing, bacterial isolates recovered from the surface of clean room garments during both the laundering and gender comparison studies were predominantly identified as skin commensal species of *Staphylococcus* and *Micrococcus* and environmental species of *Bacillus.* However, *Bacillus* species were not identified on suits having undergone terminal gamma sterilisation. In addition, the identification of isolates recovered from the surface of garments worn by female operators was found to be more diverse than those recovered from the surface of garments worn by their male counterparts.

Chapter 7:

Overall Summary, Conclusions & Future Work

7.1 Overall Summary

A brief overview of clean room use in Chapter 1 highlighted the significant role such environments play in the manufacture of sterile pharmaceutical products, which are required to be particulate and microbial free. The severity of sterile pharmaceutical product contamination was discussed, with reports that such events can not only lead to facility shut downs and loss of revenue (Champagne 2008) but more worryingly, in patient disease and in worst case scenario death (Tran *et al.* 2006; MDPH 2012; Pharmacy Practice News 2013; Public Health England 2014; Sprinks 2014; Torjesen 2014). Despite clean room operators wearing specialist garments, designed to capture and contain their detritus, an investigation of previous literature identified operators as the primary source of clean room contamination (Whyte and Hejab 2007; Champagne 2008; Casser 2011a, Strauss *et al.* 2011; Chen *et al.* 2013; Sandle 2014). In addition, bacteria were shown to contaminate the outer surface of clean room operator's garments whilst they work (Grangè *et al.* 2010), furthermore, as discussed in Chapter 3, the polyester fibres, commonly used to manufacture reusable clean room garments, were also shown to provide a suitable substrate for bacteria to live upon and grow (Hsieh and Merry 1986; Neely and Maley 2000; Schmidt – Emrich 2016). Therefore, such garments were thought to have the potential to become a transmission mode for microorganisms. Consequently, it was emphasised that the bacterial bioburden of the surface of garments worn by operators in the clean room environment should remain low, preferably zero, during the donning and working period (Rhodes 2006). However, in order to achieve this, the requirement for reusable clean room garments to return from out – sourced laundering facilities suitably sterile and remain in this condition throughout the donning process, and working period, was highlighted. Therefore, the aim of this work was to investigate and examine the levels and types of bacterial contamination on the surface of clean room operators' garments post laundering, following the donning process, and post wear, using a commonly employed surface sampling technique, developed early on in the research project. The overall objective of this study was to use its findings to help to contribute towards improving contamination control standards within clean room facilities, with respect to clean room operators and their garments. A topic, which on investigation of the published literature, severely lacked in substance. This research was achieved through five distinct stages, as detailed in Chapter 1 – Section 1.2. Firstly, the recovery of bacteria from antistatic carbon

filament polyester fabric (used to manufacture reusable clean room garments) was investigated using surface sampling techniques in line with the following objective and as detailed in Chapter 2:

- **To develop a surface sampling technique which could subsequently be used to recover, enumerate and compare the levels of bacteria on the surface of reusable antistatic carbon filament polyester clean room garments during the remainder of the study.**

During this early stage of the study, *S. aureus* NCTC 6571, pre – inoculated onto antistatic polyester carbon filament squares, permeated the fabric, with the rate of its migration increasing over time. This was obviously found to be of concern, as microorganisms which evade clean room attire can contaminate the outer surface of the garment, before potentially detaching and entering the clean room environment, potentially to the detriment of the products manufactured within the facility. However, it is important to note that during this stage of our study the bacterium was only considered in its unicellular form (~ 0.8 – 1 µm) and not attached to larger skin particles, which act as a bacterial transmission mode and are reported to be ~ 33 x 44 x 3 µm in size (Sandle 2014). However, as well as in their entire form these particles have also been identified in the air as fragments, (Whyte and Hejab 2007; Whyte and Eaton 2016), reported to be 10 – 25 µm x 1 µm (Clark 1974) and 20 µm x 3 – 5 µm (Whyte and Hejab 2007) in size, which could readily penetrate the pores of the fabric investigated during our study.

