An investigation into alternative materials to silicone rubber for reducing *Candida albicans* biofilm formation in in-dwelling urinary catheters.

by

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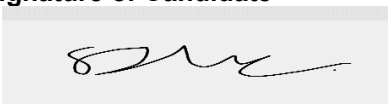

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Abstract

Candida albicans is a dimorphic yeast-like fungus that can adhere to indwelling medical devices such as catheters, produce biofilms and then disseminate. This dissemination can lead to invasive candidiasis and sepsis. Catheters are most commonly made from silicone rubber, a material that has been extensively studied for its capability for microbial growth. In this study, *C. albicans* biofilms were grown on 2mm diameter, 1.5mm thick discs made from silicone rubber, neoprene (also known as polychloroprene) and butyl rubber (also known as Isobutylene-isoprene) with of aim of investigating a potential alternative to silicone as a catheter material. The discs were inoculated for 2 hours in an orbital shaker at 37^0C at 180rpm then statically incubated at either 30 or 37^0C for 24 or 48 hours in malt extract broth or Rosewell park memorial institute 1640. Wells were stained with crystal violet to detect biomass. The greatest average growth came from the 37^0C incubated plates, with neoprene having the most overall growth in malt extract broth and butyl having the most overall growth when grown in Rosewell park memorial institute 1640. Interestingly, silicone and butyl in MEB had more growth on average at 37^0C after 24 hours incubation than after 48 hours, though dissemination could have been responsible for this reduction. Some biofilms were significantly different from each other- in particular silicone and butyl. There appeared to be a combination of factors that affected biofilm growth with varying levels of influence, but duration did not play a significant role in the amount of growth in this study. Surface topography appeared to be a factor, as did media. Overall, the results from this study showed that the difference in growth was not great enough to warrant the cost and effort required to switch materials from silicone to either neoprene or butyl.

I would like to thank; my husband, Fred Maile, for his support throughout this entire year and all the years before that. My family for everything. My supervisory team, for their guidance and support; Dr. Steve Beeton, Dr. David Wareing and Dr. Achyut Guleri. Stefanie Gilchrist and all the technicians, for their all their help and advice.

“I declare that no accomplishment has substance as great as the road used to achieve it. We are not creatures of destinations. It is the journey that shapes us.”

-Brandon Sanderson

Contents

List of abbreviations

Adenosine triphosphate enzyme	ATPase
Agglutinin-like sequence	ALS
Agglutinin-like sequence 3	ALS3
Agglutinin-like sequence 1	ALS1
brain heart infusion	BHI
Candida albicans	*C.albicans*
Catheter associated urinary tract infections	CAUTI
Cell surface hydrophobicity	CSH
Checkpoint kinase 1 protein	Chk1p
Colony forming unit	CFU
Deoxyribonucleic acid	DNA
Endoplasmic reticulum	ER
ETS related gene	ERG
Extracellular polymeric substances	EPS
Germ tube test	GTT
G-protein-coupled receptor	GPCR
Guanosine diphosphate	GDP
Guanosine triphosphate enzymes	GTPases
High affinity calcium uptake systems	HACS
Hybrid histidine kinase	HHK
Hyphal wall protein	Hwp1

Isobutylene-isoprene	IIR
Low affinity calcium uptake systems	LACS
malt extract broth	MEB
Megapascal	MPa
National Collection of Pathogenic Fungi	NCPF
National Institute for Health and Care Excellence	NICE
Neutrophil extracellular trap	NET
Phosphate Buffered Saline	PBS
Public Health England	PHE
Revolutions per minute	RPM
Roswell Park Memorial Institute 1640	RPMI 1640
α-mannan- β 1, 6 glucan complex	MGCx

1. INTRODUCTION

1.1 *Candida albicans*

Candida albicans is a dimorphic yeast like fungus that can grow *in vitro* and *in vivo*, producing budding yeast cells which can differentiate into pseudo hyphae or true hyphae, which are collectively known as the filamentous forms. The name *Candida albicans*, derived from the Latin meaning 'white', refers to its appearance when cultured on a plate see Figure 1.- (and in instances of infection such as thrush where it appears as a milky white coating/discharge). *C.albicans* is part of the human flora, colonising as a commensal organism. It can be found in/on the gut, skin, gastrointestinal tract, oral and vaginal cavities and the urinary tract (Hameed, *et al*, 2018). Pathogenicity of *C. albicans* is more common in the immunocompromised (Bruder-Nascimento, *et al*, 2014). The reasons why *C. albicans* becomes more pathogenic in the immunocompromised is not fully understood, though possibly linked to the hosts' physiological condition inducing morphogenesis (Matare, *et al,* 2017). A study by Wibawa, *et al,* (2015) examined the differences between *C. albicans* extracted from healthy individuals and those with human immunodeficiency virus (HIV). The HIV patients' isolates were faster growing (significantly so) and developed more hyphae. As mentioned above, *C. albicans* can differentiate from bud to filamentous forms. This ability to reversibly switch between the unicellular yeast and the filamentous forms is required for biofilm formation and believed to be the source of virulence (Southern, *et al,* 2008). The switch between forms occurs after one of the three main signalling cascades is activated, which then induces morphogenesis. These cascades are also involved in the co-regulation of adaption to their environment (Boyce & Andrianopoulos, 2015). The cascades are a two-component cascade, a heterotrimeric G protein and Ras signalling cascade and

calcium signalling cascade, all discussed in further detail in section 1.8. Prior to morphogenesis there is adhesion- be it to epithelial tissue or, in the interest of this experiment, to an indwelling medical device (Hameed, *et al,* 2018).

Figure 1.- *C. albicans* strain NCPF 3179 cultured on Sabouraud Dextrose agar (Taken from Hanson, 2015).

1.2 Germ tubes

Germ tube growth is what distinguishes true hyphae from pseudo hyphae. A germ tube is an elongation of a cell with parallel sides and no constrictions. Only true hyphae contain germ tubes (Sudbery, 2001). During *C.albicans* cell division, the daughter bud cell elongates and a septum ring forms which pinches off the daughter cell so it is completely separated from the mother cell. Hyphae are formed when the elongated daughter cell doesn't separate from the mother cell. This results in elongated cells of various lengths originating from the mother cell. True hyphae have no constrictions from a septum. Pseudo hyphae have a partially formed septum which causes constriction but no separation of the cells. There are numerous environmental causes for the hyphal switch which include but are not limited to, a

temperature of 37^0C (Sudbery, *et al,* 2004). *C.albicans* and *Candida dubliniensis* infections are distinguished from other Candida spp infections via a germ tube test (GTT). This test, performed after a positive blood culture, involves growing the *Candida* on a solid agar medium. A colony is removed and placed in serum for 3 hours then identified using light microscopy (Sheppard, *et al,* 2008). This technique has been used for many years and is still considered the most economical, easy and rapid way of identifying the two *Candida* strains from other species (Moya-Salazar & Rojas, 2018).

1.3 *C. albicans* adhesion proteins

Initial attachment of *C. albicans* to the substrate (either host tissue or material) is achieved by adhesins. The most extensively studied are the agglutinin-like sequence (Als) proteins. This family contains eight proteins, of which the hyphae associated Als3 is considered most important for adherence (Mayer, *et al,* 2013). Previous studies investigating the adherence properties of the Als family determined that the greatest loss of adhesion came from the deletion of the *ALS3* gene (Hoyer & Cota, 2016). The central region of Als3 are rich in serine and threonine, and these hydrophobic amino acids are exposed on the cell surface. Their hydrophobicity enables mediation of direct adherence to some substrates, an example of this being polystyrene (Frank, *et al,* 2010). Als3 is a multi-functional protein and can act as an invasin after adhesion (Liu & Filler, 2010). In a study by Finkel, *et al,* (2012) silicone was used as a substrate for *C. albicans* adhesion. Deletion of Als3 or hyphal wall protein 1 (Hwp1) did not affect substrate- *C. albicans* adherence. However, deletion of the *ALS1* gene caused significant adhesion defects, highlighting the importance of

Als1 in cell-substrate adhesion. Both Als3 and Als1 have been noted as being the most important proteins in the Als family for biofilm formation. Both code for large glycoproteins (Nailis, *et al*, 2008).

In a study by Nailis, *et al,* (2008) investigation into the roles of Als1 & Als3 in biofilm formation on silicone found that the levels of *ALS1* gene expression altered significantly when it was downregulated at 72 and 96 hours. This was measured by growing biofilms on silicone discs in a CDC reactor and at different time points, removing the discs and using confocal laser scanning microscopy. The differentiation of true hyphae, pseudo hyphae and germ tubes were performed using solid phase cytometry.

Prior to downregulation at 72 hours it had been upregulated consistently, showing a uniform need for Als1 during all stages of biofilm development. For *ALS3* gene expression, there was upregulation at 30-minute, 1-hour and 6-hour points post adhesion then there was downregulation as the biofilm matured, indicating it had a greater importance during the earlier stages of biofilm formation. The expression pattern of Als1 was unsure to be because of biofilm maturation reducing the need for the protein or because the biofilm was cultured in a CDC reactor it was a reaction specific to that environment. The expression pattern of Als3 was believed to be associated with the morphology of the cells switching- it is a hyphal associated protein and thereby overexpressed during periods of morphological transition (Nailis, *et al,* 2008).

Adherence by the filamentous forms of *C. albicans* is greater than that of yeast, however the act of yeast epithelial adherence is a stimulator for germ tube formation thereby linking hyphae formation and adherence (Naglik, *et al,* 2011).

Initial adherence can either be direct or indirect. As this experiment is only concerned with direct attachment to a substrate that will be the only method discussed though it is acknowledged not to be the only way.

1.4 Direct *C. albicans* adherence

Direct adherence begins with electrostatic and hydrophobic attraction between the yeast and epithelial cell, with adhesin- receptor mediated reactions. Once adherence is complete, one of the three signalling cascades, either a two-component cascade, a heterotrimeric G protein and Ras signalling cascade or calcium signalling cascade will be activated to induce morphogenesis. *C. albicans* can invade the epithelial tissue leading to invasive candidiasis and, ultimately, sepsis. This can be achieved one of two ways; the passive host mediated induced endocytosis, or active penetration via the filamentous forms. Induced endocytosis occurs after recognition of invasins expressed by *C. albicans* by the E-cadherin receptor on the epithelial cell surface. Endocytosis can be triggered without the involvement of the E-cadherin receptor via invasin/epidermal growth factor receptor interactions (Richardson, *et al,* 2018). A study by Nikawa, *et al,* (1992) found that with yeast-solid surface adherence, in *C. albicans*, the main mechanisms of adherence were electrostatic and specific interactions. The influence of hydrophobicity was greater on *Candida tropicalis*. Active penetration will either occur through an epithelial cell or between two epithelial cells. *C. albicans* secretes aspartic proteinases to degrade the mucosal layer. The proteinase Sap5p degrades the E-cadherin receptor on the cell and Sap2p degrades the mucins. There are other mechanisms that are utilised by the *C. albicans* during active penetration, this is just one example. After active penetration

by the hyphae, there is induced endocytosis to further penetrate the basal proliferative layers. Both passive and active invasion are required for penetrance of the stratified epithelial cells, but invasion of the outer layers is performed by active penetration. After access to the basal proliferative layer, the *C. albicans* can then migrate to the bloodstream, causing candidaemia (Richardson, *et al,* 2018). *C.albicans* expresses hydrophobic proteins on its surface, making the surface hydrophobic. This is known as cell surface hydrophobicity (CSH). A higher CSH level is linked to the ability to adhere, form biofilms and penetrate host tissue (Rodrigues & Elimelech, 2009).

1.5 Candida spp. Infections

Invasive candidiasis is a more commonly associated nosocomial fungal infection caused by various members of *Candida spp*. Invasive candidiasis is frequently manifested as candidaemia. Severe or untreated cases of infection can lead to sepsis (Pappas, *et al,* 2018). The risk factors for invasive candidiasis can be split into two groups; health care associated, and host related. Contributing factors in host related invasive candidiasis includes, but is not limited to, age and immunosuppressive conditions. Contributing factors towards health care associated invasive candidiasis include things such as antimicrobial drug use and the implementation of medical devices such as catheters (Yapar, 2015). According to Public Health England's (2017) candidaemia surveillance, the youngest and oldest age groups were the most affected, with men aged over 75 years of age most at risk. From the ages of 5-44, there were more incidents within the female population compared to males of the same age. The candidaemia mortality rate is around 30-60% (Hirano, *et al,* 2015). Sepsis, according to the World Health organisation (2018), is an adverse reaction to infection that induces tissue damage and has a global

morbidity rate of >30 million and a global mortality rate of up to 6 million people annually. Untreated, sepsis can develop into severe sepsis. This can further develop into septic shock, a condition where hypotension leads to tissue hypoxia and subsequent organ failure (Russell, *et al,* 2018). *C. albicans* has, for many years, been the predominant cause behind candidiasis and candidaemia (Falagas, *et al,* 2010). 42% of candidaemia cases in 2017 were attributed to *C.albicans* and they displayed varying levels of resistance to commonly tested antifungals, according to Public Health England's (2017) recent findings from the laboratory surveillance scheme. However this figure has decreased from 47% over the last five years. 24% of candidaemia cases were associated with *Candida glabrata* and 10% associated with *Candida parapsilosis.* Resistance to drugs, such as amphotericin B decreased (98% susceptibility in 2015 vs 99% in 2017) whereas caspofungin resistance increased (100% susceptibility in 2015, 96% in 2017). It should be noted that PHE have warned that these levels may be overestimated and not a true reflection of resistance prevalence.

1.6 Catheter associated urinary tract infections and catheters

Catheter associated urinary tract infections (CAUTI) account for nearly 80% of nosocomial infections worldwide (Jacobsen, *et al,* 2008). These infections are defined as being funguria or bacteriuria at concentrations greater than 10^5 colony forming units/ml^{-1} (CFU/ml^{-1}) (Singha, *et al,* 2016). Opportunistic pathogens can gain access to the urinary tract via catheter implementation and begin colonising (Jacobsen, *et al,* 2008). There are two main types of catheters: short term (<28 days) and long term (>28 days). Long term catheters are also referred to as indwelling. There is a link between duration of catheterisation and infection risk according to the National Institute for Health and Care Excellence (NICE) guidelines (2014). CAUTI's

caused by Candida spp. account for a third of all nosocomial CAUTI's. Some cases, wherein the Candida spp. has established a biofilm on the surface of the catheter and not migrated or invaded epithelial tissue, are asymptomatic and the only sign of the colonisation is Candiduria (Nett, et al., 2014). The most commonly used indwelling catheter is the Foley catheter, named after Frederic Foley, who created the original design.

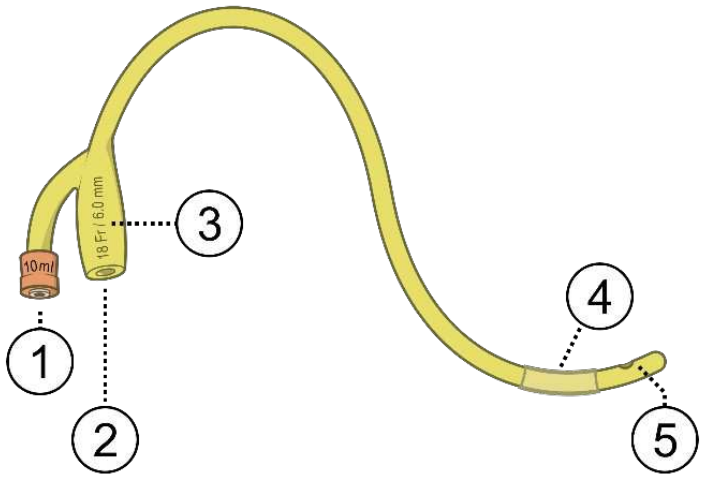

Figure 2. An example of a Foley catheter, taken from (Remesz, 2011). This is a size 16 Fr (as stated by the text on the drainage channel labelled 3 on the above image). This refers to the diameter of the catheter, in this case it is 16mm. The Fr is a reference to the French gauge system which is a common catheter measuring scale. The French gauge system is triple the diameter in mm so to find the diameter of the catheter the Fr number would be divided by 3.

A Foley catheter has two lumens. The first is the drainage funnel (labelled 1 on Fig. 2) and this is connected to the drainage eye (labelled 5 on Fig. 2) and is used for

urine expulsion. The other lumen, called the inflation connector (labelled 2 on Fig. 2) is connected to the balloon (labelled 4 on Fig. 2 and is deflated in the image), allowing it to inflate when it is inside the bladder to hold it in place. The balloon is inflated with sterile water that is injected via syringe which is administered via the inflation channel. The rounded tip of the drainage eye (labelled 5 on Fig.2) extends the balloon and has one or more holes to allow the urine to drain from the bladder. Foley catheters were originally all made from Latex. Due to the cytotoxic nature of Latex the catheters were either coated in silicone elastomer, a hydrophilic coating or, as is the most commonly the case now, made completely from silicone (Feneley, *et al,* 2015).

1.7 Urethra physiology

The urethra is a tube-like structure composed of striated and smooth muscle. The smooth muscle is divided into two layers- the inner, thicker, mainly longitudinally arranged layer, and the outer thinner circular layer (Groat, *et al,* 2015). The urethra is designed to be an outlet for the expulsion of urine from the bladder, connecting the neck of the bladder to the external urinary orifice. The urethra is formed from multiple types of epithelial cells. These are urothelium (a form of transitional epithelium), pseudostratified columnar, stratified columnar, then finally the stratified squamous cells that are located proximal to the external urethral orifice. Within the lining of the lumen of both male and female urethras are glands that produce secretions that act as protection for the urinary tract by ‘washing’ out the lumen aiding the prevention of microbe adherence (Hickling, *et al,* 2015). In females, the urethra is only used for micturition. In males the urethra is also used for ejaculation. The lengths of the urethra in males and females differ (22cm average for males, 4cm average for females). The smooth muscle cells of the urethra are mechanically coupled together

to ensure relaxation and contraction required for micturition. This is achieved via ion channels which regulate the electrochemical gradient and induce action potentials (Kyle, 2014). The urothelium acts as a physical barrier for the basal cells, aiding protection from microorganisms and urine, which has a pH range of between 5.5 and 7. The urothelium contains specialised lipids, uroplakin proteins and tight junction proteins to maintain this barrier (Birder & Andersson, 2013).

1.8 Signalling cascades

As mentioned previously, there are three main signalling cascades that once activated triggers morphogenesis. The first discussed is a two-component cascade that involves the hybrid histidine kinase (HHK) (see figure 3). These signalling cascades induce a response via phosphorylation of aspartate and histidine residues from component proteins and are part of a four step phosphorelay that induce a transcriptional and morphological response resulting in filamentation (Defosse, *et al,* 2015). One of the HHK's identified in *C. albicans* is the checkpoint kinase 1 protein, Chk1p. In research by Kruppa, *et al,* (2003), chk1p deficient *C. albicans* mutants had extensive hyphae flocculation and greatly reduced yeast cell adherence to epithelial tissue.

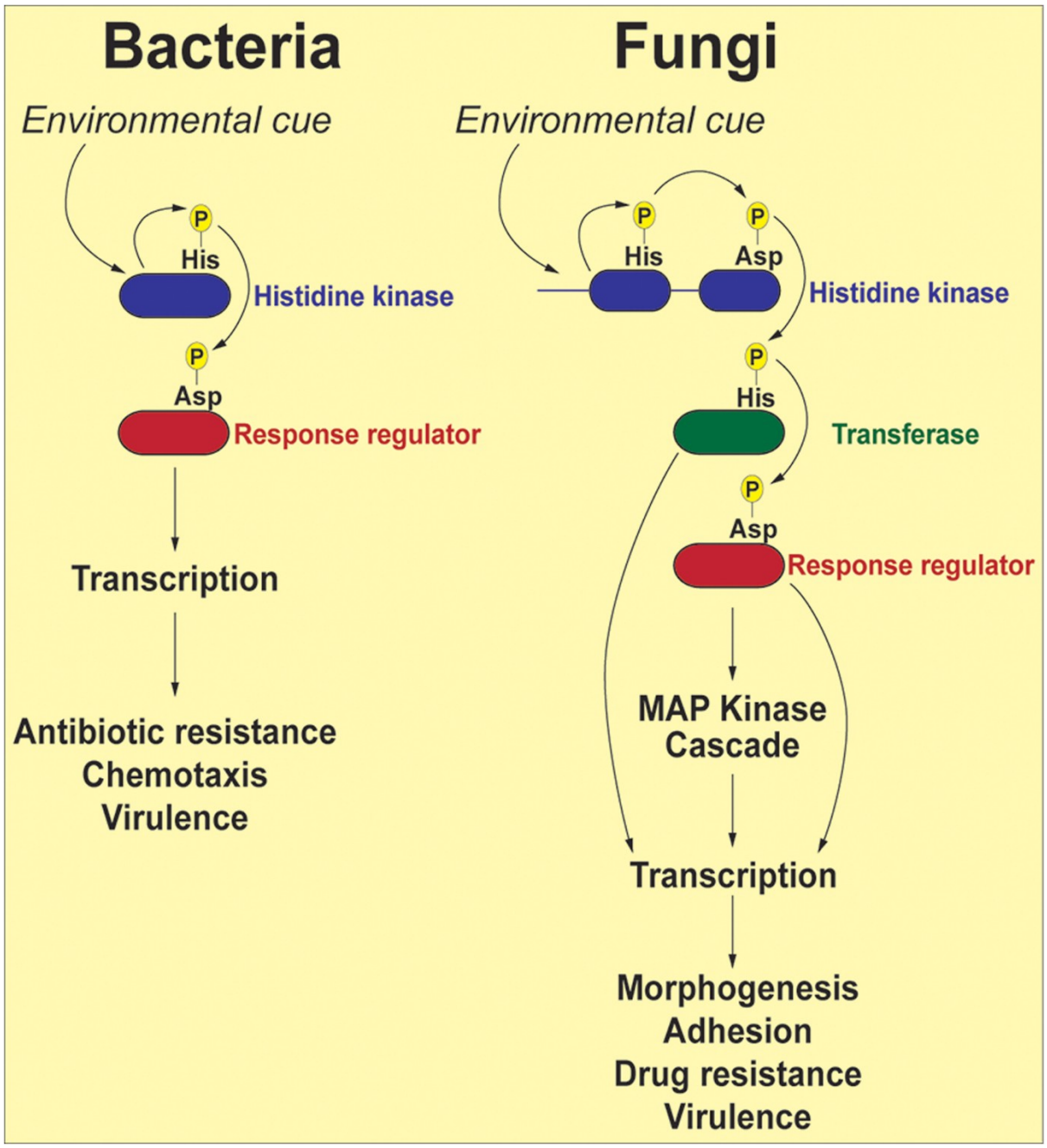

Figure 3- the environmental stimuli triggers autophosphorylation of the histidine kinase. Taken from (Shor & Chauhan, 2015). The phosphoryl group is intramolecularly transferred to the aspartate on the histidine kinase, then transferred to a histidine on a transferase protein and then transferred to an aspartate on a response regulator. This phosphorelay then causes activation of the MAP kinase pathway downstream (see figure 4 for MAP kinase pathway) (Shor & Chauhan, 2015).

