Determination of the Role of Glucose and pH in the Production of Biofilms, Phospholipase and Protease in *Candida auris*

by

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Abstract

First isolated in 2009 from the ear canal of a Japanese patient, *Candida auris* has become a growing concern. It has been found to have a similar profile of virulence to *Candida albicans.* It forms biofilms and produces phospholipase and protease activity. The virulence of *C. auris* varies according to the strain, with aggregating strains showing less virulence than non-aggregating strains, though reports find it to be less virulent overall than *C. albicans*. Alarmingly, however, it has shown greater resistance to multiple drugs from all three classes of antifungals and routine cleaning protocols. Most at risk of invasive and systemic infections are severely immunocompromised patients in intensive care settings particularly those with a urinary catheter *in situ.* Due to impaired immunity and associated comorbidities, patients with diabetes mellitus are considered a high-risk group. The study investigated whether synthetic urine mimicking the high glucose and low pH conditions found in diabetic urine would increase the production of biofilms, protease and phospholipase of an aggregating (NCPF 8977) and non-aggregating strain (NCPF 8971) of *C. auris*. *C. auris* was found to grow well in the synthetic urine media in its planktonic form where the culture medium is shaken to preventing biofilm formation, and the growth increased in line with glucose concentration and varied in respect to pH with pH 5.6 showing less growth than pH 5.2 or pH 6.3. The study found that there was no significant change in biofilm formation at any of the tested pH levels and glucose concentrations. However, variation in phospholipase and protease activity was seen when the conditions were altered, and this appeared to be in a strain dependent manner. Other studies have shown that *C. auris* produces protease

and phospholipase in a strain dependent manner, though this study has shown that this can vary with respect to glucose and pH.

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I should begin by thanking whatever muse inspired me to leave a career in nursing to become a scientist. That was five years ago, though it feels a lifetime ago, and I have learned so much and grown as a person more than I could have imagined. It has not been an easy journey and I have questioned my sanity on many occasions, but I must also thank the muse that inspires my bullheadedness and has ensured that I have made it to the end. I would also like to thank my supervisory team Dr Steven Beeton, who lit the flame under my passion for microbiology and has made me believe that I can do this even when I doubted myself, Dr David Waring for his support and proof-reading, and Dr Achyut Guleri for providing support and inspiration for where to go with this project. Many thanks are owed to Stefanie Gilchrist for her support in the lab, her endless patience, fixing things that I broke and always going to extra mile to help. I also want to thank Sarah Maile who has studied alongside me throughout our degree and MSc, and has been a sounding wall for my whining, neurosis, and love of books and video games. Journey before destination, Sarah. Finally, I would like to thank my family and friends, especially my son Harrison for his patience and understanding, and for allowing me to use my computer to write my thesis when he could have been playing Minecraft.

“My hopes and fears are alternately raised and quelled by the minutest—the most trivial circumstances—by the slightest words.”

A Diary of Thomas de Quincey, 1803.

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1: Introduction

1.1 Aim

This MSc (by research), entitled “Determination of the role of glucose and pH in the production of biofilms, phospholipase and protease in *Candida auris*” aims to determine whether there is increased virulent activity in two strains of the fungal pathogen *C. auris* in synthetic urine supplemented with increasing glucose concentrations and varying pH levels. Biofilm formation, phospholipase and protease are recognised virulence factors, which aid the *in vivo* spread and colonisation of the organism. Here we will determine whether synthetic urine mimicking the raised glucose and low pH as seen in diabetes mellitus would increase the virulence of two strains of *Candida auris.*

1.2 Diabetes mellitus

1.2.1 Brief summary

According to Zaccardi *et al* (2015), diabetes mellitus (DM), more commonly referred to simply as diabetes, is a group of diseases that occur as a result of impaired insulin production or impaired insulin sensitivity. Mayer[1] *et al* (2007) describe insulin as a 51-residue protein consisting of two chains, an A chain and a B chain, connected by disulphide bonds. It is produced in β-cells in the Islets of Langerhans in the pancreas and its main roles are to stimulate the absorption of glucose and to suppress hepatic gluconeogenesis. Gluconeogenesis is largely performed in the liver and is a method of glucose regulation whereby glycogen stored in the liver is converted to glucose during periods of fast when systemic glucose levels are depleted. This process is regulated by insulin to maintain blood