During this early stage of our study the ability of two surface sampling techniques, namely the direct agar contact method and swabbing method (using both a moist and dry swab bud), to retrieve bacteria from pre – inoculated fabric were compared. The recovery of *S. aureus* NCTC 6571 from antistatic carbon filament polyester fabric using these methods was found to be very poor, with recovery efficiencies of less than 3 % being obtained. Overall, poor percentage recovery was thought to be due to the number of variable associated with each of the procedures investigated, as well as a lack of standardised protocols, as previously discussed in the literature, and in Chapter 2. Inconsistency in swabbing materials

and methods, leading to extreme variability in recovery efficiencies obtained, has previously been reported in the literature (Moore and Griffith 2002a; Moore and Griffith 2002b; Edmonds 2009). Therefore, in a bid to increase bacterial recovery using swabbing, swab bud moisture content was studied. This factor was found to increase bacterial recovery - with a moist bud recovering significantly more bacteria than its dry counterpart. However, as found during our study, and as previously documented in the literature, the swabbing method can underestimate bacterial recovery, due to the swab buds' ability to retain microorganisms (Rose *et al.* 2004; Moore and Griffith 2007; Dalmaso *et al.* 2008).

In contrast, the direct agar contact plate method of bacterial recovery, eliminating the agar transfer stage associated with swabbing, was subsequently found to recover a significantly higher percentage of bacteria from the antistatic polyester carbon filament fabric than its swabbing equivalents. However, concerns over the recovery efficiency of this method were also discussed, and in return the ability of the process to recover bacteria was assessed in relation to the fabric's bacterial seeding density (Hsieh and Merry 1986; Salo *et al.* 2000; Probst *et al.* 2001; Frawley *et al.* 2008), agar contact time (Foshchino *et al.* 2003) and the fabric's moisture content (Marple and Towers 1978; Craythorn *et al.* 1980; Moore and Griffith 2002a; Moore and Griffith 2002b), all factors the literature had documented as affecting bacterial adherence and / or recovery but the results of which could not be compared directly to our study due to the differences in surface composition. During our study percentage bacterial recovery using contact plates was found to increase as the density of bacteria on the fabric increased, suggesting a direct relationship between surface bacterial density and recovery efficiency of the method, as previously demonstrated in past research. As observed, significantly less bacteria were recovered from fabric squares seeded with 100 colonies compared to that recovered from fabric seeded with either 1000 or 10,000 colonies of the same bacterium. This suggested that the direct agar contact method may be underestimating low levels of bacterial surface contamination, such as those found within the clean room environment. However, it is important to note that the recovery efficiency of contact plates reported during our study only considered antistatic carbon filament polyester fabric and not flat non – porous surfaces, which are commonly sampled in clean environments. As was

previously discussed, recovery efficiencies of a surface sampling method can vary between surfaces (Edmonds 2009).

Also during Chapter 2, agar contact time was increased in a bid to improve recovery efficiency, as had been found in past research (Foshchino *et al.* 2003). However, a 5 second contact time, as initially employed during the study, was found to recover significantly more bacteria than a 30 second or 60 second testing period. This decrease in the efficiency of the method at longer testing periods was thought to be due to the agar losing its initial adhesive properties over time. An increase in percentage recovery was observed with a 60 second contact time when compared to a 30 testing period, however, this was thought to be due to an increase in the moisture content of the fabric resulting from the tool, over time, as was observed. An overview of previous literature showed that moisture can aid bacterial recovery (Sattar *et al.* 2001; Moore and Griffith 2002a), however, during our assessment of bacterial recovery with respect to fabric moisture content there was shown to be no significant difference in the percentage of bacteria recovered from 'moist' and 'dry' fabrics. However, an increase in the percentage of bacteria being recovered using a 60 second time period was thought to be due to increased moisture resulting from the contact plate rather than the surface, as had previously been observed with a moist swab bud during our swabbing assay. This suggested that moisture resulting from the tool, rather than the surface itself, aided bacterial recovery. Therefore, moist contact plates may have achieved greater recovery efficiency than dried plates, as was used during this study. This factor was thought to be a limitation of our research. The continuous direct agar contact assay proved that *S. aureus* NCTC 6571 were still present on the surface of the fabric squares following their testing with the initial contact plate. Subsequent plates were found to recover equal proportions of bacteria, however, in each case (topside and underside of separate fabric squares) the 10^{th} plate was found to recover a higher percentage of bacteria than its 9 predecessors. Again, this was thought to be due to the increase in moisture content of the fabric resulting from the tool (contact plate), as was observed.