The second cascade is heterotrimeric G protein and Ras signalling (see figure 4). The three (alpha, beta, gamma) heterotrimeric G protein subunits are closely coupled to a transmembrane receptor, a G-protein-coupled receptor (GPCR) which binds an external ligand causing guanosine triphosphate (GTP) hydrolysis to guanosine diphosphate (GDP). This causes disassociation of the beta/gamma dimer from the alpha subunit. Both the dimer and the GDP bound alpha subunit can affect downstream signalling to trigger a cascade that results in a transcriptional response leading to hyphae formation (Li, *et al,* 2007). Ras proteins are highly conserved GTP enzymes (GTPases) that belong to the G protein family and are often localized to the membrane. In *C. albicans*, the RAS proteins are associated with cell morphology regulation. They are pivotal to the response to environmental cues which include but are not limited to; carbon dioxide concentration and temperature (Inglis & Sherlock, 2013). Mutant Ras1 *C. albicans* strains were found to be unable to support hyphal development in a study by Piispanen, *et al,* (2011).

The third cascade is calcium signalling (see figure 4). In *C. albicans*, calcium homeostasis is regulated by high and low affinity calcium uptake systems (HACS/LACS). Calcium is a secondary messenger pivotal for cell growth. Its release can be triggered by environmental stresses to promote survival. Spf1 is an endoplasmic reticulum ATPase that, alone with another ER ATPase is responsible for calcium homeostasis. In ScSpf1 null mutants (the *Saccharomyces cerevisiae* homologue of Spf1), hyphal growth, biofilm formation and virulence were reduced (Yu, *et al,* 2012).

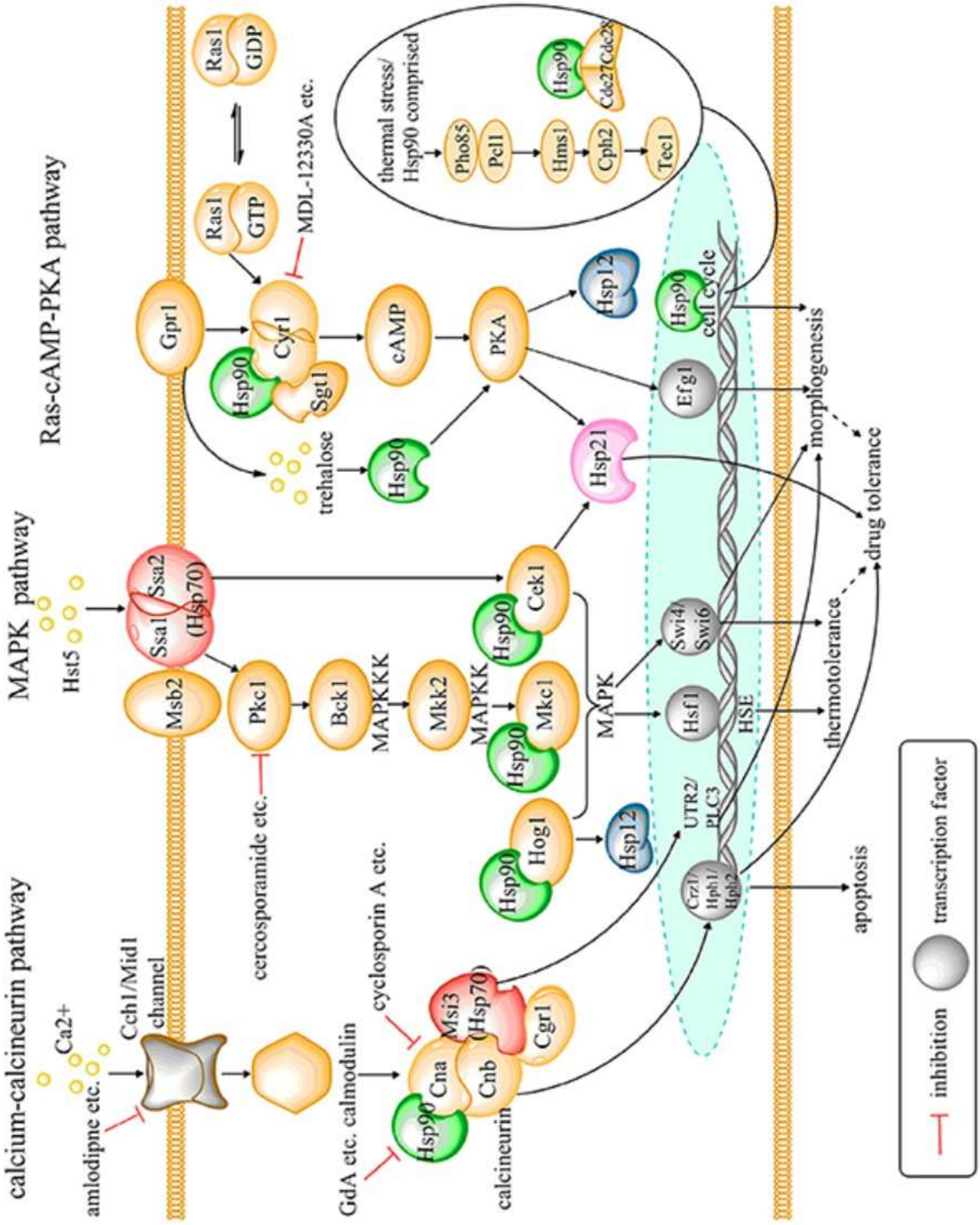

Figure 4- the pathways of both calcium signalling and the heterotrimeric G protein and Ras signalling, Taken from (Gong, *et al,* 2017) The MAP kinase pathway is following on from the

signal transduction discussed in figure 3. The heterotrimeric G protein and Ras signalling occurs as described above. The calcium signalling is induced when external stresses- such a change in pH or temperature- are encountered by the calcium influx channel located within the cell membrane (The HACS/LACS as described above). This induces a rapid influx of calcium into the cell. Calmodulin senses this intracellular calcium increase and binds 3 calcium ions. The calmodulin with the calcium still bound then binds itself to calcineurin subunit A and a calcium ion simultaneously binds to the high affinity binding site on calcineurin subunit B, causing calcineurin activation. This acts on downstream targets such as Crz1 (in the grey bubble on figure 4) by dephosphorylation and translocation from the cytoplasm to the nucleus which then induces a response, one of which includes morphogenesis (Liu, *et al,* 2015).

1.9 Biofilm formation

According to Nobile & Johnson (2015), an acceptable definition of a biofilm is a cluster of aggregated cells adhered to a surface that display different properties to their planktonic counterparts. A *C. albicans* biofilm contains both bud and filamentous forms. It has been shown that there are numerous stages in biofilm development which are; initial adherence by yeast cells, proliferation of the anchored cells to form the biofilm's basal layer, morphogenesis of the two filamentous forms alongside production of the extracellular matrix which is comprised of extracellular polymeric substances (EPS) and yeast cell dispersal (Gulati & Nobile, 2016).

The EPS consists of polysaccharides, glycoproteins, glycolipids and extracellular DNA. This polymer network is highly hydrated and interwoven in a matrix formation to provide a stable environment for cell growth. The hydrated state of the EPS allows for dissolution of molecules and nutrient supply to the biofilm (Flemming, *et al,* 2007). The matrix can respond to environmental stimulus such as pH and temperature and protect the biofilm from host immune cells. Roughly 55% of the dry weight of a *C. albicans* biofilm can be attributed to the protein concentration,

with the predominant proteins being heat shock and glycolytic enzymes. Carbohydrates constitute around 25% of the matrix dry weight and for *C. albicans* biofilms, the most common type of carbohydrates is the mannans. 15% is attributed to the lipids and 5% to the DNA. The structure of the biofilm's matrix differs to that of the cell wall composition (Pierce, *et al,* 2017). An example of this variation is the α-mannan- β 1, 6 glucan complex (MGCx). The mannan in the MGCx is found in much higher quantities in biofilms and the glucan is linear as opposed to branched as it is found in cell wall structures (Mitchell, et al, 2015). The extracellular matrix is able to protect the cells from host immune cells. During a *C. albicans* infection, neutrophils are the primary responders from the host. Under normal circumstances the neutrophils encounter the pathogen and trigger a series of responses which include; phagocytosis, degranulation, the production of reactive oxygen species and the release of neutrophil extracellular trap (NET). NETs are histones, DNA and antimicrobial protein structures used to attack larger organisms and in particular, hyphae which cannot be ingested via phagocytosis. However, these measures fail to eradicate an intact *C. albicans* biofilm. A study by Johnson, et al, (2016) found that in the presence of a *C. albicans* biofilm these NETs fail to release. It is believed to be linked to the extracellular matrix production as prior to this, or if extracellular matrix production is halted leading to an improperly formed matrix, NET release is successful. The reason behind the inhibition is understood to be associated with the concealment of cell surface components as mannan, secreted aspartic proteases and β-glucan in planktonic *C. albicans* cells trigger NET release. This resistance to neutrophil attack varies by strain and species, with *C. glabrata* exhibiting a reduced inhibition when compared to *C. albicans*. (Kernien, *et al,* 2018). Previous studies compared the wild type *C. albicans* against two strains- one incapable of yeast

formation the other incapable of hyphae formation. The hyphae null strain could only form the basal layer and the yeast null only the outer layer (Kojic & Darouiche, 2004). The results from that study compounded the idea that dimorphism is essential for successful biofilm establishment (James, *et al,* 2016). A major concern with biofilm growth is increased antimicrobial resistance. There are numerous ways in which the biofilm can resist, including the extracellular matrix acting as shield for the internal cells by binding the antifungal drugs to reduce penetrance, the use of the membrane transporter systems to reduce internal concentrations of the drugs, gene mutations (such as the ETS related gene- ERG) that lead to increased resistance and activation of the calcineurin pathway via antifungal drug induced stress response (Ramage, *et al,* 2012). This resistance makes it harder to treat the infection and increases the risk of candidaemia.

There are numerous influences for biofilm growth and development. These include, but are not limited to, environmental factors such as the glucose availability - high concentrations encourage biofilm growth- (Pereira, *et al,* 2015). The chemical structure of the contact surface, as demonstrated in a study by Hawser & Douglas (1994). Their results showed that the material used for establishment of a biofilm, such as PVC or polyurethane can influence growth. Temperature is another factor, with 37^0C being a (Sudbery, *et al*, 2004).

1.10 Properties of silicone

Silicone, as a material for catheters, has a tensile strength between 2.4-7 megapascal (MPa). Its molecular structure can be seen in figure 5. Its elongation percentage is between 350-600%. It has excellent UV resistance and good chemical

resistance but moderate adherence properties (Lawrence &Turner, 2005). One issue with silicone rubber is its mechanical strength- there is a risk of the balloon rupturing either during insertion or through prolonged use. There is also a high risk of elongation deformation. These issues are thought to be caused by the weak intermolecular forces between the polymer chains of the compound. The risk of these incidents occurring are reduced via vulcanization. Though it is an effective process it does not entirely remove the less desirable properties of the silicone (Liu, *et al,* 2015). This process is performed most commonly with sulphur and can be applied either by steam/hot air (known as free vulcanization) or via pressure. The sulphur molecules reacts with the carbon- carbon double bonds in an ionic vinyl polymerisation reaction that results in the formation of cross links that ultimately strengthen the silicone rubber (Joseph, *et al*., 2015). In a study by Liu, *et al,* (2015), they investigated the mechanical properties of silicone rubber after vulcanization and its durability after immersion in artificial urine. They imaged the rubber sheets before and after immersion (14 days). From the images there were surface changes to the silicone but no internal structural alterations. The surface alterations they noted appeared to make the material surface area less dense. The surface area of an object can be a factor in *C.albicans* adherence. In particular the roughness, with the rougher surfaces providing more opportunities for bud anchorage, as shown in a study by Da Silva *et al,* (2016).

The issues with catheterisation and infection have been recognised within the medical field for many years (Lawrence & Turner, 2005).

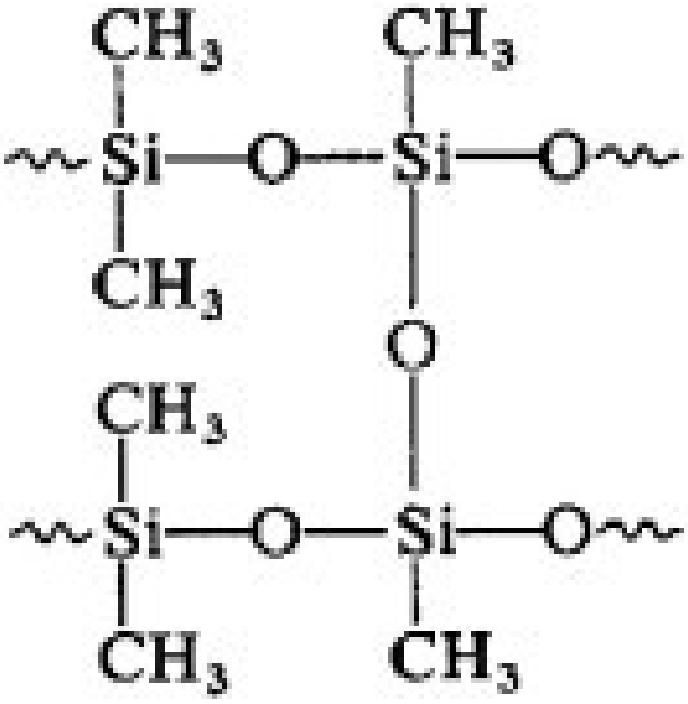

Figure 5- the molecular structure of silicone rubber, taken from *(*Coran, 2013)

1.11 Properties of neoprene

Neoprene is the commercial name for polychloroprene and is the name used throughout this thesis. It is the polymerised form of chloroprene. Its molecular structure can be seen in figure 6. Neoprene was one of the first synthetic rubbers to be produced. It is known for its resistance to oils, flame and oxidation when compared to natural rubbers. It can withstand heat of up to 125^{0}C although they lack the low temperature flexibility of natural rubber. Neoprene has a maximum tensile strength of 27.5 MPa, a maximum elongation percentage of 600% and excellent abrasion resistance (Schaefer, 2002). It has many uses, including insulation, wetsuits and as an alternative material to latex for gloves (Obrecht, *et al,* 2011). Neoprene can be found in various forms which include foam or rubber. This is due to vulcanisation. By introducing the sulphur cross links within the carbon-carbon double bonded structure it can strengthen and harden the structure. Vulcanisation of

neoprene is not only used to strengthen the material but to alter its physical and chemical properties depending on what its intended purpose is (Desai, *et al,* 2007).

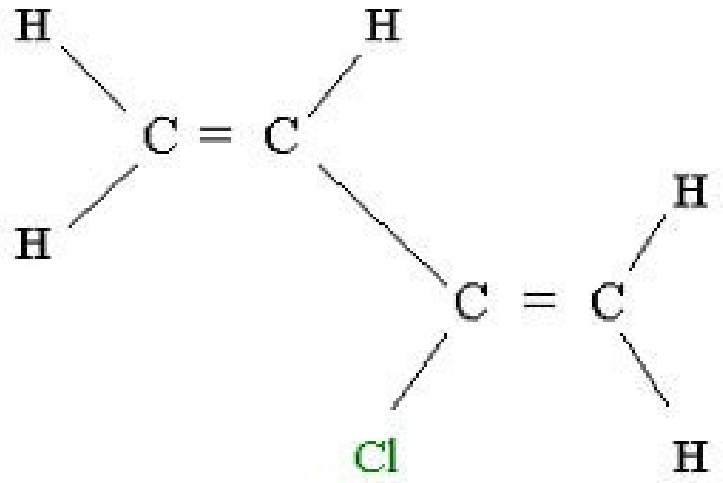

Figure 6- the molecular structure of neoprene, containing carbon-carbon double bonds, taken from (American Chemistry council, 2004). One of the double bonds in this chain will be cross linked to the chlorine atom on another chain and this is what gives neoprene its elasticity (American Chemistry council, 2004).

1.12 Properties of butyl

Butyl rubber, also known as Isobutylene isoprene (IIR) is a synthetic elastomer known for its resistance to liquid, oxygen, chemicals (both organic and inorganic) and a very low gas and liquid permeability. Its molecular structure can be seen in figure 7. It can withstand temperatures up to around 120^0C (Wang, *et al,* 2006). Some applications of butyl include; rubber stoppers and seals for bottle in the pharmaceutical industry, inner tubing of tires and for tubing exposed to high pressures (Thomas, 2010). Butyl is also able to be vulcanised and there are three methods of doing so. The choice is dependent on the intended function of the

material. The structural alterations remain the same, with the addition of cross links to strengthen the molecular structure (Struck & Hanser, 2014). It is considered one of the most robust rubber polymers available (Razzaghi-Kashani, *et al,* 2007).

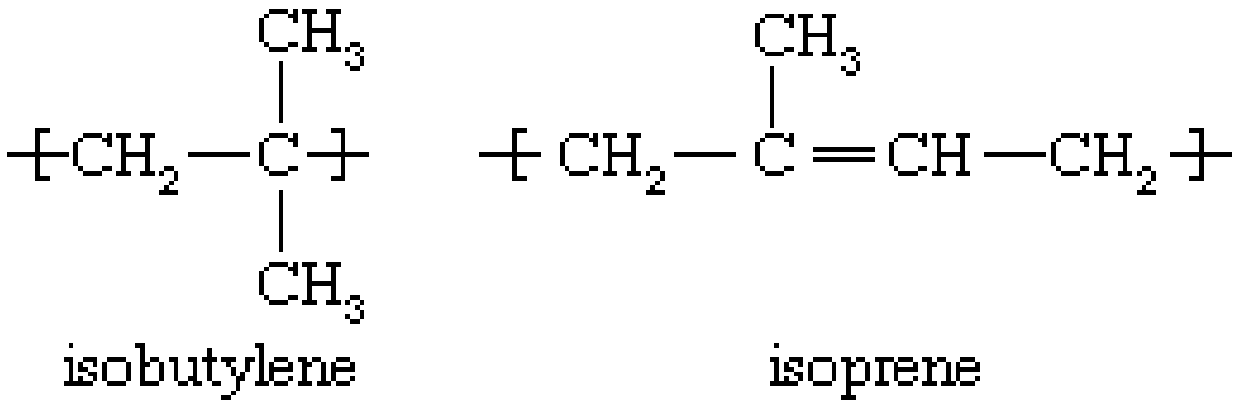

Figure 7- the structure of butyl taken from (Hosch, 2019). Butyl is produced by polymerisation of isobutylene and isoprene to form a copolymer. There are only small amounts of isoprene within the compound as it is found as a volatile liquid at normal temperatures (Hosch, 2019).

There have been no studies previously performed that investigated the growth of *C. albicans* on neoprene or butyl.

1.13 The search for a reduced infection rate

The issues with catheterisation and infection have been recognised within the medical field for many years (Lawrence & Turner, 2005). With the introduction of a foreign object to the body, there is always a risk of infection. The need for implanted medical devices is unavoidable, so there have been many efforts to investigate means of reducing this infection risk (Von Eiff, *et al,* 2005). Some methods have

investigated a coating for the catheter, such as silver which is biocompatible and was shown to reduce antimicrobial activity (Rupp, *et al,* 2004). However not only is it costly, when one patient study compared annual cost of Foley catheters-silver coated and non-coated- the difference in price totalled over £23,000 (Dikon & Olah, 2006), it has had variable results across many studies (Lawrence & Turner, 2005). There has been investigation into alternative materials, such as polyvinylchloride, but this was deemed inappropriate due to additive leaching *in vivo* (Singha, *et al,* 2017). Investigations into possible methods of reducing infection rates are ongoing, and the aim here was to investigate previously untested materials (butyl and neoprene) as potential alternatives to silicone with an aim to reduce these infection rates of indwelling catheters that currently account for almost 80% of nosocomial infections globally (Jacobsen, *et al,* 2008) by measuring the average growth of *Candida albicans* biofilms for up to 48 hours' incubation. Neoprene, shares similar properties to silicone and is biocompatible, being used for wetsuits and gloves (Obrecht, *et al*, 2011). Butyl is a very dense material that has previously shown issues with biocompatibility when used a fracture fixation device (Vasenius, 1988) but has been adjusted to demonstrate excellent biocompatibility in more recent studies (Kun, *et al,* 2012).