glucose levels (Hatting *et al*., 2017). DM includes type I (DMI) which usually has its onset in childhood and is characterised by the failure of the pancreas to produce insulin or to produce it in sufficient quantities leading to insulin deficiency. Type II DM (DMII) usually has its onset in adulthood and occurs generally as a result of obesity, a high fat and sugar diet, and lack of exercise. DMII typically involves a resistance or reduced sensitivity to insulin. Gestational diabetes is a form of DM that occurs in pregnancy and is usually resolved following delivery of the baby. The insulin resistance or deficiency seen in DM leads to poor regulation of gluconeogenesis and raised blood glucose levels (hyperglycemia) (Zaccardi *et al.,* 2015) above the normal fasting blood glucose of around 0.6 – 1.0 mg/mL (Güemes *et al*., 2015). Long-term hyperglycemia can have implications for health and can lead coronary heart, cerebrovascular, nephropathy, retinopathy and neuropathy complications. Prolonged high blood glucose levels can lead to the excretion of glucose into the urine, this process is regulated by the kidneys (Lofty *et al*., 2016). The kidneys are two bean shaped organs situated in the left and right retroperitoneal space. The kidney is highly vascularised and receives around 25% of cardiac output. It participates in the removal of toxins and balance of fluid and constituents of blood entering the kidney via the renal arteries and exiting via the renal veins. The nephron, of which there are around 1 million, is the functional unit of the kidney and is formed of a filtering unit called the glomerulus, which filters water and small solutes from plasma, and a tubular region made up of the proximal tubule, the loop of Henle, and the distal tubule which are responsible for the reabsorption of electrolytes, amino acids, proteins and glucose, any remaining water and solutes are excreted as urine (Hoenig & Zeidel., 2014). In a healthy person, the glomeruli filter around 180 g of glucose

from plasma each day and virtually all of this glucose is reabsorbed. The reabsorption is mediated by sodium-glucose linked transporter (SGLT) proteins present in the cell membrane of the proximal tubule and a healthy individual will have a zero-net excretion of glucose in the urine. The transporter proteins are only able to reabsorb approximately 375 mg glucose/min and when the blood glucose level exceeds 1.6-1.8 mg/mL and once the threshold for reabsorption is exceeded, the excess glucose is excreted in the urine (glucosuria) as seen in DM (Zaccardi *et al*., 2015).

1.2.2 Urinary infections in diabetes

Patients with DM are at an increased risk of urinary tract infections. The reason for the increased risk is largely unknown though it is proposed that glucosuria, impaired immune response and leukocyte function are amongst the possible risk factors (Nicolle., 2014). Tandogdu and Wagenlehner (2016) consider DM a primary risk of UTIs alongside age, previous history of UTI and sexual activity. UTIs are more common in women, though age increases the risk of UTI in men. DM is associated with comorbidities and according to the Public Health England (PHE) (2018), DM is the largest single cause of end-stage renal failure and excluding accidents, the greatest cause of lower limb amputations, in addition to higher rates of cardiac disease, foot ulcers and nerve damage. McAllister *et al* (2014) found that patients with high blood glucose levels of >11.1 mmol/L admitted to Intensive Care Units (ICU) for cardiovascular and respiratory diseases had prolonged hospital stays, poorer prognoses and higher rates of mortality than those with a blood glucose level of <6.1 mmol/L. According to the Health Innovation Network (2016), 56% of UTIs are associated with indwelling urinary catheters. Shackley *et al* (2017) report that catherization rates across the

UK vary amongst hospital sites and range between 12-26%, though the highest catheterisation rates are seen ICU settings where over 70% of patients have an *in situ* indwelling urinary catheter. Catheterisation is more prevalent in males than females is more common in patients aged >70 in a hospital setting. The rationale for catheterisation varies and detailed statistics are unavailable, though Shackley *et al* (2014) offers an explanation for gender variation and suggests that males are catheterised more than females due to their susceptibility to prostate disease and the increased likelihood of urinary retention following surgery. However, the presence of a urinary catheter increases the risk of a catheter associated UTI (CAUTI). Maxwell *et al* (2014) states that each day a catheter is left *in situ* increases the risk of CAUTI by 5%. In-dwelling medical devices such as urinary catheters are associated with biofilm formation and have been widely studied in relation to *Candida albicans*. Reports vary, though the use of urinary catheters is implicated in over 60% of cases of infection with *C. auris* (Snyder & Wright., 2019). To date, there is little evidence to determine if *C. auris* is associated with increased risk of infection with respect to increasing concentrations of urine glucose.

1.3 Structure of a typical yeast cell

Yeasts are unicellular eukaryotic organisms and they contain almost the same organelles as those found in more complex eukaryotic organisms which include endoplasmic reticulum, Golgi apparatus, mitochondria, cytoplasm, ribosomes, cytoskeleton *etc.* Yeast cells also have a cell wall. The cell wall is mostly comprised of polysaccharides, proteins and lipids, and provides the cell with

rigidity and structure and protection from shearing forces. The cell wall plays a role in the adhesion of the cell to surfaces and in pathogenesis (Murray., 2016).

1.4 *Candida auris*

Candida auris, is a newly emerging yeast pathogen which was first identified in 2009 in the ear canal of a Japanese. It can colonise the skin and mucosa and lead to invasive and systemic infections in immunocompromised patients (Satoh *et al*., 2009). The Center for Disease Control (CDC) (2018), the World Health Organisation (WHO) (2018), PHE (2017[1]) and many other health organisations all consider *C. auris* to be an emerging concern as it has shown resistance to multiple drugs from all three classes of antifungal drugs used in *Candida spp.* and to standard cleaning regimens (Ku *et al*., 2018).