During this stage of the study concerns were also raised that the poor recovery efficiencies observed may have been a result of agar composition and / or agar incubation time, as suggested in the literature. The issue of agar composition was

assessed in Chapter 3, where on comparison of two general purpose agar types (NA and TSA) there was shown to be no significant difference in recovery efficiencies. It was discussed that a selective agar may have increased the percentage of the bacteria recovered, however, it was emphasised that a general purpose agar should be used throughout this study, which could cultivate the wide range of organisms which may be present on the surface of clean room operators' garments. Therefore, it was decided that contact plates to be used throughout the remainder of our study would be filled with general nutrient agar. Agar incubation time was assessed in Chapter 4. Although not shown to be statistically significant, during an assessment of both a 24 and 48 hour incubation period, an increase in the percentage of plates displaying low, moderate and high levels of growth following a further 24 hour incubation period (taking total incubation time to 48 hours) suggested that an insufficient incubation period may have contributed to the low recovery efficiencies observed during Chapter 2. Therefore, it was decided that for the remainder of our study only contact plates incubated for 48 hours should be considered. However, maximum recovery efficiency was not the overall aim of this early stage of the study, but rather to develop a method which could subsequently be used to recover, enumerate and compare the levels of bacteria on the surface of reusable clean room garments – as was achieved throughout the remainder of this study using the direct agar contact method. However, it is important to consider that the levels of bacteria recovered and reported throughout the duration of this thesis may only be a representation of a garments' bacterial population (~ 1 – 3 % dependent upon bacterial density).

Research has shown that bacteria will contaminate the outer surface of clean room garments whilst operators work (Grangè *et al.* 2010), therefore, following wear garments must undergo decontamination (European Commission 2008). However, as reported in Chapter 3, clean room specialists have previously raised concerns over the sterility of reusable clean room garments returning from out - sourced laundering facilities (Larkin 2009). This was an area, which on further investigation, was found to severely lack in published literature. Therefore, the second stage of our research was to use this direct agar contact method, as investigated in Chapter 2, to compare the surface bacterial bioburden of clean

room suits following out - sourced laundering under the following objective in Chapter 3:

- **To compare the surface bacterial bioburden of reusable clean room suits following their laundering either with or without terminal gamma sterilisation.**

Using the aforementioned surface sampling method, developed in Chapter 2, bacteria were recovered from the surface of clean room suits having undergone either laundering with or laundering without terminal gamma sterilisation, reiterating previously documented concerns raised over the insufficient laundering of reusable clean room garments returning from out – sourced laundering facilities (Larkin 2009). However, a reduction in the bacterial bioburden of clean room suits was observed in those having undergone terminal gamma sterilisation. This was predominantly at the chest and umbilicus region of suits having undergone laundering only, where higher levels of bacteria had been observed. Initially, this suggested that garments to be worn in clean rooms where the product is at increased risk of microbial growth should undergo additional gamma sterilisation following laundering. However, on investigation of previous research, it was found that whilst gamma sterilisation of garments can reduce their surface bacterial bioburden, as reported during our study, this process can significantly decrease the barrier efficiency of the garments (Galvin and Vyas 2016), which is a critical feature of garments to be worn in a pharmaceutical clean room. Furthermore, on assessment of the limited literature, multiple laundering cycles were also shown to reduce garment viability (Leonas 1998; Ljungqvist and Reinmüller 2003; Lee *et al.* 2012). Therefore, in conjunction with the results of our study, we suggested that reusable clean room garments could be substituted for disposable garments. However, the efficiency of disposable clean room garments was also found to lack in published literature, and therefore would require further investigation before their use. In addition, the bacterial bioburden of such garments was not found to be something which had been previously studied. Furthermore, it was reported that whilst disposable garments were found to provide a predictable performance (Larkin 2012), their occlusive fabric may result in these garments being

uncomfortable for the operator to wear (Hao *et al.* 2004; Chen *et al.* 2013). Therefore, it was suggested that clean room garments constructed of fabric with an antimicrobial finish could be employed. However, despite such finishes having been shown to reduce the microbial load of textiles (McIlvaine and Tessien 2006; Bajpai *et al.* 2011), on review of the literature there was found to be a lack of research with regard to the use of anti - microbial fabrics to manufacture clean room garments. Unfortunately limitations during the laundering comparison stage of our research were found to exist, with one of the biggest restrictions encountered being the inability to sample garments under the same environmental conditions. Although every precaution was taken to counteract any effects this may have had on the outcome of the research it was suggested that this study could be replicated to produce reliable and reproducible results.