2. METHODOLOGY

The following experiment were carried out with approval by the Biological Safety Committee and with ethical approval from the School of Pharmacy and Pharmaceutical Sciences Ethics, Health and Safety Committee.

2.1 *C. albicans* stock preparation

C. albicans strain NCPF 3179 (Public health England culture collections) on a parent plate of malt extract agar was used to create a 20% frozen glycerol brain heart infusion-BHI- (VWR chemicals, Prolabs, Leicestershire) (calf brain 12.5g/L, beef heart 5g/L, proteose peptone, 10g/L, disodium phosphate, 17.6mM, glucose 11mM, sodium chloride 85mM pH 7.4). stock that would be used for the entire experiment. The 20% glycerol stock were stored in a -80 freezer. The stock plate was incubated overnight at 37^0C (GenLab prime incubator) then refrigerated for the week before being discarded.

2.2 Preparation of materials

A 2mm cork borer were used to obtain the discs. The sheets of the material (all 1.5mm in thickness to ensure even discs) were laid flat on the bench, the cork borer was sharpened prior to use (a dull cut would increase the risk of jagged edges which would affect adherence) and the discs freshly cut for each test. Each disc post cutting had a thickness of 1.5mm and a diameter of 2mm.

The three materials selected for the experiment were silicone elastomer (Camthorne Industrial Supplies, Staffordshire), neoprene (T & P Supplies, Bolton)

and butyl rubber (White Cross Rubber Products Ltd, Lancaster). After each disc were cut, they were placed into a sieve where detergent (household) was added and then they were scrubbed, in the sieve, with the brush and thoroughly washed with distilled water to remove all detergent traces. The materials were washed in their groups (silicone discs for that experiment altogether etc). As the butyl and neoprene were the same colour and the silicone and neoprene tended to stick together, this was done to avoid confusion of materials and making sure all surfaces were evenly washed.

All three materials had to be sterilised in the same manner in order to create an equal and fair comparison of *C. albicans* growth and to reduce variables that could impact the experiment. Originally, research into the materials showed that although neoprene and silicone could withstand high temperatures such as autoclaving, butyl could not as it was prone to blister (Wang, *et al,* 2006). Based on this information a cold sterilisation technique was applied. The technique was based on the one used by Samaranayake, *et al.,* (2013) with minor adjustments.

A 10% (v/v) sodium hypochlorite solution in distilled water (Fisher scientific, Loughborough) was used. The discs were prepared as above and then submerged into the sodium hypochlorite solution. They were washed twice in sterile distilled water with agitation for 3 minutes before transfer to nutrient broth (Sigma Aldrich, Dorset) incubated at 37^0C for 48 hours. This is all took place within a class 2 microbiological safety cabinet with sterile materials (BioMat 2, Contained air solutions, Middleton) to maintain a sterile environment. After this the universals were removed and checked for turbidity, which would be a sign of microbial growth. This

technique was not successful, so another sterilisation method was sought, autoclaving.

After washing, the materials were submerged into distilled water and autoclaved and once cooled, transferred to the media for inoculation.

2.3 Optical density

Optical density readings were carried out at 600nm to produce a standard curve which would be used to determine the cells per ml of the *C. albicans.* An overnight culture in malt extract broth-MEB- (Sigma Aldrich, Dorset) (malt extract 17g/L, mycological peptone 3g/L pH 5.4). were used. MEB was made according to manufacturer's guidelines.

The culture was then centrifuged (MSE Micro Centaur) at 1,330gfor 3 minutes. The spectrophotometer (Jenway 7315, Staffordshire) were blanked with ¼ strength Ringer's. Further readings were taken at 600nm.

The first sample were read and then diluted to the desired absorbance. This desired absorbance was 0.8 to obey Beer- Lamberts law. Once the cells were read at 0.8, 0.1ml were taken out and placed on a haemocytometer. They were then counted using a Nikon Eclipse E200 microscope (Nikon, UK). The number of cells were determined by counting cells from the top right, left, bottom right, left and central square then the following calculations were carried out:

Number of cells ÷ 80 X 1÷0.00025 = answer x dilution factor

This gave the cells per ml. This were repeated three times. The results were plotted on a graph to produce the standard curve.

2.4 Disc inoculation

Cells were standardised to 10^7 cells/ml in the spectrophotometer, read at 600nm. This high density of cells was selected partly due to the studies this methodology was based on using this amount, like Nweze, *et al,* (2012) and Mateus, *et al, (*2004*).* Also this high density of cells was not going to be placed in a well with the discs and incubated the discs were exposed to this density of cells for a short period then removed so the issues with using high densities of cells, such as quorum sensing and reduced filamentation (Kruppa, 2009) would not impact this investigation.

Cells were harvested from an overnight culture (cells at stationary phase) and washed as described in section 2.3. Cells were diluted to the corresponding absorbance based on the standard curve prepared above and then the ¼ strength Ringers were exchanged for the same volume of appropriate media (either MEB or Roswell Park Memorial Institute 1640- Sigma Aldrich, Dorset-) (Glycine, 0.13mM, L-Arginine, 1.14mM, L-Asparagine, 0.37mM, L-Aspartic acid, 0.15mM, L-Cystine2HCl, 0.20mM, L-Glutamic Acid, 0.13mM, L-Glutamine, 2.05mM, L-Histidine, 0.096 mM, L-Hydroxyproline, 0.15 mM, L-Isoleucine, 0.38 mM, L-Leucine, 0.38 mM, L-Lysine hydrochloride, 0.27 mM, L-Methionine, 0.10 mM, L-Phenylalanine, 0.09 mM, L-Proline, 0.17 mM, L-Serine, 0.28 mM, L-Threonine, 0.16 mM, L-Tryptophan, 0.024 mM, L-Tyrosine disodium salt dihydrate, 0.11 mM, L-Valine, 0.17 mM, Biotin, 8.1967213E-4mM, Choline chloride, 0.021 mM, D-Calcium pantothenate, 5.24109E-

4mM, Folic Acid, 0.0022 mM, Niacinamide, 0.0081 mM, Para-Aminobenzoic Acid, 0.0072 mM, Pyridoxine hydrochloride, 0.0048 mM, Riboflavin, 5.319149E-4mM, Thiamine hydrochloride, 0.0029 mM, Vitamin B12, 3.690037E-6mM, i-Inositol, 0.19 mM, Calcium nitrate (Ca(NO3)2 4H2O), 0.42 mM, Magnesium Sulfate (MgSO4) (anhyd.), 0.40 mM, Potassium Chloride (KCl), 5.33 mM, Sodium Bicarbonate (NaHCO3), 23.8 mM, Sodium Chloride (NaCl), 91.3 mM, Sodium Phosphate dibasic (Na2HPO4) anhydrous, 5.6 mM, D-Glucose (Dextrose), 11.1 mM, Glutathione (reduced), 0.0032 mM, HEPES, 25 mM, Phenol Red, 0.013 mM pH 7.2).

and the resulting volume were added to sterile universals containing the discs to ensure all discs were exposed to the same amount of cells. The total volume of media in the universal was 2.5ml. This volume of media was enough to ensure that during inoculation the discs would remain submerged. Each disc was placed in an individual bottle to ensure that all discs were exposed equally. The universals were secured in the 37^0C orbital shaker for 2 hours at 180rpm for the initial adhesion period. After this the discs were removed, loosely adherent cells washed off with sterile Phosphate Buffered Saline (PBS, 137 mM NaCL, 10 mM phosphate, 2.7 mM KCL, pH 7.4) and the discs transferred to a 96 well plate with fresh media. Washing of the discs were performed by sterile tweezers removing the discs from the universals and being held over a glass beaker and gently washed with 1ml of PBS. A control for each material for each test carried out were prepared in the same manner as described above without the addition of the *C. albicans*. The controls were placed in the same well plate but on an opposite side to their inoculated counterparts. The adhesion period of 2 hours was selected due to the studies used as basis for this methodology. For example, studies like those carried out by Nweze, *et al,* (2012) and Lee, *et al,* (2016) inoculated for 90 minutes after coating the

substrate with foetal bovine serum to aid adherence. In the study by Mateus, *et al,* (2004) they did not coat the discs prior to inoculation and extended the incubation period by 30 minutes to 2 hours. The adhesion period was to enable the first phase of biofilm development, the initial adherence by C. albicans cells, forming the basal layer of the biofilm which would further develop during the 24/48-hour period.

The purpose of this method was to replicate the incidence of infection via implantation of a catheter. The plates were either placed in the 30^0C incubator or the 37^0C incubator for either 24 or 48 hours.

2.5 Crystal Violet stain

The supernatant was aspired from the wells of the plate and washed with distilled water. As the discs in the wells were not fixed to anything, the plate could not be washed following commonly used methods (submerging the whole plate into a bowl of water and tipping the plate upside and down and tapping the bottom gently). Instead, the wells were washed using a pipette and gently releasing 300µl (maximum volume of the well) of distilled water into the well and then aspiring it out. 125µl of 0.1% crystal violet were added to each well and incubated at room temperature for 15minutes. The crystal violet was removed, and the plate washed again in distilled water in the same manner described previously to remove excess stain and this was carried out 3 times in total. The discs were transferred to another well in the same plate (the purpose of this was to ensure only the biofilms on the discs would be solubilised and that any *C. albicans* that had adhered to the plate wall were dead). The plate was then placed overnight in the 30^0C incubator to dry. 150 µl of glacial acetic acid (30%) were added to each well and left to incubate at room temperature for 15 minutes. After this, 125 µl were aspired out and transferred to a new plate which was then read by the FluroOmega star plate reader ((BMG tech,

Aylesbury, Buckinghamshire) at 550nm. Results over 0.8 were diluted to obey Beer-Lambert's law. All results were multiplied by 1.2 to account for the original volume of glacial acetic acid added to each well. The controls were processed as described above and a blank well was also used each time to subtract the glacial acetic acid from the results (after the dilution factor had been accounted for).

2.6 Synthetic urine preparation

The original design of the experiment had a synthetic urine that would be made and used as the media for the experiment to replicate physiological conditions as much as possible. The recipe used was based on a synthetic urine recipe created by Brooks & Keevil, (1997), (recipe peptone, 1.37g, yeast extract, 0.1ml, lactic acid, 1.1mmol, citric acid, 2mmol, urea 170mmol, uric acid 0.4mmol, creatinine 7mmol, $CaCl_2$ 2.5mmol, NaCl 90mmol, $FeSO_4$ 0.005mmol, $MgSO_4$ 2mmol, Na_2SO_4 10mmol, KH_2PO_4, 7mmol, NH_4Cl 25mmol, K_2HPO_4 7mmol, $C_6H_{12}O_6$ 5.5mmol). The synthetic urine were made as a ten times concentrated stock and diluted down using sterile distilled water.

The strain of *C. albicans* used for the experiment were selected due to its ability to form buds, hyphae and biofilms. This was tested by visually examining the growth of *C. albicans* in a 96 well plate using an inverted microscope (Nikon Eclipse E200 and Nikon Digital Sight camera attachment). The plate was inoculated with cell standardised to 10^7 cells per ml as described above. The cells were cultured overnight in malt extract broth. The plate was left overnight in a 37^0C incubator to grow (statically). After 24 hours the plate was removed and washed three times with water and tapped gently on the bottom to remove excess water and loosely adherent

cells. The plate was then stained with 0.1% crystal violet and washed three times after a 15-minute incubation period to remove excess stain. The plate was left to dry overnight and then examined under the inverted microscope. A two tailed unpaired Student's test were used for the statistical analysis of the data. The main purpose of the experiment was to compare the growth of the two new materials (neoprene and butyl) to silicone, the current standard, to see whether there were any significant differences in the growth of *C. albicans*. As a Student's T test compares the means from two groups it was deemed appropriate to use this method of analysis.

3. Results

3.1 *C albicans* adhesion and biofilm development on materials

The ability of the yeast like fungus *Candida albicans* to colonize on the surface of a variety of materials has been extensively investigated in studies such as those by Kojic and Darouiche (2004) and (Singha, *et al,* 2017). Which investigated different materials such as silicone, polyvinylchloride (PVC) and polypropylene. In the study by Kojic and Darouiche (2004), the highlighted the importance for dimorphism for successful biofilm establishment of materials by *C. albicans*. The study by (Singha, *et al,* 2017) investigated polyvinyl chloride as an alternative catheter material but found it was not biocompatible. There has been investigations into what encourages *C.albicans* adhesion and biofilm development on materials including the role of the Als protein family (Nailis, *et al,* 2008) which found that on silicone both Als1 & Als3 had important functions for both adhesion and biofilm establishment. Hawser & Douglas (1994) discovered that there are numerous factors

that influence the development which included the environment and the structure of the contact surface. A study by Da Silva et al, (2016) highlighted the surface texture was also a factor, with rougher surfaces providing a more hospitable anchor for adhesion but this was not always a linear relationship.

To investigate a potential alternative to silicone as an indwelling catheter material, three materials, silicone, neoprene and butyl were selected and cut to 2mm discs with a diameter of 1.5mm.the discs were submerged into the three media selected for the experiment in order to assess any damage or changes the media may cause to the materials as neither neoprene or butyl have been used for this type of experiment previously and so there was no data on the media effect on the material. The discs were originally submerged in the media for one week at 37^0C and then examined using light microscopy. After this experiment, discs were inoculated with stationary phase cells and submerged into the media for either 24 or 48 hours (statically). Control discs were also submerged and stained but not inoculated. The average growth of the biofilm, as determined by a crystal violet stain and spectrophotometry reading, was analysed using a Student's T test (two tailed).

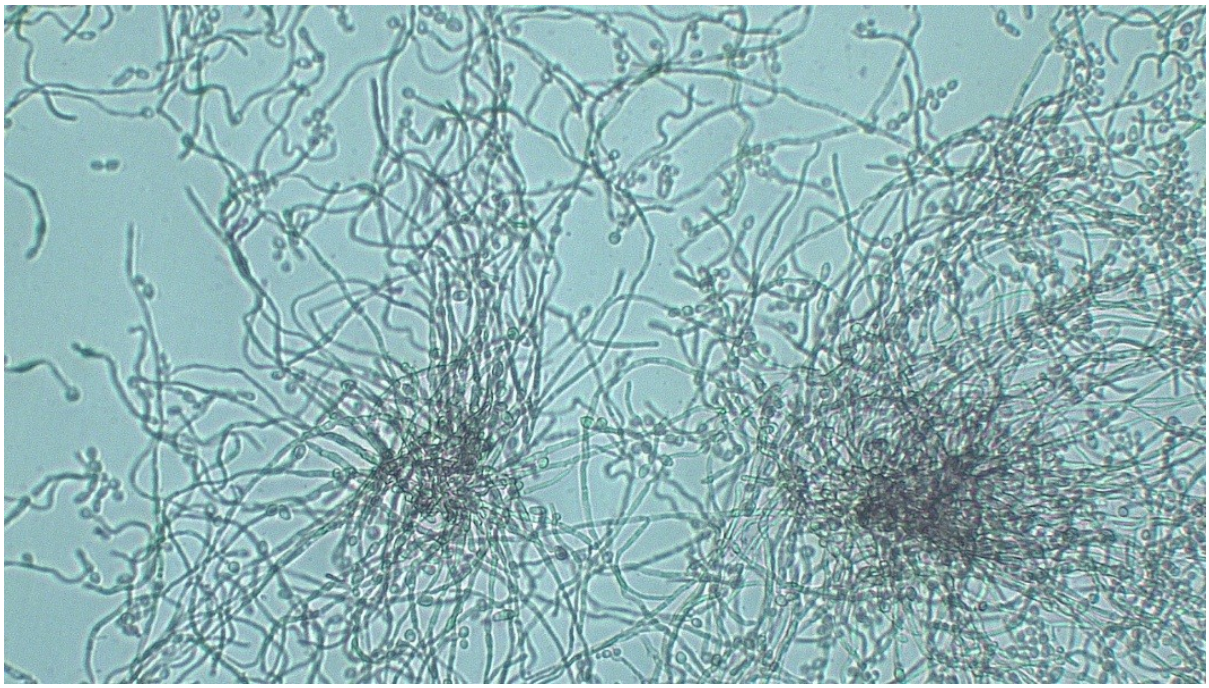

Figure 8 - A X20 magnification of *C.albicans* grown in MEB for 24 hours, incubated at 37^0C. There is a mixture of both budding yeast cells and filamentous forms and the formation of two biofilms. Cells were grown statically overnight in a 96 well plate. After 24 hours they were washed with sterile distilled water and left to dry overnight in a 30^0C incubator. They were stained with 0.1% crystal violet and examined using inverted microscopy.

The purpose of this imaging was to identify that the strain that would be used for the experiment, NCPF 3179, was in fact able to produce both buds and filaments (both types of the filamentous forms, as **figure 10** demonstrates) and was able to form biofilms. As this was simply to confirm the strain used was able to produce the above, it was deemed acceptable that all the perimeters of the experiment did not need to be undertaken for this and so only cells grown in MEB at 24 hours growth at 37^0C were used.

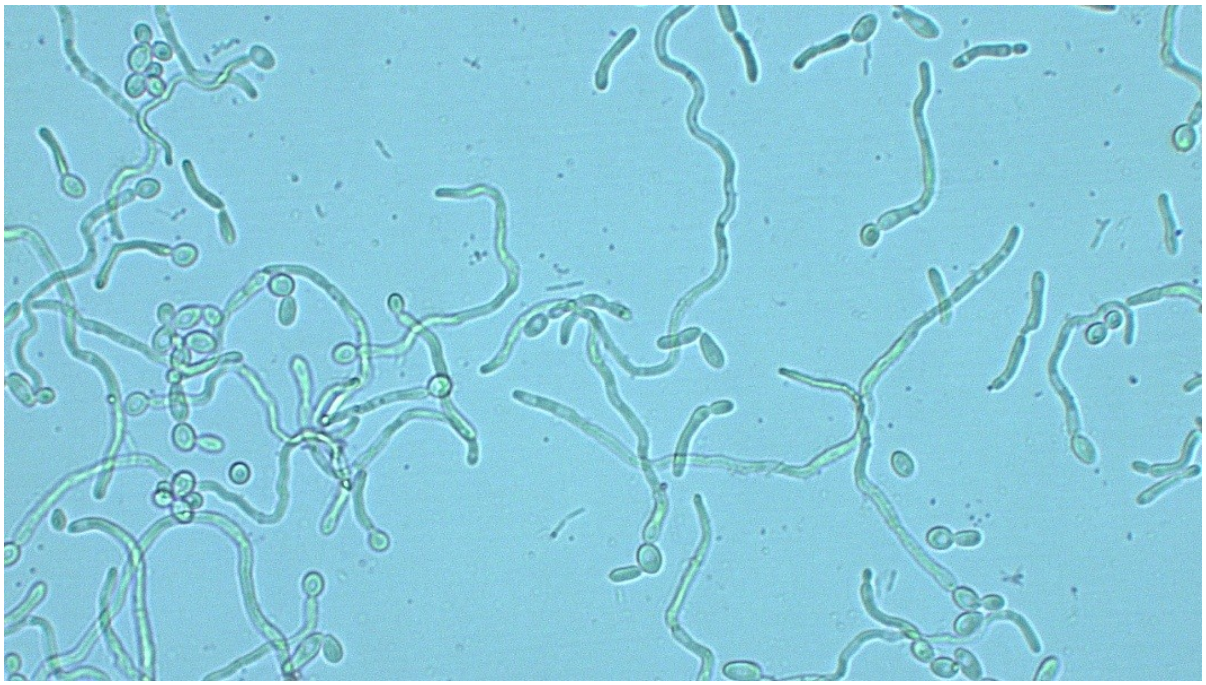

Figure 9- A X40 magnification of *C.albicans* grown in MEB for 24 hours, incubated at 37^0C. Cells were grown statically overnight in a 96 well plate. After 24 hours they were washed with sterile distilled water and left to dry overnight in a 30^0C incubator. They were stained with 0.1% crystal violet and examined using inverted microscopy.

There are mainly filamentous forms in this image although there are some single cells remaining. Some pseudo hyphae are distinguishable from true hyphae at this magnification.

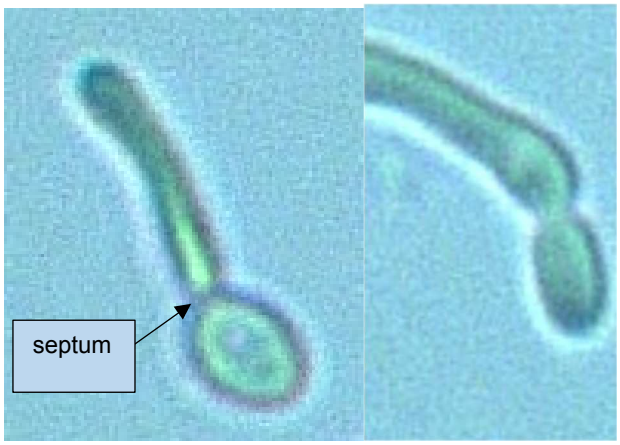

Figure 10- Enhanced images from the previous image. *C.albicans* grown in MEB for 24 hours, incubated at 37^0C. Cells were grown statically overnight in a 96 well plate. After 24 hours they were washed with sterile distilled water and left to dry overnight in a 30^0C incubator. They were stained with 0.1% crystal violet and examined using inverted microscopy.