1.4.1 Antifungal drugs

Antifungal drugs are grouped into three main classes based on their site of action: azoles, polyenes and echinocandins. Azoles, such as clotrimazole and miconazole, inhibit the synthesis of ergosterol, a vital component in fungal cellular membranes. Polyenes, which include amphotericin B and nystatin, disrupt the formation of fungal cell membranes by binding to ergosterol. Echinocandins like capsofungin and micafungin inhibit the synthesis of (1,3)-beta-d-glucan synthase, a vital cell wall component of many fungi. Allylamines are another class of antifungal drugs which also target sterol synthesis, though they are not used in the treatment of *Candida* infections (Owens *et al*., 2010).

According to the CDC, around 90% of *C. auris* isolates were found to show resistance to fluconazole, about 30% are resistant to amphotericin B and <5% have been resistant to echinocandins.

Analysis of the *C. auris* genome by Rossato and Colombo (2018) has revealed many uncharacterised proteins, though it has been shown to be closely related to four other *Candida* species: *Candida heamulonii, Candida duobushaemulonii, Candida pseudohaemulonii* and *Candida lusitaniae.* Around 40% of *C. auris* proteins are orthologus to those of *C. lusitaniae* which also has limited susceptibility to amphotericin B. *C. haemulonii* also has limited susceptibility to amphotericin B and azoles. The CDC (2019) also report that isolates have shown a mutation in the *Erg11* gene responsible for encoding the azole target enzyme 14-demethylase (Erg11p), which suggests that *C. auris* would be unlikely to respond to fluconazole. *Erg11* mutations have long been associated with azole resistance in *C. albicans* (Xiang *et al*., 2013). The *C. auris* genome demonstrates genes well-characterised in the role of biofilm formations, the production of proteases, phospholipases and transporter proteins which likely contribute to its azole resistance as well as to its virulence (Cortegiani *et al*., 2018).

1.4.2 Epidemiology of *Candida auris*

There have been multiple confirmed cases of *C. auris* reported throughout the world including over 1500 cases in South Africa (Govender *et al.,* 2018). By October 2018, there had been 433 confirmed cases in the United States of America with a further 30 probable cases (CDC, 2018). In April 2018, the European Centre for Disease Prevention and Control (ECDC) published the Rapid Risk Assessment, until that time there had been 620 reported cases of infection or colonisation throughout Europe. Of these, 221 incidences were reported in the UK at 35 hospital sites in patients who had been transferred from 3 hospitals where the largest outbreaks were seen. Most of the cases of infection

or colonisation were detected through screening of swabs and samples taken from commonly colonised areas of the skin and mucosa. Approximately a quarter of the positives screens led to a clinical infection with 27 patients developing blood stream infections (candidaemia). In July 2017, PHE launched a pilot survey at 5 hospital sites to screen patients admitted to intensive care units to determine the origin of colonisation with *C. auris* and inform future surveillance (PHE., 2017[2]). It is acknowledged that *C. auris* is likely to be under reported due to the diagnostic limitations available in routine microbiology laboratories with *C. auris* often misidentified as other species or identified only to genus level (Snyder and Wright., 2019). According to Chowdhary *et al* (2017), the risk of a DM patient developing an infection from *C. auris* is similar to other *Candida spp.* at around 18%.

1.4.3 Aetiology

Patients can become immunocompromised through a number of acquired and congenital mechanisms including DM (Zhou *et al.,* 2018). Impaired immunity typically includes reduced immune surveillance, reduced capacity to clear pathogens and an increased susceptibility to infection (Nicholson., 2016). Commensal microbiota can be disturbed by the use of antibiotics, which, while targeting some bacterial species, allows other microbes to thrive due to reduced competition for resources (Langdon *et al*., 2016). In-dwelling medical devices, such as urinary catheters and central lines commonly used in intensive care units, and also provide a point of entry for microorganisms to invade the host as well as providing a substrate for them to proliferate (Raman *et al*., 2016).

1.4.4 Pathogenesis of *C. auris*

Once a pathogen like *C. auris* has established colonisation, it has the various virulence factors which can cause damage to the host tissue. The host immune defences can detect infectious agents on the surface or within cells of the host mucosal epithelium and can activate the innate and adaptive immune response to clear pathogens (Caffrey and Obar*.,* 2016). The immune system responds using an escalating manner to address threats posed by invading pathogens. The response includes symptoms associated with infection such as fever and increased white cell production. However, in the event of a systemic infection, an overzealous effort by the immune system to clear a pathogen can also lead to sepsis, a serious and often life-threatening complication of infection (WHO., 2018).