A review of standards highlighted the importance of the outer surface of clean room garments remaining sterile following the donning process (Rhodes 2006; European Commission 2008). However, despite contaminated hands being recognised as one of the main transmission modes for microorganisms (World Health Organisation 2009), communication with clean room specialists ascertained that there was no standardised approach to glove use during the clean room garment donning process (C. Alexander 2012, Personal Communication; C. Dawson 2012, Personal Communication). Therefore, the third stage of our research involved using the direct agar contact method, developed in Chapter 2, to investigate the surface bacterial bioburden of clean room garments following the donning process, as described under the following objective in Chapter 4:

- **To compare the bacterial bioburden of the surface of clean room garments donned by operators dressing wearing either no gloves, non – sterile gloves or sterile clean room gloves.**

During this stage of our research bacteria were recovered from the surface of clean room garments donned by operators wearing either no gloves, non – sterile gloves or sterile clean room gloves, reiterating that hands act as a transmission mode for bacteria whether gloved or not. However, there was shown to be no significant

difference in the resultant bacterial bioburden of clean room garments, at all sites tested, following their donning with no glove, non – sterile gloves or sterile clean room gloves. This suggested that expensive sterile clean room gloves could be substituted for either their non – sterile equivalents or no gloves during the donning process without increasing the bacterial bioburden of the garments. Chapter 4 discussed how this could provide a significant cost saving in the clean room industry, especially in the NHS where budgets are imperative. Additionally, it may also be important to consider these results with respect to clean room glove use for other tasks. However, it is important to note that this stage of the study did not consider the transfer of non – viable particles, which on review of the literature were previously discussed as being to the detriment of a pharmaceutical product and patient (Tran *et al.* 2006).

Clean room suits donned with non – sterile gloves were shown to exhibit a greater degree of bacterial surface variability between sites than those garments donned with non gloves or their sterile counterparts. Furthermore, a review of previous research highlighted that non – sterile gloves are prone to a higher percentage of holes and / or tears than their sterile equivalents (Zinner 1994). Furthermore, it was reported that skin commensal bacteria have been recovered from opened non – sterile gloves boxes (Hughes *et al.* 2013). Therefore, suggestion was made that the omission of gloves, in line with a standardised hand washing protocol, may be more effective at minimising the transfer of hand borne bacteria during the clean room garments donning process than wearing non – sterile gloves. With respect to garment site, higher levels of bacteria were detected on the chest region of suits and the oral cavity of hoods donned under the variable. As discussed in Chapter 4, this was thought to be due to hand borne transmission of bacteria at the chest region, due to the method of donning, as well as the predominantly high levels of bacteria housed in the oral cavity. A percentage of these bacteria were also thought to be the result of inefficient laundering processes, as previously found in Chapter 3.

It was emphasised throughout this study that the levels of bacteria on the surface of specialist garments worn by operators working in the clean room environment should remain low, ideally zero, during the working duration (Rhodes 2006). However, previous research had shown that although such garments will reduce

the particulate dispersion from operators, they will not contain all sources of human detriment (Ramstorp *et al*. 2005; Whyte and Hejab 2007). Furthermore, the surface of such garments can become contaminated with bacteria during wear (Grangè *et al.* 2010). Therefore, in the fourth stage of this investigation, discussed in Chapter 5, the direct agar contact method was used to assess the surface bacterial bioburden of clean room garments following their wear in the clean room environment, under the following objective:

- **To investigate the bacterial bioburden of clean room garments following their wear in a clean room environment with respect to gender.**

During this stage of our study bacteria were recovered from the surface of garments worn by male and female operators working in the clean room environment. In addition, gender was shown to play a role in a quantity of bacteria released between individuals, with the surface of garments worn by males being shown to be more highly contaminated at all sites tested, than those worn by their female counterparts. It was suggested that male operators should undertake additional measures to reduce any differences between microbial dispersion observed between genders. However, the recovery of bacteria from garments worn by both male and female operators suggested that additional measures should be undertaken by both genders. It was recommended that, to reduce the dispersion of microorganisms, this could include the examination of everyday clothing worn under clean room garments, and potentially substituting this with specialist clean room garments, or by operators wearing garments with a more occlusive fabric or that treated with an antimicrobial finish. However, limitations with these options were found to exist, as discussed in Chapter 5, and therefore further work would be required to investigate these choices. The biggest limitation of this part of our study was found to be the disproportionate number of female operators tested in comparison to the small pool of male operators tested - due to the high prevalence of female students taking part in our study. Therefore, replication of this study using equal numbers of male and female operators should be undertaken to produce reproducible results. Furthermore, it is important to remember that

although supervised, and abiding by written protocol, this study was undertaken with novice operators.