Left to right; image one shows an example of pseudo hyphal *C.albicans* with an arrow pointing towards the septum that defines the filament as a pseudo hyphae. The second image, on the right, appears to be a true hyphal extension. There is narrowing of the cell but the septum has not formed and therefore the filament is an extension of the mother cell with no separation. Both true and pseudo hyphae are considered the filamentous forms of *C.albicans* (Hameed, *et al*, 2018).

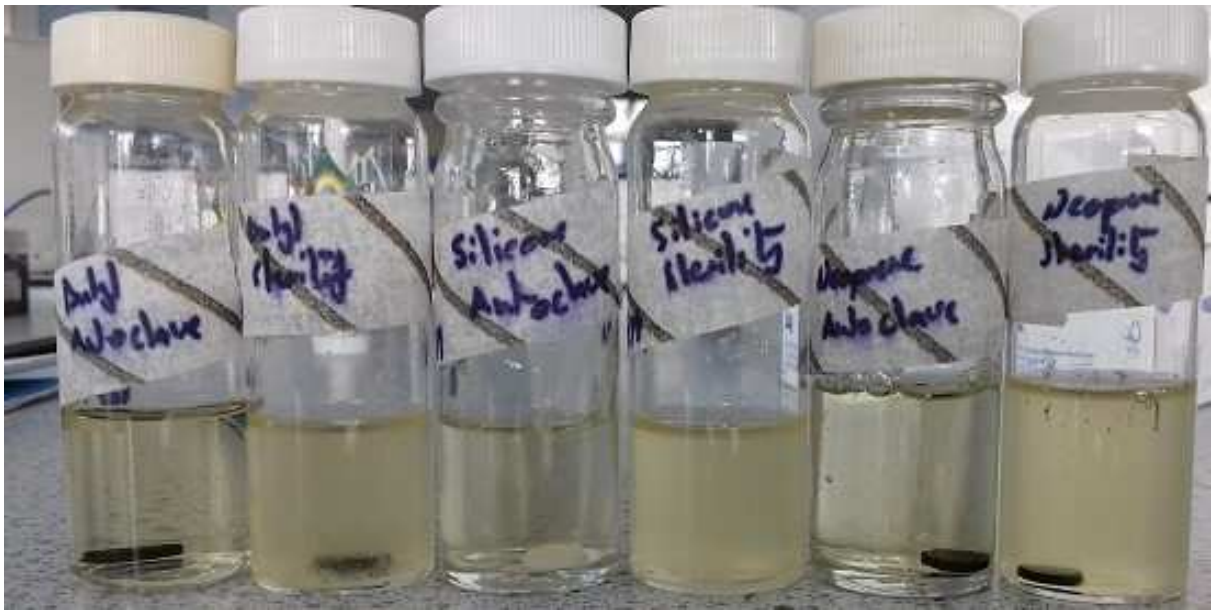

Figure 11 The testing of the sterility methods. One disc per universal suspended in Nutrient Broth for 48 hours in a 37^{0}C incubator. The labels identify what material is in the bottle and what method was used- either autoclaving or cold sterilisation using 10% Sodium Hypochlorite (labelled as sterility on the universals). From left to right; butyl, autoclave, butyl cold sterilisation. Silicone autoclave, silicone cold sterilisation. Neoprene autoclave, Neoprene cold sterilisation. The discs were transferred to the universals containing sterile nutrient broth using sterile tweezers which were ran through a blue flame after each transfer. The nutrient broths containing the discs were then incubated.

This method proved variable, sometimes the discs were sterile and other times were not with butyl being the material most commonly not sterile. There were numerous reasons why this technique proved so variable with the most likely cause being human error. The nature of this technique required more stages at which contaminants could be introduced such as the transference of the discs than autoclaving which is why autoclaving showed itself to be the much more reliable technique as shown above with the discs sterilised via autoclaving having no turbidity (repeatedly, as this comparison test was carried out over 3 times on separate days). As autoclaving reaches a temperature that butyl is not able to withstand (Wang, *et al,* 2006), the materials were all autoclaved and visually

examined with light microscopy to check for surface changes (see figures 12, 13, 14) to ensure that autoclaving would be a suitable technique to use going forward. A possible reason why butyl was the most common material not to be sterilised may be its textured surface. During the washing stages, all materials were brushed gently to remove anything stuck to the surface but not harshly brushed to avoid causing scratches or opening pores on the material which would then affect the experiment. It is possible that this technique was not adequate enough to remove all contaminants from the butyl thus leading to failure of sterilisation. Whereas when using the autoclaving, there is no chance of any contaminant being left as it reaches far too high a temperature to allow any contaminant to remain (Rutala & Weber, 2004).

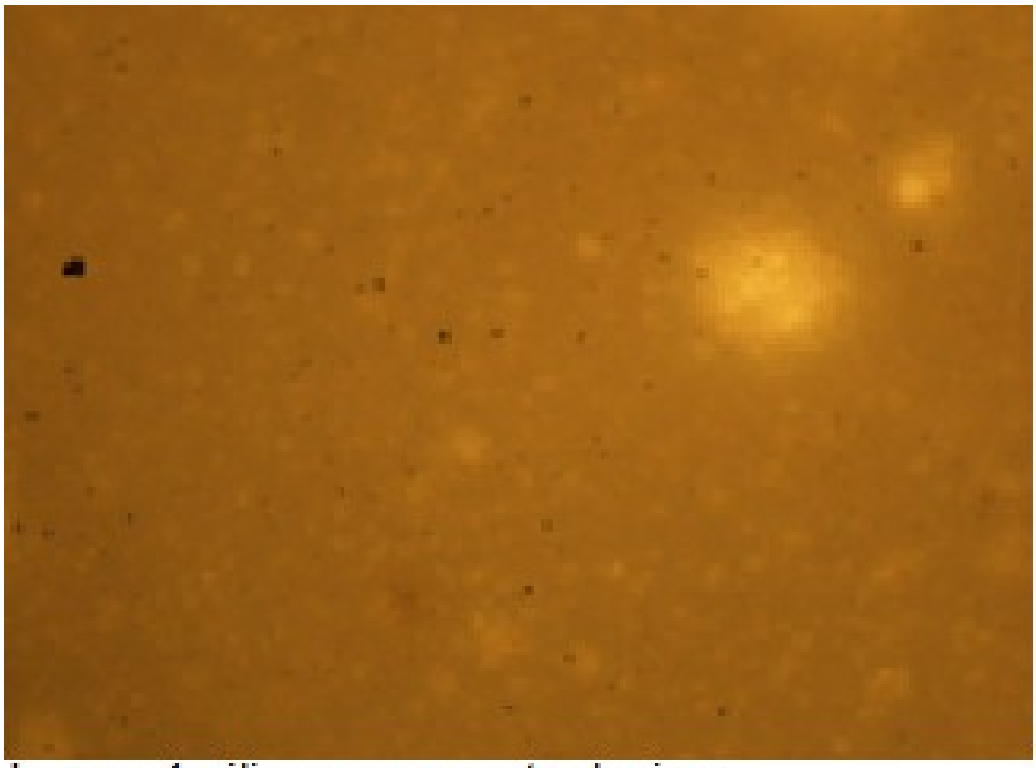

Image 1 silicone pre-autoclaving

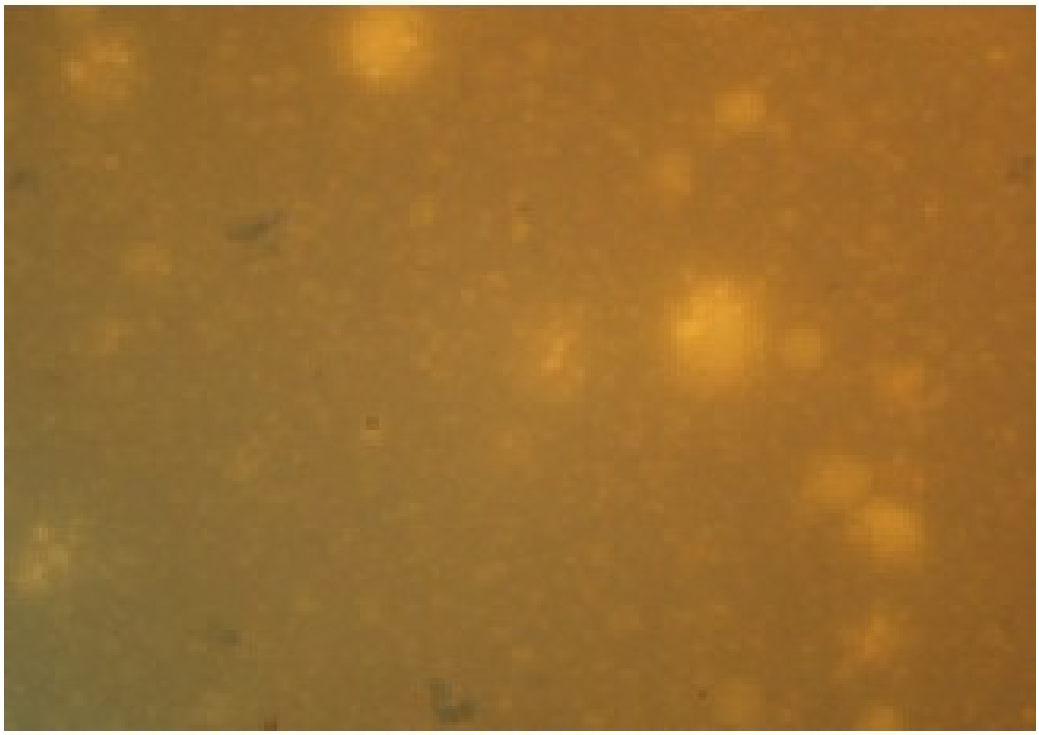

Image 2 post- autoclaving

Figure 12- The autoclaving images for silicone. Autoclaved to 121^0C and visually examined with a Nikon Eclipse E200 camera and image captured with a Nikon Digital Sight camera attachment at x200 magnification.

Although both silicone and neoprene can withstand this temperature, all three materials were subject to this examination to check for any surface topography changes. The dark spots on the material, seen more clearly on image1 were part of the material- it wasn't removed with washing or scrubbing. On both images there appears to be areas that are thinner, which allow more light through and appear to be bright spots under the microscope. The apparent varying thickness of the silicone was taken into account when analysing the growth on the discs. As it was a part of the material and present prior to any testing it was deemed to be a part of the normal structure. It was determined that there was no damage from autoclaving. The material was not stained in order to visually examine.

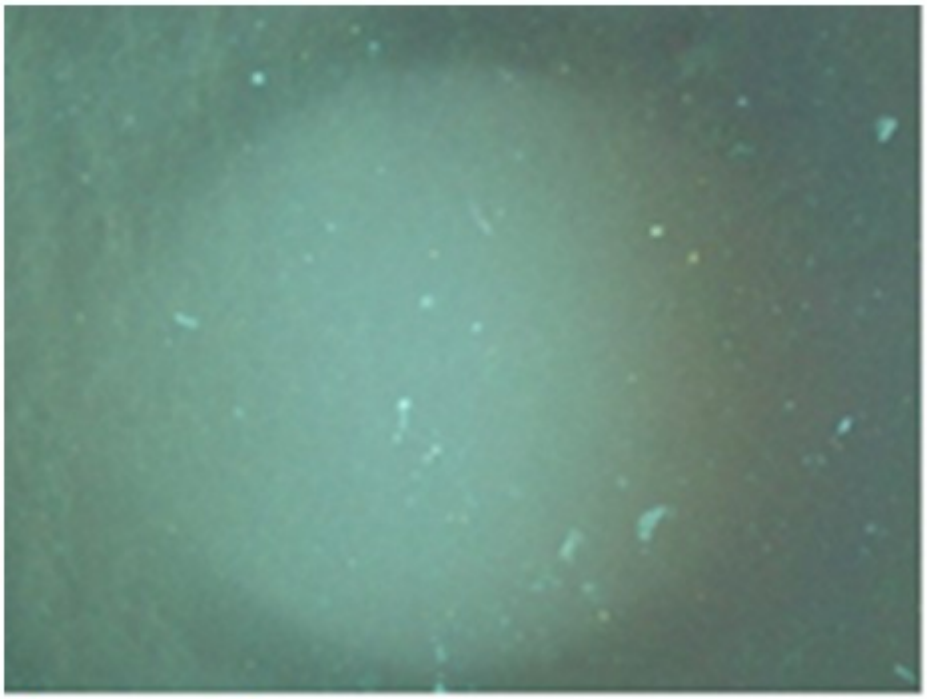

Image1 neoprene pre - autoclaving

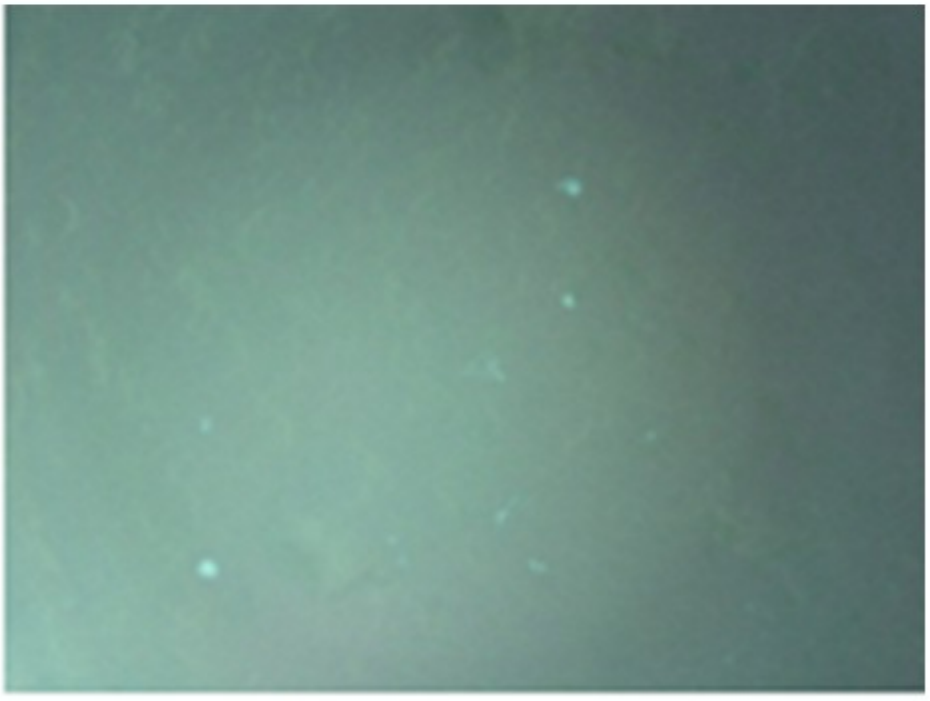

Image2 neoprene post autoclaving

Figure 13 Neoprene autoclaving test. Autoclaved to 121^{0}C and visually examined with a Nikon Eclipse E200 camera and image captured with a Nikon Digital Sight camera attachment at x200 magnification.

Bright dots on both image1 & 2 appear to be holes but this is the normal topography of this material used in the experiment (determined by cutting sections from the sheet at different points to assure that this was a uniform occurrence). It was determined that there was no damage from autoclaving. The material was not stained in order to visually examine.

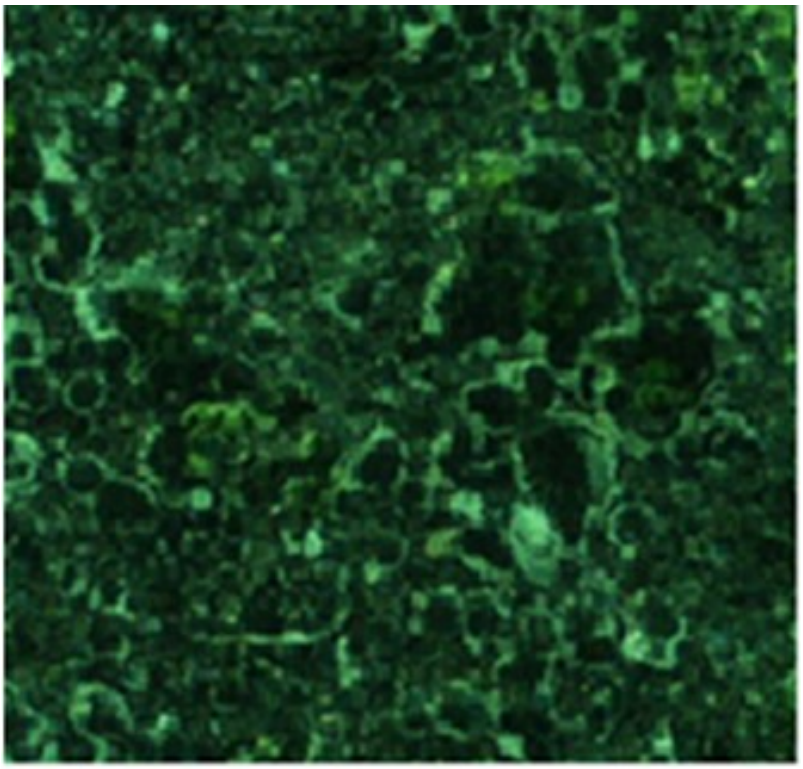

Image1 butyl pre autoclaving

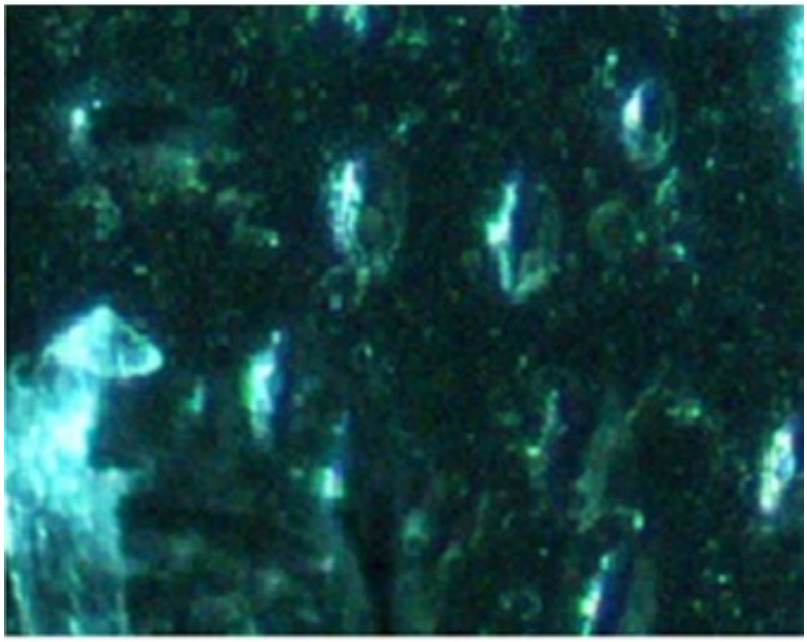

Image2 butyl post autoclaving

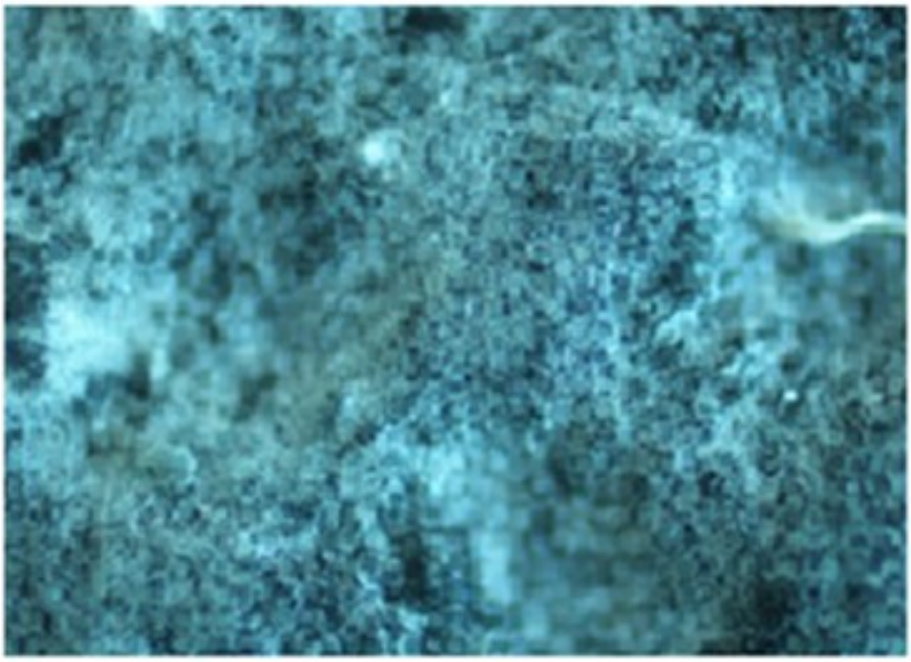

Image3 butyl post autoclaving

Figure 14 Butyl's autoclaving test. As this material is known to withstand temperatures of 120^0C and the autoclave is 121^0C the butyl was imaged to assess any possible damage. The butyl in image 3 was autoclaved and removed from the universal with sterile tweezers then placed in a 96 well plate containing *C.albicans* cells suspended in malt extract broth and incubated at 37^0C overnight then visually examined.