1.5 Pathogenic *Candida*

1.5.1 How does *Candida* cause infection?

Fungi are a diverse group of eukaryotic organisms that includes mushrooms, moulds and yeasts. Yeast typically grow as single cells and primarily multiply asexually occasionally via binary fission (fission yeast) as seen in the brewers' yeast *Schizosaccharomyces pombe* whereby cells divide through mitosis similar to the cells of multicellular animals. Yeast cell division is most commonly via budding (budding yeast) where daughter cells (buds) emerge from the mother cell, enlarge and separate (Martin and Arkowitz, 2014). *C. auris* is an example of a budding yeast. The morphology of yeast varies amongst species, though they can exist as single cells (unicellular) or grow as filaments known as hyphae which can intertwine to form a mycelium. There are an estimated 1.5-5.0 million fungal species, about 1500 are yeast species, most of which are harmless to humans

and only a few hundred are considered to be pathogenic (Köhler *et al*., 2015). Many are considered medically or commercially valuable and are used in the manufacture of a range of products including antibiotics, biodiesel, wine and bread. According to Brunke *et al* (2016), pathogenic fungi typically arise from two sources: environmental sources and host niches, with the majority coming from the environment. Brunke *et al* (2016) explain that environmental fungi are intermittently exposed to conditions similar to those found within human hosts which likely lead to evolutionary adaptations. Host microrelationships can be separated into two subgroups: obligate human pathogens and commensals. Obligate human pathogens can affect immunocompetent patients (patients with a healthy immune response) though the commensals usually colonise harmlessly in humans and are opportunistic pathogens which usually affect immunocompromised patients (Brunke *et al*., 2016). *Candida spp.* are opportunistic pathogens, the most common being *Candida albicans* (Borman *et al*., 2016). Larkin *et al* (2017) have determined that *C. auris* has similar mechanisms of infection to *C. albicans. C. albicans'* ability to colonise and invade host niches is largely due to its cell wall. The C. *albicans'* cell wall contains specialised sets of proteins known as adhesins which mediate cell-cell adherence, and adherence between the host cell and abiotic surfaces, such as medical devices, which allow the *C. albicans* to colonise the skin and mucosa as well as materials such as urinary catheters (de Groot *et al*., 2015). Other specialised proteins known as invasins mediate induced endocytosis when they bind to host ligands triggering the host cell to engulf the yeast cell. Some yeasts such as *C. albicans* can alter their morphology and switch from yeast to hyphal cells, which can puncture the surface of the host cell and invade it in a process

known as active penetration (Mayer *et al.,* 2013). The cell wall plays many roles in the virulence of *Candida spp.* Virulence describes the yeasts' potential to cause disease in humans and *Candida* show many virulence factors or mechanisms to cause disease. Examples of these include the ability to secrete enzymes from within the cell wall, such as phospholipases and proteases (Larkin *et al*., 2017); which actively target attributes of the host cell membrane to facilitate the invasion of host cells

1.5.2 Biofilms

An important factor in the virulence of fungal pathogens is the ability to produce a biofilm. A biofilm is a consortium of microbes that can adhere to a surface such as epithelial cells or the surface of an in-dwelling medical device such as a urinary catheter and proliferate across the surface within an extracellular matrix (ECM) (Richardson *et al*., 2018). The ECM is comprised of water and extracellular polymeric substances (EPS) namely polysaccharides, proteins and DNA (Di Martino., 2018). The cells within the biofilm have a distinctive phenotype compared to their planktonic peers (free-floating cells) and a cell's capacity to adhere to a surface to form a biofilm is mediated by regulatory adherence proteins. According to Gulati and Nobile (2016), the formation of a biofilm is largely influenced by the nature of the available surface, composition of environmental nutrients and quorum-sensing molecules. Quorum sensing is a method of microbial communication which utilises chemical signalling to regulate cell behaviours such as virulence and biofilm formation (Albuquerque and Casadevall., 2012). The development of a yeast biofilm begins with the seeding process. This starts with the initiation of a basal layer when a single yeast cell(s) begins to adhere to a solid surface such as an epithelial layer *in vivo* or a