Drawing no comparison to gender, during this chapter of the research, a comparison was also made between the levels of bacteria on each garment site tested, within each gender, under each working condition. Levels of growth on the chest and posterior cervicis, observed in garments worn by male and female operators in the Grade C clean room, were shown to be reduced with the addition of a hood over the head, as worn in the Grade A / B clean room. This suggested that donning a hood reduces the dispersion of head borne bacteria onto these neighbouring sites. However, it was noted that further work should be undertaken to ensure that this finding wasn't a factor of time spent working in the clean room rather than garment selection as such – an issue which could not be assessed during our study. Overall, throughout the clean room stage of our study (Chapters 3, 4 and 5) the chest, umbilicus and oral cavity region of garments were generally found to be more contaminated than the other sites tested. The reasons behind this were largely explained. The chest was thought to be more contaminated than the other sites tested due to billowing or pumping action of the suits during wear, with particles travelling up the suits in the direction of the head (Eudy 2003). Furthermore, it was discussed, that the site was in close proximity to the head, and contamination may be due to the bacteria residing in the oral cavity (Dewhirst *et al.* 2010). These factors in addition to the transfer of hand borne contamination at this site during the donning process, as previously discussed in Chapter 4, were thought to cumulatively affect the levels of bacteria at this site. The high levels of growth detected on the hood worn by operators were predominantly thought to be due to the sheer number of bacteria in the oral cavity. Contamination of clean room suits at the umbilicus region largely remains undocumented. However, it may be due to the area being in close proximity to the direct working area, as well as becoming contaminated during the donning process, as also discussed in Chapter 4. Higher levels of bacteria on the chest and umbilicus region of suits post laundering suggested that this process did not fully decontaminate areas prone to higher degrees of bacterial contamination. However, a limitation of this study was that hoods were not assessed during the laundering comparison study. It was discussed that bacteria recovered during the garment donning study (Chapter 4)

and gender study (Chapter 5) may be the direct result of the laundering processes, rather than the task in hand. However, although this may be the case with a small percentage of the colonies recovered, the higher percentage of plates displaying growth observed during the subsequent chapters of this thesis suggest they are a direct result of the task being undertaken.

Finally, having investigated the surface bacterial bioburden of clean room garments prior to, following donning and wear, the identification of a representative pools of isolates recovered during the laundering and gender comparison studies was undertaken, in a bid to determine their source, under the following objective in Chapter 6:

- **To seek the identification of a representative selection of bacterial isolates recovered from the surface of clean room garments during the laundering and gender comparison studies.**

Initially during this stage of the research first stage phenotypic identification assays and 16S rRNA gene sequencing were undertaken on self – selected representative selection of isolates recovered from the surface of clean room suits during the laundering comparison study. 16S rRNA gene sequencing was found to be more reliable and accurate at identifying these isolates than traditional phenotypic methods of identification, successfully identifying 87 % of these isolates to the genus level and 73.9 % of these to the species level. In comparison, first – stage phenotypic identification methods were initially found to identify 33.3 % of the same isolates to the genus level, of which, using 16S rRNA gene sequencing, > 85 % were subsequently proven to be misidentified. As discussed in Chapter 6, this was thought to be due to accuracy and reliability of phenotypic testing being based upon the isolates' behaviour and morphology, which are not static. Using 16S rRNA gene sequencing isolates were predominantly identified as skin commensal species of *Staphylococcus* and *Micrococcus*, which further reiterated an inefficient laundering process following wear. In addition, *Bacillus* species were also identified on suits having undergone laundering without sterilisation, but not within the pool of isolates recovered from suits having

undergone gamma sterilisation. This suggested that gamma sterilisation was a successful tool in eliminating such environmental species, potentially immune to laundering process alone. However, it was important to remember that a limitation of our study was that identification was only undertaken of a representative pool of the isolates recovered during the laundering comparison study.