Although there does appear to be some warping of the butyl from its original topography, there were no holes so it was agreed that it would continue to be used and sterilised using autoclaving. To prevent blistering, the materials were all submerged in distilled water prior to autoclaving. Image2 contains some water molecules that have attached to the butyl, which are the crystalline type structures. Image3 was taken after autoclaving and after submergence in MEB for 24 hours

incubated at 37^0C. The MEB was inoculated with *C.albicans*. The purpose was to try and capture some images of the *C.albicans* on the materials. It was not possible using the equipment- Nikon Eclipse E200 camera and image captured with a Nikon Digital Sight camera attachment- as clear images were almost impossible to obtain. However, the butyl dried out during the image capture process and image3 is what was eventually caught. Unfortunately, this could not be performed with the Neoprene as it was only the Butyl that dried like that- its surface wasn't smooth like the neoprene, it had some texture so when it- and the *C.albicans* that was adhered to it- air dried this was the result. The purpose of including image3 is to show that the autoclaving effect was not as great as it appeared to be during the autoclave test, as the contrast of the white *C.albicans* on the black Butyl which then air dried to produce the image gave a much clearer picture of the state of the Butyl after autoclaving. And as the material was black, there wasn't a stain available at that time that could be used to demonstrate this. A x400 magnification was used for all 3 images.

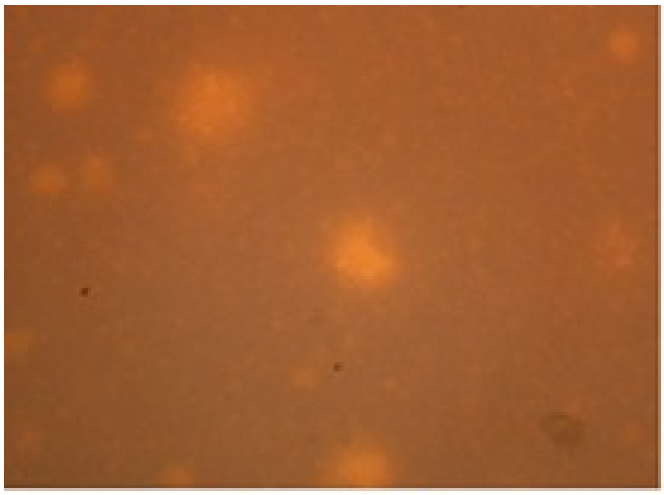

Image 1 silicone MEB

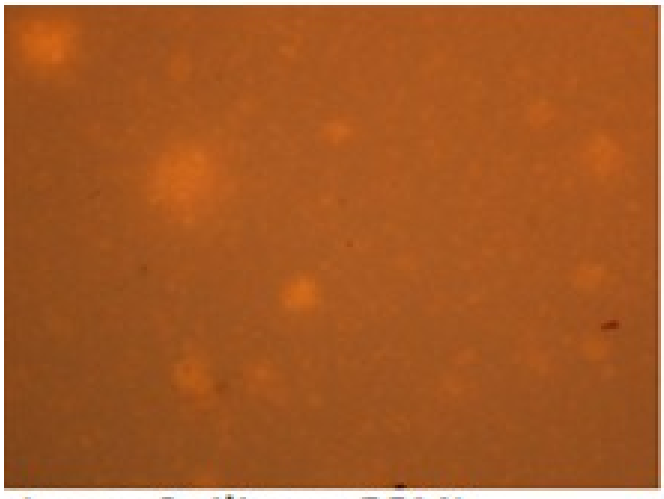

Image 2 silicone RPMI

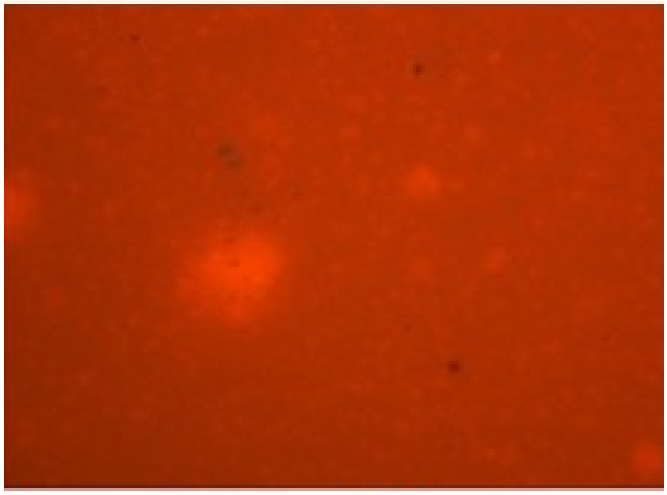

Image 3 silicone SU

Figure 15 Originally there were three media that would be used for growing the *C. albicans;* MEB, RPMI 1640 and synthetic urine. In order the ensure there was no damage to the materials from the media, all three materials were submerged for 1 week in universal bottles at 37^0C in the three medias.

Comparing images1 & 2 to images 1 & 2 from figure 12, there's no difference, so MEB and RPMI 1640 have had no effect on silicone. However the synthetic urine has caused discolouration on the disc on image3. The two most likely compounds from the synthetic urine recipe that could have caused this are; ferrous sulphate and urea**.** The reason it would have discolouration silicone and not the other two materials is because the silicone was white and the other two materials were black. Other than a discolouration there was no damage to the material by any of the media.

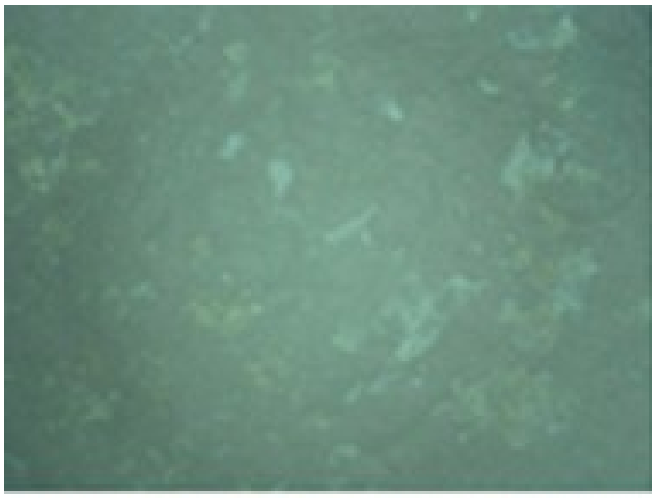

Image1 butyl MEB

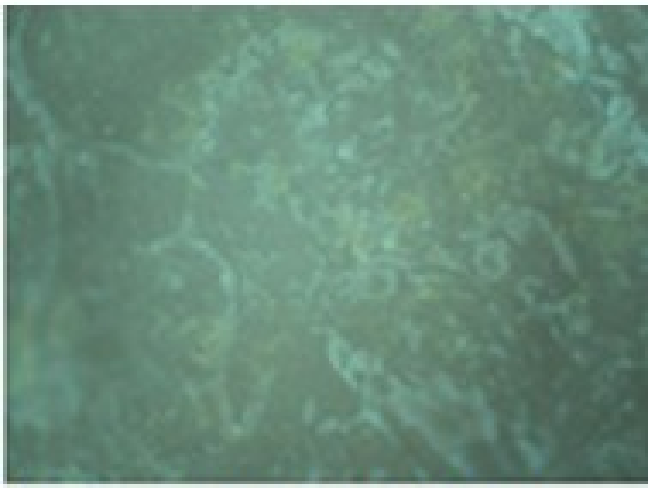

Image 2 butyl RPMI

Image3 butyl SU

Figure 16 Butyl imaged at x200 magnification using a Nikon Eclipse E200 camera and image captured with a Nikon Digital Sight camera attachment. The materials were all submerged for 1 week in universal bottles at 37^0C in the three medias selected for the experiment.

On all three discs, some of the media has adhered to the discs, which are the discoloured (pale yellow/beige) spots on the discs. The white residue on the materials is the result of air drying. The butyl used in this experiment came with a chalky residue on its surface and, despite washing with detergent and scrubbing with a soft bristled brush, some residue would remain if the material was dampened and then dried.

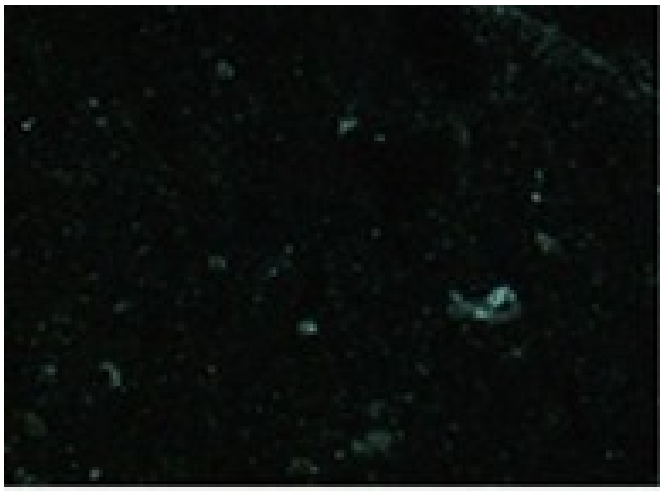

Image1 neoprene MEB

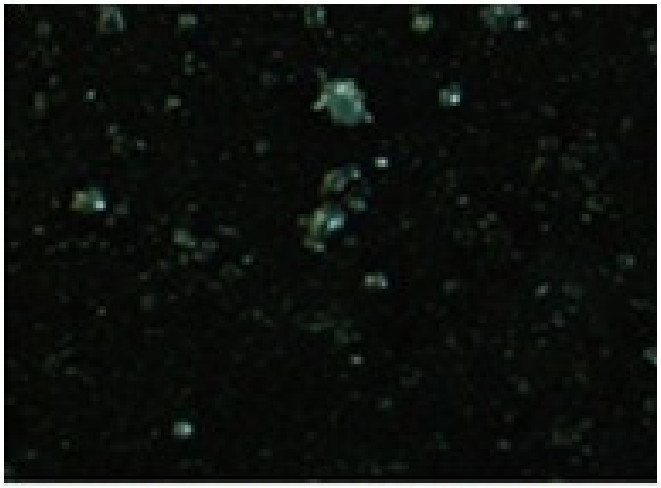

Image2 neoprene RPMI

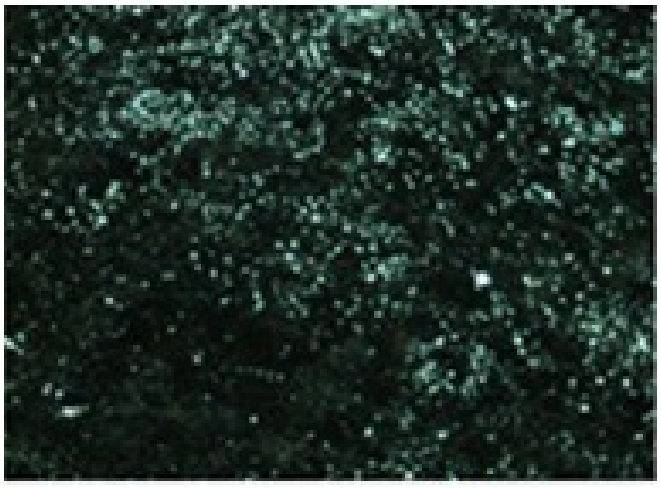

Image3 neoprene SU

Figure 17 Neoprene imaged at X200 magnification using a Nikon Eclipse E200 camera and image captured with a Nikon Digital Sight camera attachment. The materials were all submerged for 1 week in universal bottles at 37^0C in the three medias selected for the experiment.

There are no changes between MEB and RPMI (Images 1&2), the material has reacted the same in both media and they look similar. Image3 however is full of holes. It was decided to investigate this further by submerging the neoprene (in triplicate) into synthetic urine for a total of two days incubated at 37^0C and examine the discs. This would only be with the neoprene as the other two materials did not have holes caused by the synthetic urine like neoprene did. There has been no study previously undertaken to examine the effect of urine or synthetic urine on this type of neoprene (the solid sheet) and it required further investigation. So this test was repeated again, only with neoprene and using three discs submerged at 37^0C. The discs were removed once every 24 hours and examined using light microscopy to gain a better understanding of how long the holes took to develop.

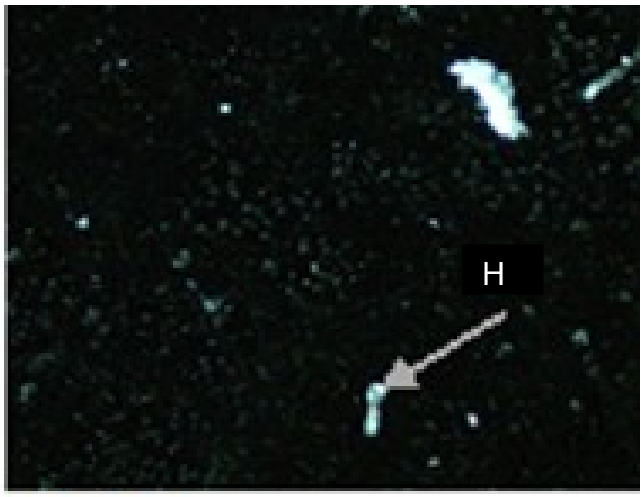

Image 1

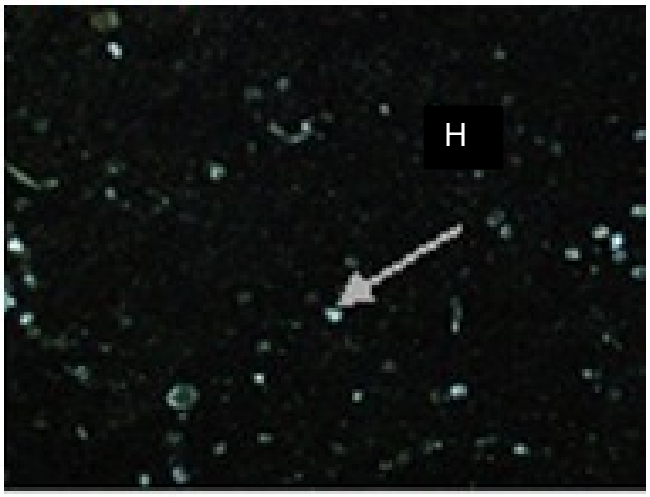

Image 2

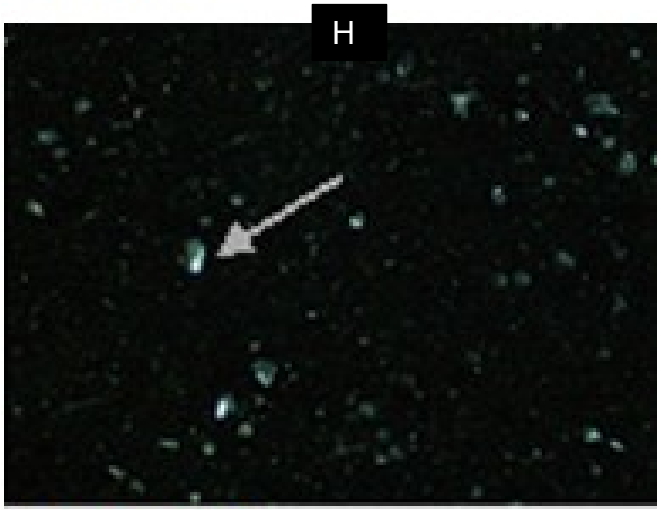

Image 3

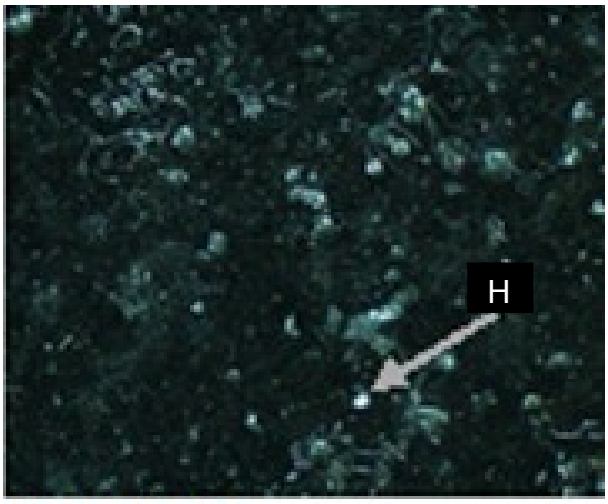

Image 4

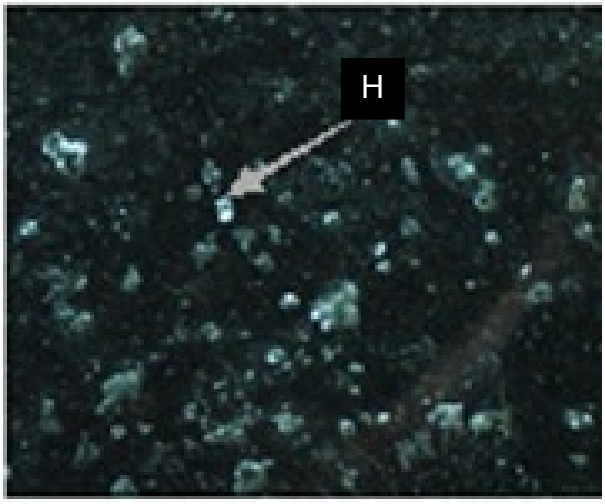

Image 5

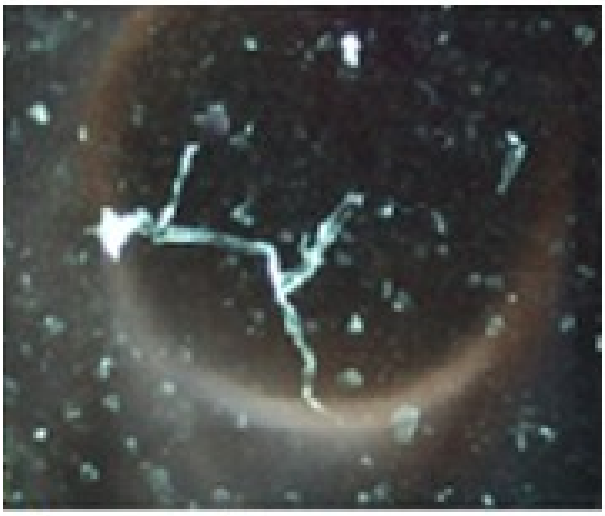

Image 6

Figure 18- H= hole. The test for neoprene in synthetic urine. As the media test showed damage sustained by neoprene in the synthetic urine, new neoprene discs (x3) were submerged in synthetic urine and taken out every day and visually examined. Images 1-3 are the three discs on day 1 and images 4-6 are the same three discs on day 2

The arrows indicated holes caused by the synthetic urine (as annotated). After 24 hours, there were visible holes which worsened after 48 hours. As the media, a replicate of a physiological system, this would have ruled out neoprene as a material for catheters however it was still used as if the growth were significantly reduced compared to silicone, then ways of strengthening the resistance of neoprene or looking for a material with similar properties could have been investigated. The synthetic urine contained citric, lactic and uric acid which are most likely responsible for the neoprene breakdown.

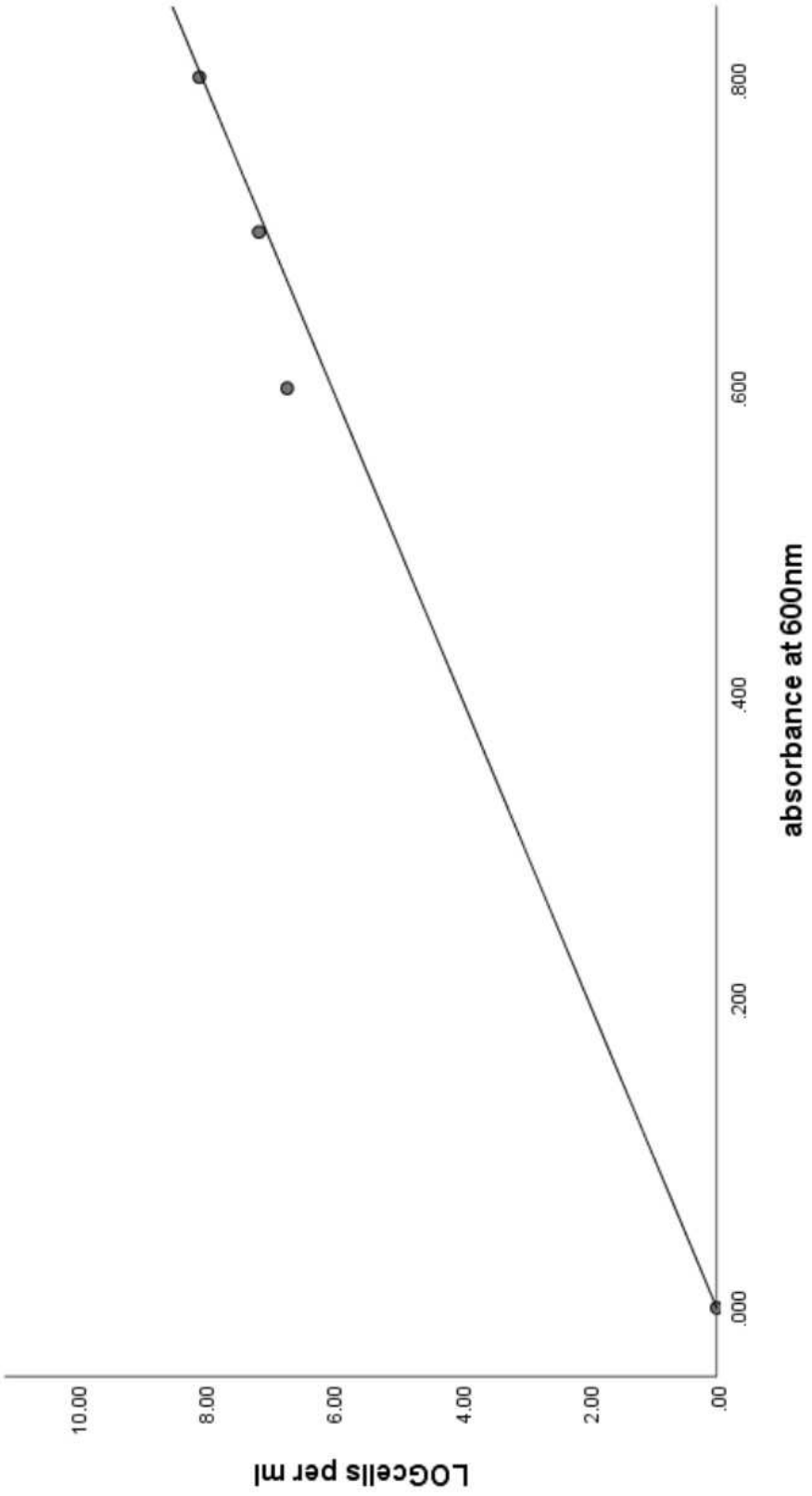

Figure 19 The standard curve described in section 2.2**.** As soon as the readings were taken and cell count undertaken the calculations were performed.