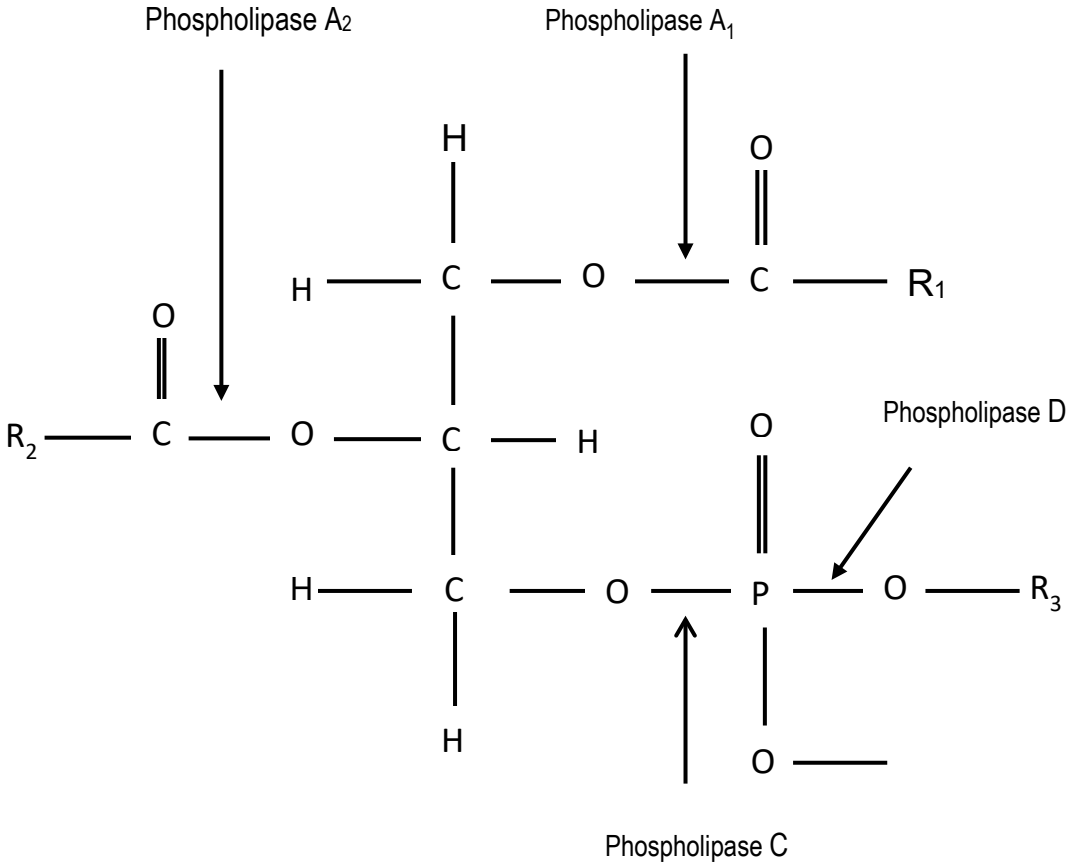
microtitre plate *in vitro*. In *C. albicans*, adherence typically takes around 60-90 minutes (Fox *et al*., 2015). The cells then begin to proliferate, usually through asexual processes, and the early biofilm begins to form. In some species, such as *C. albicans*, early stage filamentous growth can be observed which becomes more prominent as the biofilm matures (Gulati and Nobile., 2016). The mature biofilm contains layers of yeast cells and, depending on the species, hyphal (elongated thread like filaments) cells and pseudohyphal (formed of newly divided cells through budding) cells, which are surrounded by an ECM (Fox *et al.,* 2015). A mature biofilm of *C. albicans* typically takes 24 hours to form. The final stage of biofilm development is the dispersal stage, where yeast cells are dispersed in order to seed new biofilm sites (Gulati and Nobile., 2016). The ability to form hyphae within a biofilm is considered important to provide structure and stability for the biofilm. This process is considered critical to the overall development and maintenance of the biofilm. The ECM is largely produced by the biofilm cells, though it also contains some environmental aggregates including some intracellular components of host cells, and the composition can vary depending upon the conditions (Fanning & Mitchell., 2012). A typical composition for a *C. albicans* biofilm is around 55% glycoproteins, approximately 25% carbohydrates, 15% lipids and around 5% is nucleic acids. It is worth noting that biofilms *in vivo* are not usually comprised of a single species and are typically a consortium of fungal and bacterial species (Alim *et al*., 2018). *C. auris* has not been reported to develop hyphae in its biofilm, though its biofilm is comprised of an ECM with a similar composition to *C. albicans* (Dominguez *et al*., 2019). Despite the absence of this critical biofilm component, Borman *et al* (2016) found the pathogenicity of non-aggregating strains comparable with *C. albicans* when they used the *Galleria*

mellonella infection model, a model which uses wax moth larvae as a host for infection. This contrasts with Larkin *et al* (2017) who found that *C. auris* showed weaker virulence in terms of biolfim formation and enzyme production than *C. albicans* and advise that the clinical significance of *C. auris* is largely due to its multidrug resistance. Biofilm formation in *C. auris* is also considered strain dependent with non-aggregating strains showing a greater propensity to form biofilms than aggregating strains (Singh *et al*., 2019). Another term for aggregation is flocculation which describes cell-cell adhesion where cells form clumps that settle out of suspension under the influence of gravity. Flocculation is a particularly sought-after quality in brewers' yeasts like *Saccharomyces cerevisiae* which flocculate after the fermentation process is complete and settle to the bottom leaving the product clear of cells (Verstrepen & Klis., 2006).

1.5.3 Phospholipase

Phospholipases are a family of ubiquitous enzymes involved in a diverse range of biological processes. They are critical to cell survival and their actions are involved in cell membrane homeostasis, the digestion of nutrients, the formation of bioactive molecules, and they play a role in intracellular signalling pathways. Commonly, phospholipases also share the action of catalysing the hydrolysis of ester linkages in glycerophospholipids, the polar molecules which form the bilayer in cell membranes (Haas & Stanley., 2007). All phospholipases target phospholipids though they are categorised as A, B, C or D based on the specific bond they target (see Fig. 1.5.3.1). It is thought that phospholipases are employed by *Candida spp.* to facilitate invasion of host cells due to their ability to cleave phospholipids and disrupt the phospholipid bilayer (Ghannoum., 2000).