Due to the superior identification efficiencies achieved with 16S rRNA gene sequencing on isolates recovered during the laundering comparison study, this method was undertaken on 47 of the isolates recovered from the surface of clean room garments during the gender comparison study. As discussed in Chapter 6, 16S rRNA gene sequencing successfully identified 93.6 % of these isolates to the genus level and 76.6 % of these to the species level. Again, these isolates were predominantly identified as species of skin commensal *Staphylococcus* and *Micrococcus*, as previously discussed, Sandle (2014), Martín *et al*. (2012), Park *et al.* 2013 and Moissi – Eichinger *et al.* (2015) all found that the majority of clean room isolates were of the same genus'. This helps reiterate the theory that clean room operators are the primary source of clean room contamination, as previously discussed in the literature (Whyte and Hejab 2007; Strauss *et al.* 2011; Chen *et al*. 2013; Sandle 2014). Also identified were environmental species of *Bacillus*. This suggested that contamination of suits was not only the direct result of wear but that environmental bacteria were either transferred into the environment via the garment itself or picked up from the environment during the working duration. Interestingly, the pool of isolates identified, which were recovered from the surface of garments worn by female operators, were found to be more diverse than those recovered from their male counterparts. However, it is important to remember that in this case identification was only undertaken of a representative pool of the isolates recovered during the study. Of which more isolates were recovered from garments worn by female operators during our study – due to the higher prevalence of woman taking part in the study – as previously discussed as a limitation of our study.

7.2 Overall Conclusions

- Despite the efficiency of the direct agar contact method at recovering bacteria from antistatic carbon filament polyester fabric being poor (< 3 %), this method is a successful tool to recover, enumerate and compare the levels of bacteria on the surface of reusable clean room garments of the same composition.
- Bacteria are present on the surface of reusable clean room suits which have undergone out – sourced laundering either with or without terminal gamma sterilisation.
- Terminal gamma sterilisation reduces the surface bacterial bioburden of reusable clean room suits, predominantly at the chest and umbilicus regions.
- Bacteria are transferred onto the surface of reusable antistatic carbon filament polyester clean room garments via the hand borne route during the donning process, primarily at the chest region, whether operators wear either no gloves, non – sterile gloves or sterile gloves whilst dressing.
- Expensive sterile clean room gloves can be substituted for their cheaper non – sterile equivalents or no gloves during the garment donning process without subsequently increasing the surface bacterial bioburden of the garment.
- Antistatic carbon filament polyester clean room garments, of the composition studied during our research, will not contain all human sources of contamination. Microorganisms will evade and contaminate the outer surface of such garments whilst operators work.
- The surface of clean room garments worn by male operators are more highly contaminated than the surface of those worn by their female counterparts.
- The donning of a clean room hood can reduce the levels of bacterial contamination at the chest and posterior cervicis regions of clean room suits.
- 16S rRNA gene sequencing is a successful tool to identify unknown isolates to the genus and where appropriate the species level.
- Skin commensal species of *Staphylococcus* and Micrococcus, as well as environmental species of Bacillus are present on the surface of garments following their wear in the clean room environment and following their

laundering without gamma sterilisation. *Bacillus* species are absent from garments having undergone terminal gamma sterilisation.

- Clean room specialists can use the knowledge contained within this thesis to help to contribute towards controlling contamination levels within the clean room environment, with respect to clean room operators and their specialist garments.

7.3 Future Work

Whilst the work contained in this thesis has identified the use of the direct agar contact method as a successful tool to recover, enumerate and estimate the surface bacterial bioburden of antistatic polyester carbon filament clean room garments prior to and following donning and wear, further work is necessary to address the limitations encountered during our study. Firstly, the constraint faced during the laundering study, which meant garments were tested under different conditions, should be addressed. Similarly, if sterile clean room garments were to be substituted for their non – sterile equivalents during the clean room garment donning process, a full investigation should be carried out into non – sterile options including variability between brands and their storage options. Furthermore, assessment should be made of a suitable common method for either the use of non – sterile gloves or omission to wear gloves during the donning process, both in conjunction with a standardised hand washing protocol. In addition, if reusable clean room garments are to be substituted for disposable clothing, those constructed of an antimicrobial finish or more occlusive fabric, or if such garments are to be worn in conjunction with specialist clean room underwear, all of which were suggested during our study, an assessment of their bacterial bioburden prior to donning, and following donning and wear should be fully investigated prior to their use in the clean room environment. This assessment should consider the bacterial bioburden of sites below the waist of operators, which were not considered during the glove and gender comparison study stages of our research, and therefore a limitation of our work. A full investigation into reducing bacterial levels at the chest region of suits should also be considered, this should include reflection of the donning process to reduce hand borne contamination levels in this area, as well as assessing whether a hood significantly reduces levels of bacteria at this site during wear. This would also allow for determination of whether this observation was the result of time spent working in the clean room or as the result

of hood use. Furthermore, if practical all future work in this area should be undertaken using a larger pool of trained operators and where this considers gender, using an equal proportion of male to female operators.

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