The plot points above are the $\log^{10}$ of the cells/ml which represent the following cell densities in numerical order; 0 10^6, 10^7 and 10^8. These data points are all based on 3 separate results performed on separate days (average result). The purpose of this test was to establish the cells per ml and their associated absorbance so that the cells used in the disc inoculation stage would all be equal and would be using the cells per ml as decided earlier. The main purpose of this was to establish a uniform method for treating the discs so that all the discs would be exposed to the exact same amount of cells for the same length of time in order to ensure a fair and equal opportunity for adherence and the ensure a fair comparison later after data analysis.

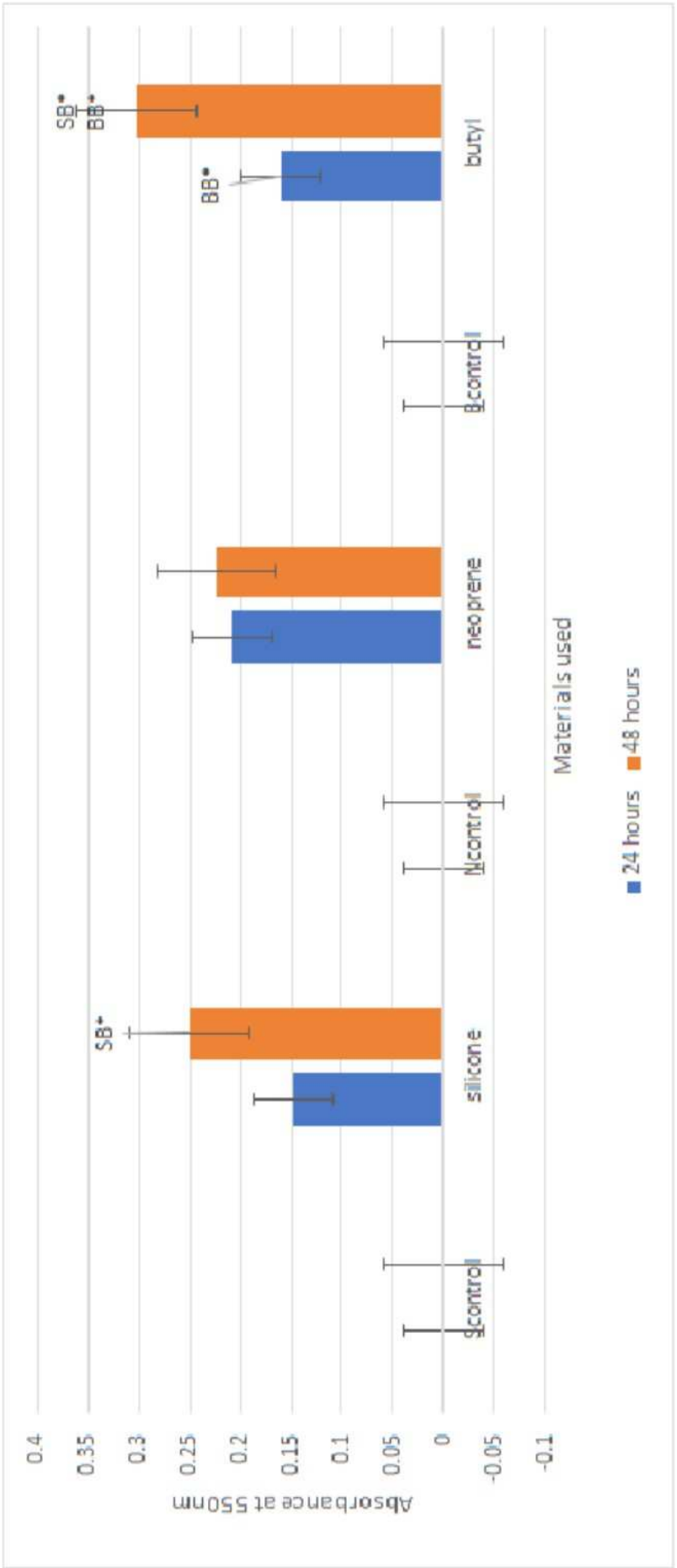
Absorbance at 550nm
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
-0.1
SB*
BB*
BB*
SB*
gcontrol
silicone
Ncontrol
neoprene
gcontrol
butyl
Materials used
24 hours
48 hours

Figure 20 Average release of crystal violet from *Candida albicans* biofilms grown statically on silicone, neoprene and butyl discs (2mm diameter and 1.5 mm thick) grown in malt extract broth statically at 30 °C for both 24 and 48 hours. Scontrol = silicone control, Ncontrol= neoprene control, Bcontrol= butyl control. SB= silicone and butyl BB= butyl and butyl. Discs were inoculated with cells with a density of 10^7cells/ml and incubated at 180rpm for 2 hours. Discs were removed and washed with sterile PBS and placed in a 96 well plate

The sample size for each experiment was three discs per test, this was repeated four times in total, so the average represented on this figure is from twelve data points. From these results, using a two tailed Students T test, only the silicone and butyl results (SB) at 48 hours were significantly different between the materials (alpha=0.5, P value= 0.02). Between the materials themselves only butyl had a significant difference between 24/48 hours (BB) (alpha= 0.5, P value= 0.04). The sample size for each experiment was three discs per test, this was repeated four times in total so the average represented on this figure is from twelve data points (n=12).

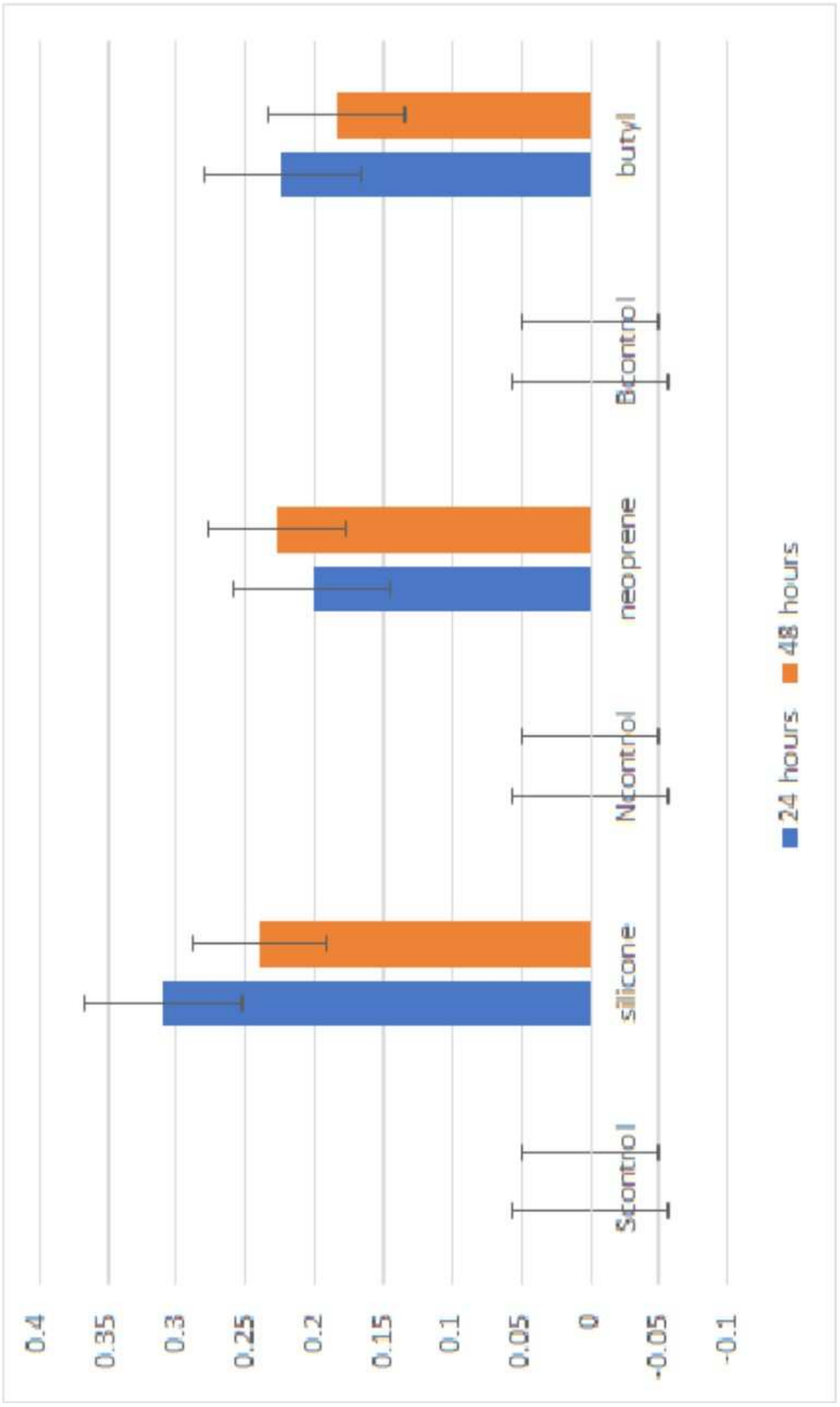
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
-0.1
Scontrol
silicone
Ncontrol
neoprene
Bcontrol
butyl
24 hours
48 hours

Figure 21 Average release of crystal violet from *Candida albicans* biofilms grown statically on silicone, neoprene and butyl discs (2mm diameter and 1.5 mm thick) grown in malt extract broth statically at 37 °C for both 24 and 48 hours. Scontrol = silicone control, Ncontrol= neoprene control, Bcontrol= butyl control.

From these results, using a two tailed Students T test, unlike the results from the 30°C tests, none of the results were significantly different from each other (alpha= 0.5). The sample size for each experiment was three discs per test, this was repeated four times in total, so the average represented on this figure is from twelve data points (n=12). For these results, temperature ort time doesn't seem to be a factor in *C.albicans* growth.

When stating that time is not a factor this does not include the initial adhesion period of 2 hours at the start of the experiment. The reason this period is not included is because this period of time was just to enable adherence to the discs, whereas the 24/48 hour time periods were to measure how much biofilm would develop on the discs. The only way the adhesion period could have impacted the results overall were if the discs were not incubated long enough for adhesion to occur. Initial adhesion is up to 2 hours, according to Shinde, *et al,* (2012), and this was the time period of incubation for disc inoculation in this experiment.

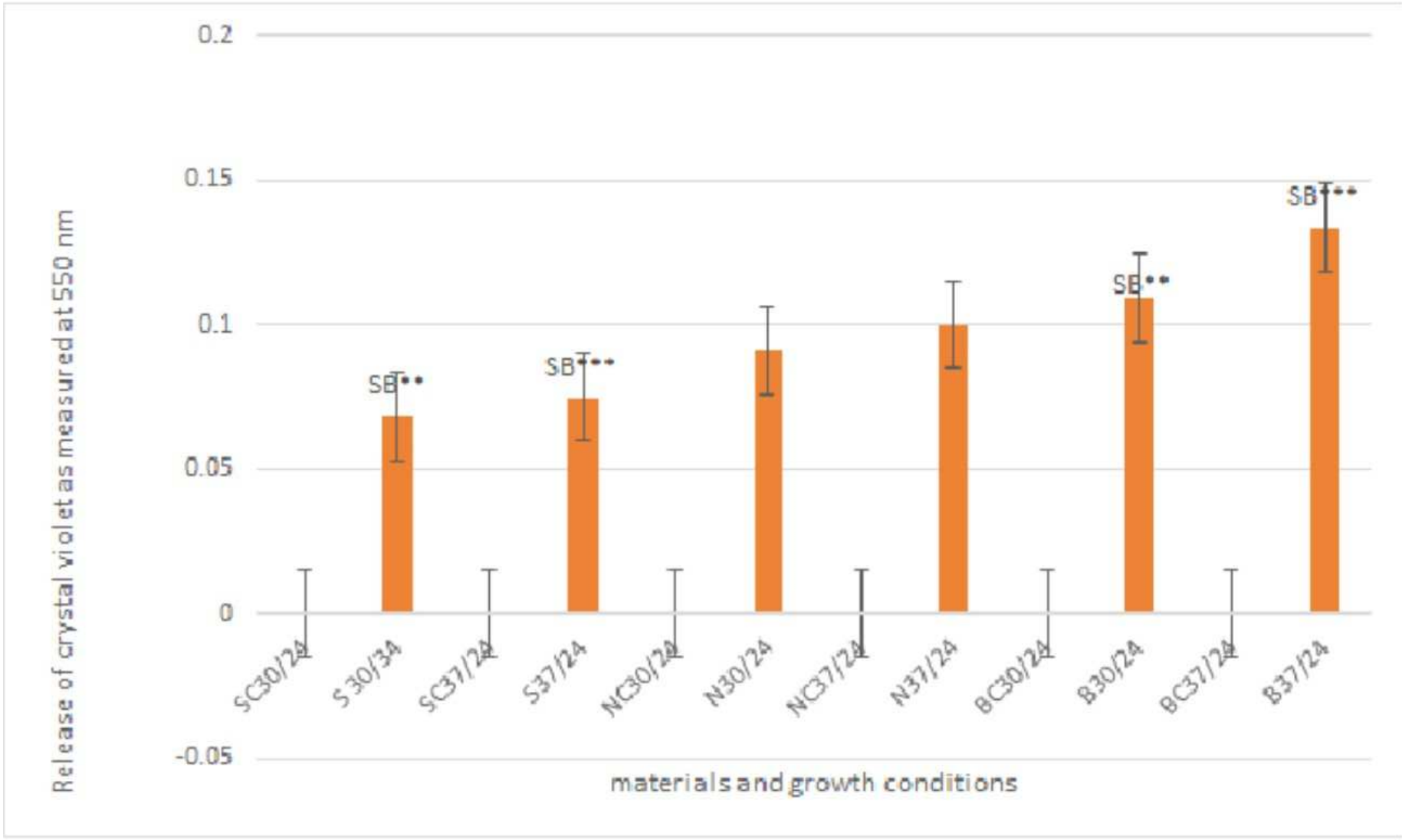
0.2
0.15
0.1
0.05
0
-0.05
Release of crystal violet as measured at 550 nm
SB**
SB**
SB**
SB**
SC30/24
S 30/34
SC37/24
S37/24
NC30/24
N30/24
NC37/24
N37/24
BC30/24
B30/24
BC37/24
B37/24
materials and growth conditions

Figure 22 Average release of crystal violet from *Candida albicans* biofilms grown statically on silicone, neoprene and butyl discs (2mm diameter and 1.5 mm thick) grown in Roswell Park Memorial Institute 1640 medium statically at either 30°C or 37 °C for 24 hours. The key for the labels are as follows; S= silicone N= Neoprene B=butyl. C=control. 30/24 = 30^0C, 24 hours. 37/24= 37^0C, 24 hours. SB= silicone and butyl

From these results, using a two tailed Students T test, only the growth on silicone and butyl discs at both temperatures were significantly different from one another (silicone and butyl at 30^0C alpha= 0.5, P value= 0.006, silicone and butyl at 37^0C alpha= 0.5 P value= $1.9x10^{-5}$). This includes testing within groups- there was no significant difference between the growth on materials. The sample size for each experiment was three discs per test, this was repeated four times in total so the average represented on this figure is from twelve data points (n=12).

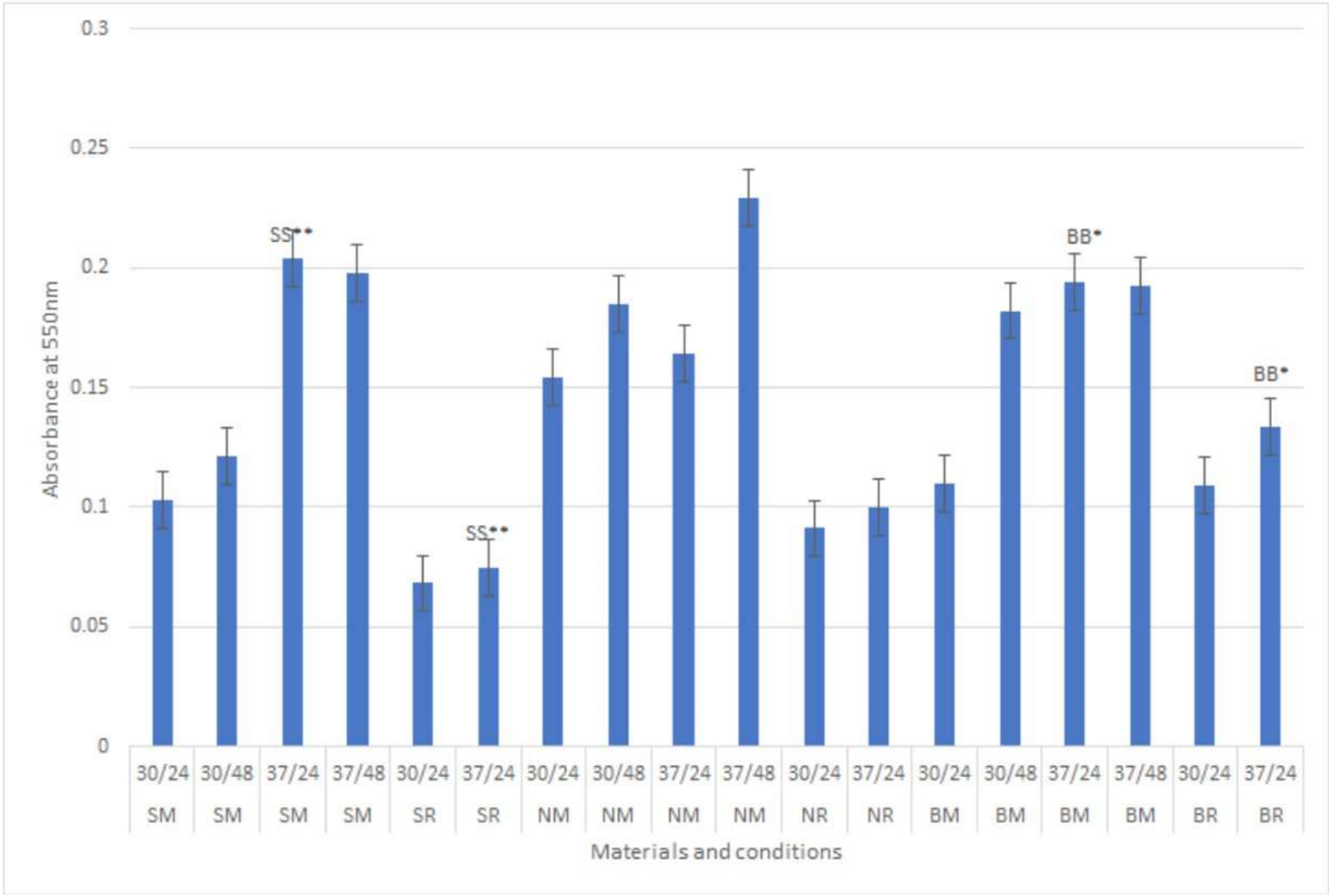
0.3
0.25
0.2
0.15
0.1
0.05
0
Absorbance at 550nm
SS**
SS**
BB*
BB*
30/24 SM
30/48 SM
37/24 SM
37/48 SM
30/24 SR
37/24 SR
30/24 NM
30/48 NM
37/24 NM
37/48 NM
30/24 NR
37/24 NR
30/24 BM
30/48 BM
37/24 BM
37/48 BM
30/24 BR
37/24 BR
Materials and conditions

Figure 23 Average release of crystal violet from *Candida albicans* biofilms grown statically on silicone, neoprene and butyl discs (2mm diameter and 1.5 mm thick) grown in malt extract broth and Roswell Park Memorial Institute (RPMI) 1640 medium statically for either 30°C or 37 °C for 24 hours (or 48 hours for the MEB only). Key= SS- silicone and silicone. BB= butyl and butyl. The controls are not featured on the graph as when they were all placed, the graph was far too small to be able to distinguish any text, data points or labels clearly.

A two tailed Students T test were performed, comparing the materials in the different media with each other. The only significant results were; silicone at 37^0C for 24 hours in RPMI 1640 and malt extract broth (alpha=0.5, P value=0.004) and butyl at 37^0C for 24 hours in RPMI 1640 and malt extract broth (alpha= 0.5, P value= 0.01). Throughout the entire experiment, it has only been silicone and butyl that have had significant differences between each other and between themselves.