Figure 1.5.3.1



Cleavage sites of phospholipase A1, A2, C and D. Phospholipases are enzymes that hydrolyse phospholipids into fatty acids. Each class of phospholipase targets a specific bond within the phospholipid.

1.5.4 Protease

The term protease refers to enzymes whose function is to degrade proteins, though proteases can be divided into two subgroups: exopeptidase and endopeptidase (also called proteinases). Exopeptidase show specificity for small peptides as the substrate (Rao *et al*., 1998). Proteases are ubiquitous and play a role in DNA replication, transcription, cell proliferation and differentiation. Their action regulates the activity of many proteins and generates amino acids and bioactive molecules. Proteases are essential in cell biology and are involved in

all areas of metabolism (López-Otín & Bond., 2008). *Candida spp.* produce many proteases and show proteolytic activity at a broad range of pH values which is likely to support survival in different host environments. Amongst the proteases produced by *C. albicans* are the secreted aspartyl proteases (SAP). There are 10 forms that have be shown to have different optimum proteolytic activity in pH ranges from pH 2 up to pH 7 (Modrzewska *et al.,* 2016). *Candida spp.* use protease to facilitate host invasion by degrading host mucins and extracellular membrane components such as the ECM and host endothelial cell tissues such as keratin and collagen. *Candida spp.* can also employ proteases to evade host immunity by degrading proteins involved in the immune response such as immunoglobulins, complement proteins and the proteins present in macrophages (Marcos., 2016).

1.6 Diabetes and Candiduria

Candida spp. can be recovered from urine and their presence can indicate colonisation of the urinary tract. Their presence in urine can be transient and asymptomatic, though the risk of infection is greater amongst immunocompromised patients (Falahati *et al.,* 2016). DM is associated with immunological impairments and poorly controlled DM with glucosuria is associated with increased infection (Zhou *et al*., 2018). According to Falahati *et al* (2016), high blood glucose levels (hyperglycaemia) leave the gastrontestinal tract, urinary tract and mucous membranes vulnerable to infection. Mandal *et al* (2014) also state that high blood glucose reduces the effectiveness of anti-fungal agents. Their study found that glucose had a high affinity to bind with voriconazole thus decreasing its antifungal activity and indicating that poor glucose regulation in DM patients could result in a reduced response to anti-fungal treatment.

Mandal *et al* (2014) also suggest that the presence of glucose in urine may promote the growth of *Candida,* as glucose is a major carbon source for fungal species. In addition to a typically low pH, diabetic urine may provide a favourable medium for fungal propagation. Nyirjesy *et al* (2012) state that poor glycaemic control is associated with increased *Candida* species in urine, though it is not known whether this is a direct relationship whereby the excess glucose provides a carbon source for yeast species or if this is an indirect relationship as a result of the reduced immune response in the presence of high blood glucose in DM patients.

1.7 Urine biochemistry

The urine of DM patients typically has a lower pH (<5.5) than non-diabetic patients, though the mechanisms for this are not fully understood. Maalouf *et al.,* (2010) considered a metabolic basis for low urine pH and found that the low pH persisted when factors such as diet, body size and age were controlled and concluded that the low urine pH was caused by a combination of greater net acid excretion and the impaired use of ammonia buffers in DM patients. A comparison of the urine of DM and non-DM patients within the study found there were significant differences in magnesium, ammonia, bicarbonate and citrate (Maalouf *et al.,* 2010). The urine of diabetic patients is complex, though for the purposes of this investigation, only a change in pH and glucose will be considered though it is important to recognise that the other biochemical differences between DM and non-DM patient urine as discussed above which may impact upon the growth and virulence of *C. auris.*

1.8 Urine as a culture medium

Microbial culture media is a solid (agar) or liquid (broth) which provides nutrients to support the growth of microbes. Culture media come in many forms, though they are broadly split into complex or defined media. Complex media contain a carbon source and a source of amino acids, though their exact composition is unknown. In a defined media, the exact chemical composition is known. For the purpose of this experiment, a defined synthetic urine (SU) medium was used. In order to support fungal growth, a culture medium must contain the following macroelements: carbon, hydrogen, oxygen, nitrogen, sulphur, calcium, iron, magnesium, phosphorus and potassium. Fungal species use these elements for numerous metabolic roles and in the synthesis of carbohydrates, proteins, lipids and nucleic acids. Fungal species also require growth factors in addition to some microelements (such as Mn, Zn, Co, Mo, Ni and Cu), which *in vivo*, form part of enzymes and cofactors (Basu *et al.,* 2015). The SU was prepared to the specifications from Brooks (1997) and adapted to vary the pH and glucose levels. The SU contained yeast extract as a source of microelements and sources of all ten macroelements. Glucose provided the main carbon source, and urea and ammonia provided a source of nitrogen. In addition, peptone provided a source of proteins, peptides and amino acids (Abelovska *et al*., 2007).