4. DISCUSSION

4.1. Media

The selection of media for the experiment was primarily dependant on what effect the media had on the materials. Silicone has been used in many *C.albicans* experiments prior to this one and had been submerged in different types of media, such as yeast nitrogen base medium (Mateus, *et al,* 2004), synthetic urine (Liu, *et al,* 2015) and RPMI 1640 (Leonhard, *et al,* 2019). Butyl does not tend to be used in a manner that would expose it to any of the media selected for this experiment. Neoprene's use that does expose it to a substance similar to one of the three media used here are wetsuits, but these are of a different structure (sponge) to that used in this experiment (solid rubber). Therefore, all materials were first subject to media submersion for seven days at 37^0C (this was the highest temperature used for the experiment and the duration as longer than that of the experiment so should the materials show no damage then they would not be damaged during the experiment's incubation periods) to assess the material durability.

This preliminary media experiment caused the synthetic urine to be removed as a choice. This was due to the damage neoprene sustained, see results figures 17 & 18. Silicone was also affected by the synthetic urine, but it was discoloured and no structural, or topographical changes were noted with the visual examination. Silicone is routinely exposed to urine (as a catheter) and the effects are well documented such as in the study by Liu, *et al*, (2015). In that study, different vulcanisation techniques were examined to improve silicone's durability in urine (37^0C for 14 days submersion). The control silicone sheets (ones vulcanised using the current technique which is used for silicone catheters), were

found to have reduced elongation, tearing strength and tensile strength, making the control sheets weaker than the experimental sheets in the study by Liu, *et al*, (2015).

For the duration of this study, the discolouration would have been the only effect the synthetic urine would have had on the silicone discs. This will have been caused by the ferrous sulphate, due to the orange/reddish discolouration seen on figure 15 image 3. Its ability to discolour urine is well known (Souza, *et al,* 2009). This would have affected the other two materials also but due to their dark colour it was not visible. There are currently no studies detailing any damage caused by ferrous sulphate to these materials and the visual examination only showed the discolouration of silicone and no microtears or wrinkling.

The other two media, the MEB and RPMI 1640 had no effect at all on the discs. There were no physical changes to the discs that would have affected the outcome of the experiment.

MEB was determined to be the main media for the experiment even though it is slightly more acidic than RPMI 1640 which is a known factor in reduced filamentation (Nadeem, *et al*, 2013). This was a decision made in part by cost. Preferably, the synthetic urine would have been the media used in order to truly replicate the physiological conditions that the materials would have been under. As that was ruled out, one of the two remaining media needed to be selected. The pH of urine varies, and the normal range can be between 5.2 and 7 according to Birder & Andersson, (2013) and both medias, according to the manufacturer's guidelines, were within the known range without any adjustment.

A study by Weerasekera, *et al*, (2016) investigated culture media effect on growth, adhesion and biofilm development on; *C. albicans* and *Candida tropicalis*

using three media; RPMI 1640, Sabouraud dextrose broth and yeast nitrogen base. From their results, there was significant facilitation of adhesion from *C. albicans* in RPMI 1640 compared to the other media and increased planktonic growth. Despite the results from that study, during this experiment overnight cultures of MEB produced more growth than the RPMI 1640 overnight cultures did. This was later to have been determined to be human error caused by a lack of familiarity with the media (RPMI 1640). How the difference in growth was assessed was by visual examination of the flasks after removal from the orbital shaker.

Using MEB despite its lower pH was not seen as an issue as the investigation was into a potential new alternative to silicone as a catheter material based on *C. albicans* growth and all the discs would be cultured in the same media, therefore given equal opportunity for growth. After the data from the MEB tests were gathered the 24 hour 30/37^{0}C tests were repeated with RPMI 1640 to act as a comparison and investigate if the trends in the previous experiments continued.

4.2 Sterilisation techniques

For this experiment, there needed to be a reliable sterilisation technique that all the materials would be able to endure without damage or distortion in order to create a uniform method and reduce variability by using one singular sterilisation technique for all three materials. More than one technique was tested as there were issues with the cold sterilisation method. This issue could have led to contamination of the discs which then would have impacted the results, as bacteria has been shown to both facilitate *C.albicans* biofilms (Bamford, *et al,* 2009) and inhibit them (Graf*, et al.,* 2019).

Butyl, as mentioned in section 2.2, was autoclaved. As also discussed in that section, another technique was initially chosen because of butyl's inability to withstand high temperatures. This was the cold sterilisation technique which proved unreliable during this study. This technique was repeated numerous times and there was never complete sterilisation as at least one universal had growth in. In an article by Rutala & Weber, (2004) they discussed that manual sterilisation can be unreliable unless all prerequisites are met and they were not all met here, based on the variability of the sterility test results. They also discussed the appropriateness of using this cold sterilisation technique for items that are defined as critical of which catheters are classed and state that it should be a last resort and to seek other forms of sterilisation.

Based on the images of butyl post autoclaving (Figure 14) it was decided that the effect on adhesion by autoclaving altering the surface of butyl was less than the effect the non-sterile material may cause. This decision was made in part, based on the results from Sousa, *et al*, (2009), and (Verran, Lees & Shakespeare, 1991). In their results they found that there was not a linear relationship between the texture of the surface and adhesion. There have been many studies on surface roughness and adhesion but none comparing the sterility of the material and *C.albicans* adhesion that would provide any better insight here.

Later on, in an attempt to capture images of the *C. albicans* growth on the discs, an image was obtained of the butyl after air drying and with *C. albicans* adhesion and the contrast of the *C. albicans* against the black background of the butyl was able to provider a clearer image of the surface of the disc post autoclaving and showed that there were actually very little surface changes if any at all. This

meant that the autoclaving did not damage the butyl as extensively as assumed prior to the examination. Regarding this experiment, the surface changes to butyl caused by autoclaving was another factor in *C. albicans* adhesion. The surface of the material is a factor in adherence for *C. albicans*, with rough surfaces providing more opportunity for cell anchorage than a smooth surface. Studies have been performed that investigated this trend, but the materials used was usually acrylic resin, used for dentures. In one study, adhesion by *C.albicans* on differently textured surfaces was investigated and the results were the surface that had been roughened with the mid-range emery paper had significantly higher adhesion than the smoothest and roughest of surfaces (Verran, Lees & Shakespeare, 1991). This is also highlighted in a study by Sousa, *et al*, (2009) that found there was not always a linear relationship between surface roughness and adhesion when comparing adhesion to the coarser silicone surface and the smoother acrylic surface.

In this study, hyphal invasion of the material was not investigated due to a lack of resources to measure this. All comments made about the surface topography are assumptions based on current understanding from studies mentioned in the above section concerning the roughness of the substrate material and based on the results found here as the texture on the material's surfaces were not measured against each other.

The purpose of the preliminary tests was to establish any damage, reduce any possible variables and to set a standard for the experiment to follow so that all materials were treated uniformly to make for a more even comparison of results.

4.3 data overview

From this data, only results from silicone and butyl were significantly different. Neoprene, despite demonstrating a lack of durability in the preliminary media tests, consistently yielded no growth difference throughout the experiment. The properties of silicone and neoprene were very similar and both had a smooth surface (see figures 12 & 13). The main visual difference between the two was the varying thickness of the silicone. There were thinner areas of the silicone that was only visible with the microscope as the light shone brighter through the thinner sections creating bright spots which can been seen on figure 12. The full effect of this varying thickness is not known at this time as there have been no studies that focused on the density of the substrate material for *C.albicans* biofilms.

4.4 Silicone

Concerning the silicone (and butyl) results, the most growth on average was in the 24-hour tests and for neoprene it was the 48-hour incubation that provided the most growth. Concerning the silicone discs at 30^0C, the growth increased from 24-48 hours with the 48-hour result having more growth on average than the 24-hour results. The opposite is true for the 37^0C tests and the average growth for both the 24- and 48-hour tests are very similar when comparing within their temperature groups. Student T tests within the silicone groups found that only the 30^0C and 37^0C tests at a 24-incubation period were statistically significant ($P<0.01$). There is almost double the growth on average in the 37^0C compared to the 30^0C in MEB. In the RPMI 1640 results, there were no significant differences between the two temperatures, with the 37^0C results having slightly more growth on average. Both silicone and butyl at 37^0C had more growth after 24 hours than after 48 hours. This could be due in part, to upregulation of the *ALS1* gene. Although both *ALS1* and

ALS3 are considered important genes for *C. albicans* adhesion and biofilm development as discussed in section 1.3. The study by Nailis *et al,* (2006), found that *ALS1* was upregulated in a 24-hour time period, up to five-fold greater than ordinary housekeeping genes in a *C. albicans* biofilm compared to their planktonic counterparts. Having this level of upregulation combined with the more hospitable environment for biofilm growth (37^0C) could have caused an increased rate of biofilm development, resulting in a greater production of biomass when compared to the lower temperature. As demonstrated in a study by Nadeem, *et al*, (2013) where biofilms grown at higher temperatures developed earlier than those grown at lower temperatures. A mature biofilm will disseminate, and some cells will relocate to another area and begin a new biofilm (Desai, 2018). Only the cells adhered to the discs were stained and measured, so any disseminated cells adhered to the plate walls were discarded. It is possible that the biofilms in the 37^0C tests were more mature than the 30^0C counterparts due a delay in growth which could have resulted in the lower growth average (due to the discarded cells not being counted). This would be applicable to the silicone and butyl 37^0C results as both of those saw a reduced growth after 48 hours when compared to their 24 hour counterparts. But neoprene did not follow this trend and yielded more growth after 48 hours on average than its 24 hour counterpart.

4.5 Neoprene

For the neoprene results, there was no difference between the growth of cells at 37^0C or 30^0C. The highest average growth came from the 37^0C 48-hour tests, although the error bar for this test (see figure 21) is rather large which would indicate that set of data was more variable than the others, so reproducing this data would be incredibly difficult and therefore this data is less reliable. Although all the data

gathered was rather varied, most likely due to the inoculation method, which, although ensured all discs were exposed to the same amount of cells for the same length of time did not assure that the same amount of cells would adhere to the disc at that time. There are larger, non-significant differences between the growth in the two temperatures in MEB than in the RPMI1640. If the facilitation of *C. albicans* adhesion by RPMI 1640 *(*Weerasekera, *et al*, 2016) played a role then this could possibly be the reason behind the smaller difference- the influence of the media on the adhesion led to a greater number of cells for the basal layer of the biofilm. The influence of media could have been greater than the environmental trigger of temperature leading to a similar result.

A factor that could have encouraged growth that the other two materials lacked is the properties of the material itself. None of the results were statistically different for neoprene within its results group, for all growth conditions. This includes comparisons of the materials; neoprene had no significant difference in *C. albicans* growth compared to butyl or silicone in any experiment undertaken here.

4.6 Butyl

The butyl results were very similar to the silicone results. 30^0C at 24 hours incubation provided the least amount of growth on average and 37^0C at 24 hours incubation provided the most (in both media). There was a small difference between the 48-hour and 24-hour incubation periods at 37^0C. This difference was only significant for the 30^0C tests ($p<0.05$). Like the silicone result, the average growth between 30 and 37^0C after 24 hours incubation is statistically significant ($p<0.001$). This could be caused by the previously discussed factors including gene

upregulation but also could be related to the material itself and how hospitable it was for the *C. albicans* biofilm's development.

The properties of the substrate material for *C. albicans* bud anchorage and subsequent biofilm development has been investigated numerous times, with a tendency to focus on surface roughness. Studies such as Al-Fouzan, *et al,* (2017), found that rough surfaces caused increased adhesion. And this was also the case in the Gleiznys, *et al, (*2015) study that found basal cells could fit into the depressions caused by a rougher surface and dislocation of the basal cells proved more difficult. For butyl it was possible for this to have occurred due to its textured surface when compared to the smoother surface of the silicone and neoprene. As mentioned in section 4.3, the density of the material has not been investigated but it is possible that it could affect development when regarding hyphal invasion of the material. As Leonhard, *et al,* (2009) demonstrated, hyphal invasion of substrate materials is possible, and it most likely utilising a mechanism like that of active penetration. A denser material would more than likely require more secretion of the aspartic proteinases used in active penetration and would take longer than a less dense material. However, this is only speculation as a study has not been performed to investigate this. As *C.albicans* is a more negatively charged organism, a positively charged surface would increase adhesion rates (Ördögh *et al,* 2014). Silver coated catheters, which are used to reduce infection rates, are negatively charged (Rupp, *et al,* 2004). In this experiment all three materials were negatively charged but to varying degrees.

There are some differences between the silicone and butyl results. One of which being the size of the increase of growth between 30^0C and 37^0C. Both materials had an increased average growth between the two temperatures but the

difference in growth was greater in butyl for both media (MEB and RPMI 1640). In the RPMI 1640 results there is no significant difference between the two butyl averages, but significant differences between the silicone and butyl results at both temperatures. 30^0C ($p<0.5$) 37^0C ($p<0.01$).

Unlike the previous results in MEB, in the 30^0C tests, duration did make a significant difference for butyl. It may be related to initial adherence (if we were to consider that the butyl 24 hour 30^0C result was low due to a reduced *C. albicans* adhesion rate which would mean less cells able to form a biofilm and therefore reduce overall growth, particularly at the temperature which is not optimum for *C. albicans*). Butyl yielded the highest average growth for the three materials overall in the RPMI 1640 unlike the MEB results where neoprene had the highest average growth.

The difference between the 24/48-hour butyl results could be explained by stating that the *C. albicans* cells are older and therefore the biofilm created by those cells will be denser after 48 hours compared to 24 hours *(*Angelaalincy, *et al*, 2018*).* This was a trend that did not continue in the 37^0C results for butyl and silicone. There are a few possibilities for this occurrence, the first being the temperature itself. Higher temperatures, between 37-42^0C, are known environmental triggers for morphogenesis, and it is said in the study by Sudbery, *et al,* (2004), that there will only be unicellular growth in temperatures below 30^0C. Filamentous growth in temperatures of 30^0C is possible, the percentage and type of filament is dependent on the media used, like the study by Kadosh & Johnson, (2005). In that

study they examined the transcriptional response of hyphae associated genes regarding the filamentous growth of *C. albicans* at 30^0C and 37^0C with and without serum. At 30^0C with serum there were filaments, as visualised by fixing the cells with formaldehyde and examining via Nomarski optics. At 30^0C without serum, there was some filamentation, but it was ~95% blastophores.

Another potential explanation as to why it only occurred it the 30^0C results are the stages of biofilm formation. Considering the rationale behind the silicone results and applying them here, the maturation of a biofilm begins 38 hours (and up to 72 hours) after initial adhesion according to Chandra, *et al,* (2001). The delay in development compared to the 37^0C plates would have been greater due to the reduced filamentation. And butyl itself will be factor under this assumption considering that the silicone results do not follow the same pattern.

4.7 Comparison of materials in MEB

Comparisons of the materials found the only significant differences were between silicone and butyl at 30^0C for 48-hours ($p<0.05$). The 24-hour MEB results at 30^0C were not significant, but they were in the RPMI 1640. This could be the media's influence encouraging more growth in the RPMI 1640 which led to a greater difference between the two materials.

The average growth on silicone discs were the lowest averages from the three materials for 30^0C in both media and butyl had the highest growth averages in RPMI 1640. To compare the results and making no assumptions about the *C. albicans* growth, it would appear that butyl provided the more hospitable surface for biofilm growth. So now attention is turned to the surfaces of the two materials. Silicone's surface was flat and somewhat sticky (as was neoprene when washing the discs

prior to inoculation, the neoprene and silicone discs would occasionally stick together and needed to be pealed apart). This was never an issue with the butyl discs; however, the butyl surface was textured which made it rough. This can be seen in the autoclaving images in figure 14. Both properties, in particular the surface roughness, are factors that can affect adhesion.

4.8 RPMI 1640 results

Regarding the RPMI 1640 results, the relationship between temperatures is similar to that of the MEB results- 30^0C yielded lower growth than its 37^0C counterparts for all materials. The RPMI 1640 results have less variation (as shown by their lower standard deviations, appendix table 26 for RPMI 1640 results and appendix table 14 for the MEB results). But the most growth came from butyl and not neoprene, like in the MEB results. It should be considered that this set of tests were only incubated for 24 hours and some factors discussed throughout this discussion have been focused after this time period (mature biofilm and the dissemination). It is possible that surface roughness of the butyl combined with the media at a more optimum pH had a greater influence on growth and that is why there is a difference in the results from RPMI 1640 and MEB. In this set of data there were two significant results: silicone 30/24 and butyl 30/24 ($p<0.05$), and silicone 37/24 and butyl 37/24 ($p<0.001$). Like the MEB results, the average growth difference between the two temperatures is greater with butyl than with silicone. With this media there were more statistically significant results when comparing materials. In these results there is no significant difference between the two butyl averages, unlike the MEB results which had two significantly different results, possibly highlighting the role of media as a greater influence in *C. albicans* growth than other factors such as surface charge.

Looking at the surface topography as being the main cause or influence, these results would follow that line of thought at first glance. As mentioned above, neoprene and silicone had a similar surface area and butyl had the rougher textured surface. Butyl yielded the most growth and there were significant differences between the three materials. But this was only in this media. As to why there was only a significant difference between silicone and butyl at 37^0C (and not neoprene and butyl) can again be brought back to a biofilm delay/ reduced growth at a lower temperature. Even at a reduced growth, there were still significant differences between silicone and butyl, with butyl being the more hospitable environment. At 37^0C this hadn't changed between the two materials, but it had for neoprene and butyl. Considering the delay, hyphal invasion and butyl's resistance to this, those factors would have closed the gap between the two materials and reduced any significance. Had there have been a 48 hour set of data for RPMI 1640 it may have followed the same pattern and neoprene succumbed to the invasion and thus yielded more growth than butyl but as there is not data for that this can only be speculation based on the results here and the possible reasons.

4.9 Surface topography

To focus on the surface roughness briefly, the textured surface provides creases and crevices which are considered favourable for colonisation, (Al-Fouzan, *et al,* 2017). This is most likely due to the fact the yeast cells that form the basal layer that adheres first can attach and fit neatly into the depressions which would make it harder to dislocate than a basal layer on a flat surface (Gleiznys, *et al,* 2015).

This would be beneficial for a biofilm formed on a catheter as if the basal layer were initiated into a depression then the flushing of urine would less likely remove it in those earlier stages, when the cells are loosely adhered, enabling the formation of a

fully mature biofilm and increasing the risk of invasive candidiasis. What this would also mean, using the results from Liu, *et al,*(2015) again is that one of the reasons that the longer the silicone catheter is in place, the greater the risk of colonisation occurs is because of the surface alterations the urine makes to the silicone and the fact that there is not always a linear relationship between surface roughness and adhesion with some cases showing a small roughness increase yielding a significant adhesion increase and larger increases in roughness showing no significant difference in growth (Sousa, *et al,* 2009).

This can be applied to butyl as the surface area changed post autoclaving however the extent of the effect this will have had is not known at this time.

In a study by Leonhard, *et al,* (2009), they investigated the growth and development of *C. albicans* biofilms on both smooth and textured (rough) surfaces using voice prostheses (two prostheses were used; one made from thermoplastic polyurethane and the other silicone rubber). Their results found that the textured surface appeared to facilitate initial adhesion, which is a possible reason why there was a thin, uniform microbial coating on the surface. Whereas the smooth surface (this experiment used a silicone voice prosthesis for this) found that initial adhesion was restricted to the edges. Despite this, there were no significant differences in the growth on either material. However, the biofilm's development did differ between the two, and this was believed to be because of the materials used. The textured surface material was thermoplastic polyurethane. On the textured surface, the biofilm that developed was unstructured and lacked hyphal forms. The silicone biofilms had a more complex EPS. This more sophisticated EPS network would undoubtedly prove much more beneficial to the silicone biofilms' survival, considering that the EPS is responsible for providing nutrients to the biofilm (Flemming, *et al,* 2007). Another

result from their study that could have implications in the results from this experiment is their scanning electron microscopy images. Using this technique, they were able to identify penetration of both materials by *C. albicans*. The invasion of the silicone was more pronounced and distinct than the thermoplastic polyurethane. In the silicone, there was invasion then excavation which then enabled yeast cells to adhere within the silicone and begin aggregating. The thermoplastic polyurethane was more resistant to invasion, though this is speculated to be related to the lack of hyphal forms. As silicone is currently used as a catheter material, there have been numerous studies investigating and comparing growth of *C. albicans* on its surface, some of those studies have been referenced in this thesis. As neoprene and butyl are not materials commonly used for instances where microbial growth would pose a persistent issue, information regarding their ability to be a substrate for microbial adherence is non-existent. So, assumptions made are based on their surface properties that could affect adherence and growth, the results from this study and understanding of *C. albicans* biofilm formation.

4.10 Surface charge

Another possible factor that could have influenced the results is the surface charge of the materials. As electrostatic interactions affect adhesion it will have had some impact. Biofilms are predominantly composed of polysaccharides (EPS) and many are negatively charged. *C. albicans* is also negatively charged (Ördögh *et al,* 2014) so a surface with a positive charge will encourage more adhesion and biofilm development. This doesn't mean there will be no adhesion if the surface is negatively charged. There has still been significant growth from a highly negatively charged surface before (Jones & O'Shea, 1994). And the als3 has been implicated in altering

the charge of hyphae in a *C. albicans* biofilm which would then reduce the repulsion between the surface and hyphae.

Concerning the surface charges of the materials, there is the Triboelectric Series. This series ranks materials by their tendency to gain/lose electrons during electron transfer when two objects come into contact with one another (Zou, *et al,* 2019). Further on, the surface charges of the materials mentioned will be according to their rating on this series.

According to Lee & Orr, (2018), neoprene has a surface charge of -98 nC/J (nanoampsec/watt of friction). So, in terms of adhesion, the surface charge of neoprene wasn't necessarily an essential factor behind the average growth. Another factor behind neoprene having the largest average growth at 37^0C for 48 hours is the presence of the chlorine atom in the chemical structure of the material. Neither silicone or butyl have this atom in their structure, and to compare sizes of all the atoms that are found in all three materials, it is the biggest-silicon is the second biggest atom from the three materials' composition- (Ospadov, *et al,* (2018). The presence of this larger atom would mean that all the layers would have small gaps between them as they would not be able to fit together very tightly. This would leave openings for the *C. albicans* post active penetration and, like a rough surface, would provide craters with these gaps for disseminated yeast cells to attach, aggregate and form more biofilms (Desai, 2018).

The issue with this rationale is that butyl is a very dense material (Wang, et al., 2006). And the significant results were between silicone and butyl. Should the structure of the materials based on atom sizes been a bigger influence then, in theory, there should have been significant results between butyl and neoprene. Butyl

rubber's surface charge is more negative than that of neoprene, at -135nC/J. Silicone's surface charge is -72 nC/J (Lee & Orr, 2018). Having a more negative charge could reduce initial adhesion rates, but by how much is not known at this time as the discs were not immediately stained and read post inoculation which would have given a clearer picture of initial adhesion. It is possible that the surface charge of butyl had a greater effect on reducing adhesion than the surface roughness had on increasing adhesion so there was less growth on butyl. This could lead to the significant results between silicone and butyl, with surface charge having a greater effect than surface roughness and molecular structure of the materials on *C. albicans* growth.

Taking this into account, and using the information that there are mainly blastophores at 30^0C (Kadosh & Johnson, 2005) then it could be plausible that the reason there was a significant difference between the materials at 30^0C for 48 hours is due to the highly negative surface of butyl causing upregulation of *ALS3* to overcome the negatively charged surface which then enabled hyphal and biofilm production post adhesion (Zhao, *et al,* 2006). As there was already a greater (compared to silicone when looking at the surface charges of both) upregulation of one of the hyphae- associated genes. This could have caused an increase in filaments not seen in silicone at the low temperature, causing a significant growth difference between the two materials.

Concerning the 30^0C at 48 hours result in MEB (the significant result between butyl and silicone), there is more growth on average on silicone than butyl, which could be due to the greater resistance that butyl could have to invasion, being the denser material of the two. There is no significant difference between any materials at 37^0C in MEB but there is in the RPMI 1640 results which would indicate that the

stimulus the material surface provides may not necessarily be the most influential factor within these results. It would also dismiss neoprene and butyl as alternatives to silicone as neoprene had more growth in MEB and butyl in RPMI 1640. When butyls growth was lower than that of silicone there was not enough of a significant difference to warrant switching.

In regards to cost, a silicone sheet measuring 8 inches X 8 inches will cost £3.95, a neoprene rubber sheet measuring 8 inches X 8 inches will cost £2.19 (Camthorne Industrial Supplies, Staffordshire), and a butyl sheet measuring 12 inches X 12 inches will cost £26.92 (White Cross Rubber Products Ltd, Lancaster).

4.11 Invasion of material

A potential explanation as to why neoprene yielded the largest growth on average after 48 hours could be found from the synthetic urine testing carried out prior to the experiment. After 24 hours the urine had managed to erode the neoprene to an extent that it put visible holes in the material which worsened after 48 hours. After this length of time post *C. albicans* inoculation, the biofilm would have been established and the hyphal invasion underway. Active penetration requires the secretion of aspartic proteinases to degrade mucosal layers in epithelial cell invasion. As shown in the study by Leonhard, *et al,* (2009), *C. albicans* can invade materials possibly using a mechanism similar to active penetration of epithelial tissue (how exactly it invades materials has not been fully determined as of yet). The invasion of the material would involve the hyphal extension and some form of secretion to degrade or erode the material. Silicone and butyl would have been more resistant to this than neoprene basing this in part to neoprene's weakness during the media tests and therefore there would have been more colonisation on the neoprene discs. In the MEB results at least.

4.12 Summary of the factors that could have affected growth within the materials

MATERIAL ASPECT	CURRENT UNDERSTANDING	RESULTS FROM STUDY
Surface roughness	Surface roughness does affect *C. albicans* adhesion, as found in the studies by Sousa, *et al*, (2009), Verran, Lees & Shakespeare, *(1991) and* Leonhard, *et al*, (2009). But this is not always a linear relationship.	Butyl is the only material with a textured surface, but it did not consistently yield a high growth average compared to the other materials, except in the RPMI 1640 were its averages were the highest of out the materials. It would appear that there are numerous factors with varying influence and surface topography is not the biggest influence on *C. albicans* growth.
Surface charge	The surface charge of the substrate for *C. albicans* growth does have some importance for adhesion and growth as electrostatic interactions between the cell and substrate are what begin the adhesion process (Richardson*, et al,* 2018). As *C. albicans* is negative, a more positively charged surface would encourage growth and these interactions have	Butyl had the most negatively charged surface and silicone had the least negatively charged surface. In MEB neoprene had the highest average growth, butyl had the lowest. In RPMI 1640, butyl had the highest average growth and silicone the lowest. Like surface roughness it would appear that there are other factors, and, in this case, media appeared to be a

	been previously described as being the main mechanisms of adherence (Nikawa, et al, 1992). Although negatively charged surfaces can host *C. albicans* growth, with als3 implicated in altering the hyphal charge (Zhao, *et al*, 2006).	bigger influence than surface charge on *C. albicans.*
Density	The substrate density has not really been investigated previously; it was an assumption made based on the hyphal invasion of substrates that a denser material would provide more of a challenge for *C. albicans* invasion than a less dense material.	As there was no scanning electron microscopy, there can only be assumptions made about how the density of the material impacted biofilm growth. Based on the results here, it could have been a factor, but like the other aspects, it would appear not to have had the biggest impact as butyl would have had the lowest growth averages, being the densest material and with the RPMI 1640 result it had the highest growth.

Table 1- summary of all factors that could have influenced growth of *C. albicans* on all three materials, current understanding from literature and a brief summary of the results from this experiment and possible implications.

4.13 Conclusion

Overall, there are so many factors that could have influenced the growth that were not tested- upregulation of the gene, dissemination, the surface roughness increasing adhesion and hyphal invasion to name a few. Some of these things could have been investigated- post disc inoculation the discs could have been stained immediately to get a truer reflection of initial adhesion for example, rather than base it from 24 hours growth. But some other tests- like hyphal invasion and gene upregulation which would have been helpful to know were not possible in the scope of this experiment and would have to be an experiment in their own right and not an extension of this one. Despite the varied results and potential flaws of the experiment overall the aim was achieved- to investigate possible alternatives to silicone as a material for catheterisation based on *C. albicans* biofilm growth and development on 2mm discs. To replace silicone with one of the materials would be costly no matter the price of the new material and neither butyl nor neoprene demonstrated an ability to reduce growth and biofilm formation to warrant serious consideration to replace silicone. Neoprene can be sourced slightly cheaper than silicone but its high growth of *C.albicans* in all conditions (growth averages of *C.albicans* on the neoprene discs were consistently higher than that on the silicone discs) and its poor resistance to the synthetic urine media ruled it out as a potential replacement for silicone. Butyl, proved to be a much more durable and resistant material than neoprene, and yielded less growth than neoprene except during the RPMI 1640 tests wherein it had the highest average growth between the three materials in that media. The only significant differences came from silicone and butyl. However, the high cost of butyl, paired with the growth averages from this experiment, would not justify changing indwelling catheter materials from silicone to butyl.

Silicone may still have its issues, such as its degeneration in urine as discussed in the study by Liu, *et al,* (2015). It may provide a more hospitable environment for *C.albicans* than other materials when compared in studies such as Leonhard, *et al,* (2009), where the EPS on the silicone was more complex and highly structured compared to that on the thermoplastic polyurethane. But finding a new material may not be the solution to the problem of *C. albicans* biofilms on indwelling catheters that cause a third of all nosocomial CAUTI's (Nett, et al., 2014). Based on assumptions made in this discussion that were made using the data collected from this experiment, it may be beneficial to investigate a coating for silicone that would not only increase the negative surface charge but also fill in some of the gaps within the molecular structure caused by the larger silicon molecules, with the aim of creating a much more hostile environment for *C.albicans* in order to discourage biofilm growth that would in turn reduce dissemination of mature biofilms and reduce the risk of candidaemia and ultimately, sepsis caused by the opportunistic yeast like fungus, *Candida albicans*.

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6. Appendix

Silicone rubber incubated at 30^0C for 24 hours	Silicone rubber incubated at 30^0C for 48 hours	Silicone rubber incubated at 37^0C for 24 hours	Silicone rubber incubated at 37^0C for 48 hours
0.074	0.073	0.094	0.087
0.096	0.143	0.078	0.094
0.134	0.069	0.108	0.125
0.174	0.17	0.448	0.083
0.191	0.07	0.274	0.058
0.234	0.097	0.272	0.065
0.118	0.188	0.112	0.183
0.049	0.122	0.29	0.126
0.044	0.107	0.188	0.12

0.048	0.14	0.13	0.196
0.049	0.136	0.147	0.418
0.06	0.141	0.142	0.664

Table 1- Raw data for the Silicone experiments. Grown in MEB and measured using a FluroStar Omega plate reader at 550nm.

Neoprene rubber incubated at 30^0C for 24 hours	Neoprene rubber incubated at 30^0C for 48 hours	Neoprene rubber incubated at 37^0C for 24 hours	Neoprene rubber incubated at 37^0C for 48 hours
0.108	0.199	0.103	0.1
0.088	0.141	0.077	0.074
0.086	0.123	0.067	0.081
0.076	0.231	0.171	0.075
0.101	0.127	0.202	0.06
0.089	0.093	0.132	0.085
0.549	0.069	0.155	0.134
0.11	0.086	0.133	0.255
0.072	0.212	0.151	0.153
0.109	0.109	0.169	0.324
0.092	0.298	0.148	0.401
0.31	0.41	0.376	0.791

Table 2- Raw data for the Neoprene experiments. Grown in MEB and measured using a FluroStar Omega plate reader at 550nm.

Butyl rubber incubated at 30^0C for 24 hours	Butyl rubber incubated at 30^0C for 48 hours	Butyl rubber incubated at 37^0C for 24 hours	Butyl rubber incubated at 37^0C for 48 hours

0.107	0.13	0.111	0.092
0.099	0.104	0.11	0.085
0.122	0.159	0.128	0.293
0.203	0.127	0.29	0.109
0.16	0.13	0.283	0.108
0.22	0.202	0.283	0.093
0.075	0.16	0.218	0.219
0.074	0.193	0.155	0.266
0.077	0.189	0.177	0.176
0.072	0.165	0.141	0.189
0.065	0.161	0.155	0.281
0.068	0.345	0.137	0.257

Table 3- Raw data for the Butyl experiments. Grown in MEB and measured using a FluroStar Omega plate reader at 550nm.

Silicone rubber incubated at 30^0C for 24 hours	Silicone rubber incubated at 37^0C for 24 hours
0.089	0.091
0.092	0.103
0.072	0.1
0.138	0.097
0.078	0.104
0.105	0.115
0.079	0.058

0.058	0.075
0.042	0.057
0.062	0.068
0.059	0.064
0.05	0.058

Table 4- Raw data for the Silicone experiments. Grown in RPMI 1640 and measured using a FluroStar Omega plate reader at 550nm.

Neoprene rubber incubated at 30^0C for 24 hours	Neoprene rubber incubated at 37^0C for 24 hours
0.117	0.154
0.086	0.081
0.088	0.063
0.063	0.082
0.107	0.09
0.073	0.08
0.091	0.162
0.094	0.168
0.096	0.093
0.092	0.103
0.113	0.091
0.135	0.076

Table 5- Raw data for the Neoprene experiments. Grown in RPMI 1640 and measured using a FluroStar Omega plate reader at 550nm.

Butyl rubber incubated at 30^0C for 24	Butyl rubber incubated at 37^0C for 24

hours	hours
0.141	0.132
0.131	0.121
0.132	0.102
0.13	0.143
0.121	0.3
0.13	0.125
0.1	0.117
0.099	0.082
0.08	0.07
0.117	0.146
0.093	0.125
0.06	0.105

Table 6- Raw data for the Butyl experiments. Grown in RPMI 1640 and measured using a FluroStar Omega plate reader at 550nm.

Silicone rubber incubated at 30^0C for 24 hours	Silicone rubber incubated at 30^0C for 48 hours	Silicone rubber incubated at 37^0C for 24 hours	Silicone rubber incubated at 37^0C for 48 hours
0.0648	0.0636	0.088	0.084
0.0912	0.147	0.069	0.088
0.136	0.0588	0.105	0.126
0.184	0.18	0.513	0.0756
0.205	0.06	0.304	0.0456
0.256	0.0924	0.302	0.054
0.117	0.201	0.110	0.195
0.0348	0.122	0.324	0.127

0.028	0.104	0.201	0.12
0.0336	0.144	0.132	0.211
0.0348	0.139	0.152	0.477
0.048	0.145	0.146	0.772

Table 7- Adjusted data for the Silicone experiments. Grown in MEB and measured using a FluroStar Omega plate reader at 550nm. The raw data were multiplied by 1.2 (the dilution factor) and then the blank from the plate reading subtracted.

Neoprene rubber incubated at 30^0C for 24 hours	Neoprene rubber incubated at 30^0C for 48 hours	Neoprene rubber incubated at 37^0C for 24 hours	Neoprene rubber incubated at 37^0C for 48 hours
0.105	0.214	0.0996	0.096
0.081	0.145	0.0684	0.0648
0.079	0.124	0.0564	0.073
0.067	0.253	0.181	0.066
0.097	0.128	0.218	0.048
0.082	0.087	0.134	0.078
0.634	0.0588	0.162	0.136
0.106	0.079	0.135	0.282
0.0624	0.23	0.157	0.159
0.106	0.106	0.178	0.364
0.0864	0.333	0.153	0.457
0.346	0.46	0.427	0.925

Table 8- Adjusted data for the Neoprene experiments. Grown in MEB and measured using a FluroStar Omega plate reader at 550nm. The raw data were multiplied by 1.2 (the dilution factor) and then the blank from the plate reading subtracted.

Butyl rubber incubated at 30^0C for 24 hours	Butyl rubber incubated at 30^0C for 48 hours	Butyl rubber incubated at 37^0C for 24 hours	Butyl rubber incubated at 37^0C for 48 hours
0.104	0.132	0.109	0.0864
0.0948	0.1	0.106	0.078
0.122	0.166	0.129	0.327
0.219	0.128	0.324	0.106
0.168	0.132	0.315	0.105
0.24	0.218	0.315	0.087
0.066	0.168	0.237	0.238
0.0648	0.207	0.162	0.295
0.0684	0.202	0.188	0.187
0.0624	0.174	0.145	0.202
0.054	0.169	0.162	0.313
0.0576	0.39	0.14	0.284

Table 9- Adjusted data for the Butyl experiments. Grown in MEB and measured using a FluroStar Omega plate reader at 550nm. The raw data were multiplied by 1.2 (the dilution factor) and then the blank from the plate reading subtracted.

Silicone rubber incubated at 30^0C for 24 hours	Silicone rubber incubated at 37^0C for 24 hours
0.082	0.085
0.0864	0.0996
0.0624	0.096
0.141	0.0924
0.069	0.1

0.102	0.114
0.0708	0.0456
0.0456	0.066
0.0264	0.044
0.0504	0.0576
0.0468	0.052
0.036	0.0456

Table 10- Adjusted data for the Silicone experiments. Grown in RPMI 1640 and measured using a FluroStar Omega plate reader at 550nm.

Neoprene rubber incubated at 30^0C for 24 hours	Neoprene rubber incubated at 37^0C for 24 hours
0.116	0.16
0.079	0.073
0.081	0.0516
0.0516	0.0744
0.104	0.084
0.0636	0.072
0.085	0.17
0.088	0.177
0.0912	0.087
0.0864	0.0996
0.111	0.085
0.138	0.067

Table 11- Adjusted data for the Neoprene experiments. Grown in RPMI 1640 and measured using a FluroStar Omega plate reader at 550nm.

Butyl rubber incubated at 30^0C for 24 hours	Butyl rubber incubated at 37^0C for 24 hours
0.145	0.134
0.133	0.121
0.134	0.0984
0.132	0.147
0.121	0.336
0.132	0.126
0.096	0.116
0.0948	0.086
0.072	0.06
0.116	0.151
0.087	0.126
0.048	0.102

Table 12- Adjusted data for the Butyl experiments. Grown in RPMI 1640 and measured using a FluroStar Omega plate reader at 550nm.

Growth conditions	Standard error of the mean silicone	Standard error of the mean Neoprene	Standard error of the mean Butyl
30/24	0.025144	0.048787	0.018751
30/48	0.015965	0.034443	0.021481
37/24	0.045195	0.027434	0.023777

37/48	0.017521	0.073963	0.028112

Table 13- Standard error of the mean from the growth under various conditions (all in MEB).

Growth conditions	Standard deviation silicone	Standard deviation Neoprene	Standard deviation Butyl
30/24	0.077475	0.174646	0.029349
30/48	0.046687	0.124157	0.117729
37/24	0.131793	0.091483	0.062039
37/48	0.214866	0.258722	0.099594

Table 14- Each material and their standard deviation for each data set. MEB tests.

Growth conditions	Average growth on silicone	Average growth on Neoprene	Average growth on Butyl
30/24	0.068233	0.091233333	0.109233

37/24	0.074817	0.10005	0.133617

Table 15- The average growth of all materials and conditions for the RPMI1640 experiments.

Growth conditions	Standard deviation Neoprene	Standard deviation Neoprene	Standard deviation Butyl
30/24	0.03173	0.02338544	0.029683
37/24	0.025579	0.043358	0.068724

Table 16- Standard deviations for all materials and conditions in the RPMI 1640.

Growth conditions	Standard error of the mean silicone	Standard error of the mean Neoprene	Standard error of the mean Butyl
30/24	0.00916	0.006750795	0.008569
37/24	0.007384	0.012516	0.019839

Table 17- Standard error of the mean for each material and condition in RPMI 1640.

Variables	**P value (alpha= 0.05)**
Silicone 30/24 and Neoprene 30/24	0.36
Silicone 30/24 and Butyl 30/24	0.42
Neoprene 30/24 and Butyl 30/24	0.46
Silicone 30/48 and Neoprene 30/48	0.1
Silicone 30/48 and Butyl 30/48	0.02
Neoprene 30/48 and Butyl 30/48	0.92
Silicone 37/24 and Neoprene 37/24	0.38
Silicone 37/24 and Butyl 37/24	0.70
Neoprene 37/24 and Butyl 37/24	0.38
Silicone 37/48 and Neoprene 37/48	0.19
Silicone 37/48 and Butyl 37/48	0.91
Neoprene 37/48 and Butyl 37/48	0.57

Table 18 The results of T tests performed on all the data gathered from the Malt Extract Broth experiments. 30/24= 30 degrees incubated for 24 hours 30/48= 30

degrees incubated for 48 hours. 37/24= 37 degrees incubated for 24 hours. 37/48= 37 degrees incubated for 48 hours.

Variables **material is Silicone only**	**P value (alpha= 0.05)**
30/24 against 37/24	0.01
30/48 against 37/48	0.22
30/24 against 30/48	0.52
37/24 against 37/48	0.94
30/24 against 37/48	0.23

Table 19- comparison within the group of materials. This is just within Silicone. Significant results are highlighted in bold. The key is the same as the previous table MEB

Variables **material is Neoprene only**	**P value (alpha= 0.05)**
30/24 and 37/24	0.83
30/48 and 37/48	0.43

30/24 and 30/48	0.62
37/24 and 37/48	0.25
30/24 and 37/48	0.34

Table 20- comparison within the group of materials. This is just within Neoprene. Significant results would be highlighted in bold, however from this data set there are no significant results. The key is the same as the previous table. MEB.

Variables **material is Butyl only**	**P value (alpha= 0.05)**
30/24 and 37/24	0.0002
30/48 and 37/48	0.7
30/24 and 30/48	0.04
37/24 and 37/48	0.96
30/24 and 37/48	0.07

Table 21- comparison within the group of materials. This is just within Butyl. Significant results are highlighted in bold. The key is the same as the previous table. MEB

Variables	**P value (alpha= 0.05)**
Silicone 30/24 and Neoprene 30/24	0.07
Silicone 30/24 and Butyl 30/24	0.006
Neoprene 30/24 and Butyl 30/24	0.2
Silicone 37/24 and Neoprene 37/24	0.2
Silicone 37/24 and Butyl 37/24	1.91157E-05*
Neoprene 37/24 and Butyl 37/24	0.2

Table 22- The Students T test results for the RPMI experiments. * this result means $1.9x10^{-5}$ and as this value is below the alpha value is it therefore significant. Data that is significant is highlighted with bold text. It was a 2 tailed equal variance test.

Variables	**P value (alpha= 0.05)**
Silicone 30/24 and silicone 37/24	0.34

Neoprene 30/24 and Neoprene 37/24	0.52
Butyl 30/24 and Butyl 37/24	0.22

Table 23- The comparison within the groups of materials. Significant results would be highlighted in bold text. It was a 2 tailed equal variance test. RPMI