1.9 Measurement of growth

This study will measure the growth of *C auris* using absorbance techniques. This technique has the advantage of being a fast way of ascertaining the bioload in a volume of liquid, however, it measures a pooled sample of cells and makes no distinction between live and dead cells (Nandy *et al.,* 2015). To measure biofilm formation, biofilms will be stained with crystal violet which binds to cell proteins

and DNA staining them purple. The colour change can be observed by reading the absorbance at 600 nm when the biofilms are solubilised with a solvent such as glacial acetic acid (O'Toole., 2011).

1.10 Assays to detect the enzymatic activity of *C. auris*

To observe the phospholipase activity of *C. auris* in SU*,* an SU agar medium supplemented with egg yolk was used. The rationale for this method is that phospholipases degrade phospholipds in the egg yolk producing insoluble precipitates which can be observed as a halo around a colony of yeast cells indicating phospholipase activity (Aryal., 2019). To detect protease activity, an SU agar was supplemented with bovine serum albumin (BSA) which would be degraded by *C. auris* proteolytic enzymes. A stain was added to the agar to bind with proteins present in the media and the area around the colony of *C. auris* where protease activity had occurred would not absorb the stain (Ozkan *et al*., 2005).

1.11 *C. auris* strains

This study used a non-aggregating strain (NCPF 8971) and an aggregating strain (NCPF 8977) acquired from PHE. The strains were selected as they had been used in a similar study of phospholipase, protease and biofilm production by Sherry *et al* (2017). The culture was managed to ensure that the same generation of the strains were used for all experiments.

1.12 Experimental plan

This study will focus on the growth and biofilm, phospholipase and protease production `of *C. auris* in a synthetic urine medium in the presence of varying concentrations of glucose and at different pH values. The glucose levels used in

the study were chosen based on those typically used in the diagnosis of glucosuria using semi-quantitative colorimetric assay or ‘dipstick.’ The glucose levels tested were 1, 2.5, 5. 10 and 20 mg/mL (Acon Labs., 2019). The pH levels used were 5.2, 5.6 and 6.3. A urine pH around pH 6 is considered normal, diabetics typically have a urine pH below pH 5.5. The pH values were selected as they were normal, at the threshold of normal and low.

2: Materials and Methods

2.1 Preparation of media

2.1.1 Preparation of agar

Malt extract agar (Sigma-Aldrich, Gillingham, UK) was prepared according to the manufacturer's instructions. 20 g of agar was dissolved in 400 mL of distilled water in a Duran (DURAN®, Mainz, Germany) bottle. The agar was then autoclaved using an electric bench top autoclave at 121 ºC for 15 minutes. The agar was tempered to 55 ºC before being poured into sterile petri dishes which were then stored in a refrigerator.

2.1.2 Preparation of broth

Malt extract both (MEB) (Sigma-Aldrich, Gillingham, UK) was prepared by adding 4 g of MEB to 250 mL of distilled water. MEB was poured into universal bottles in 10 mL aliquots or 25 mL was added to 250 mL conical flask which was sealed with a cotton wool bung and covered with four layers of aluminium foil. All preparations were autoclaved at 121 ºC for 15 minutes.

2.2 Preparation of lyophilised culture

The *C. auris* NCPF 8971 and NCPF 8977 were obtained from PHE. The capsules were broken using an appropriate tool and the paper strip containing the culture was added to 10 mL of sterile malt extract broth (Sigma-Aldrich, Gillingham, UK) in a universal bottle prepared according the manufacturer's instructions. The cultures were allowed to rehydrate at room temperature for 3 minutes before a loop of the culture was streaked onto an MEA plate. Both the universal and the MEA plate were incubated without shaking at 37 ºC for 48 hours.

Primary cultures were stored in Brain Heart Infusion Broth (BHIB) (Sigma-Aldrich, Gillingham, UK), containing 20% (v/v) glycerol. Several colonies of a culture from the MEA plates were added to microcentrifuge tubes which were then frozen as the primary culture at -80 ºC. A further sub-culture of a colony of *C. auris* from the primary MEA plate was streaked onto another MEA plate which was incubated statically at 37 ºC for 48 hours. Several colonies of this sub-culture were added to 1.5 mL microcentrifuge tubes containing BHIB with 20% glycerol and they were frozen at -20 ºC. Each week, a sub-culture sample was defrosted and streaked onto a new plate. Once the sub-cultures were used, further sub-cultures were made from a frozen primary culture.

2.3 Preparation of synthetic urine

The synthetic urine media was prepared as shown in table 2.3.1 according to the method described by Brooks (1997), though the glucose and pH was adjusted according to the experimental design for this study. The media was prepared as a 10x concentrated stock which was diluted 1 in 10 when used in all experiments.

Table 2.3.1 Components of synthetic urine media

Component	Quantity g/L
Peptone (bacteriological)	1
Yeast extract	0.005
Lactic acid	0.1
Citric acid	0.4
Sodium bicarbonate	2.1
Urea	10
Uric acid	0.07
Creatinine	0.8
Calcium chloride.$2H_2O$	0.37
Sodium chloride	5.2
Iron II sulphate.$7H_2O$	0.0012
Magnesium Sulphate.$7H_2O$	0.49
Sodium sulphate.$10H_2O$	3.2
Potassium dihydrogen phosphate	0.95
Di-potassium hydrogen phosphate	1.2
Ammonium chloride	1.3
Distilled water	Topped up to 100 mL for 10x concentrated stock which was diluted to make 1 L

The components were added as shown in table 2.3.1 to a Duran (DURAN®, Mainz, Germany) bottle, their total weight was calculated as 27.21 g. 60 mL of distilled water was added. The pH was then adjusted. Due to the buffers used in the SU, hydrochloric acid (HCl) 10M was required to alter the pH. The solution was then topped up to 100 mL with distilled water. Following preparation of the 10x stock, the mixture was separated into universal bottles. Glucose was added to each to give concentrations of between1-20 mg glucose/mL. A control stock was prepared containing no glucose. The stock SU was then stored in a refrigerator.

2.4 Establishing growth of *C. auris* in synthetic urine

The growth of both strains in SU was established by adding 1 mL of the control SU to a universal bottle and diluting with 9 mL of sterile distilled water. This was prepared in duplicate and each universal was inoculated with a loop of either *C. auris* NCPF 8971 or NCPF 8977. The process was repeated for each of the glucose concentrations. The bottles were incubated aerobically and without shaking for 18 hours at 37 ºC. Growth was confirmed visually after 18 hours in the control and all glucose concentrations. The process was repeated varying the pH between pH 5.2- 6.3 and growth was confirmed visually in the control at all pH levels and glucose concentrations.

2.5 Overnight culture (OVC)

2.5.1 OVC in MEB

A 250 mL conical flask of MEB was prepared as above and inoculated with a loop of one colony of either *C. auris* NCPF 8971 or NCPF 8977. The flask was then incubated in an orbital shaker set at 180 rpm and 37 ºC for 18 hours.

2.5.2 OVC in SU

A 250 mL conical flask was used for each OVC. 22.5 mL of distilled water was added to a flask which was sealed with a cotton wool bung then covered with several layers of aluminium foil. The flasks were then autoclaved at 121 ºC for 20 minutes. Following autoclaving, 2.5 mL of SU containing 1 mg glucose/mL was added to the flasks. The flask was then inoculated with a loop of one colony of the required strain of *C. auris* from an MEA plate. The flask was then incubated in an orbital shaker set at 180 rpm and 37 ºC for 18 hours.

2.6 Planktonic growth

250 mL conical flasks were prepared as described above. To the autoclaved flasks, 2.5 mL of the required SU from the 10x concentrated stock was added. The flasks were inoculated with 1 mL of OVC in SU. Using a clean sterile tip, a 1 mL sample was immediately taken and added to a cuvette and read at 600 nm using a spectrophotometer against a blank of diluted SU. The results were recorded, and the flasks were incubated in an orbital shaker set at 180 rpm and 37 ºC for 24 hours. The flasks were sampled again. The initial absorbance was deducted from the final absorbance and recorded.

2.5 Assays

2.5.1 Biofilm crystal violet assay

An OVC was centrifuged and diluted to a concentration to the equivalent of 10^7 cfu/mL. This was an adaptation from the protocol by O'Toole (2011). Sterile filtered SU from the 10x concentrated stock at each glucose concentration was diluted with sterile distilled water with 1 part SU and 9 parts distilled water. Six wells of a 96 well plate were selected for each glucose concentration and an additional six wells for the positive control. To each well, 0.95 mL of the appropriate reconstituted SU was added. Each of the wells were then inoculated with 0.05 mL of the 10^7 cfu/mL culture. The plates were covered with an adhesive lid and incubated statically at 37 ºC for 24 hours. Following incubation, the supernatant was aspirated gently using a multipipette. 0.2 mL of distilled water was added to each well, the plate was agitated to dislodge loose cells and the water was then aspirated. This process was repeated three times. The biofilms were then stained with 0.125 mL of 0.1% (v/v) crystal violet solution. The plates were left to incubate at room temperature for 10-15 minutes before the crystal

violet was removed by aspiration and three rounds of washing with water. The plates were left to dry at room temperature for 18 hours. Once dry, 0.125 mL of 30% (v/v) glacial acetic acid in distilled water was added to each well and left to incubate for 20 minutes at room temperature. The contents of each well were then transferred to a new plate using a multipipette. 0.125 mL of 30% (v/v) glacial acetic acid was added to an empty well to serve as a blank. The plates were then analysed using a Fluostar Omega optical density plate reader (BMG Labtech, Aylesbury, UK) at 550 nm. The absorbance of the blank well was deducted from the assay absorbance readings.

2.5.2 Phospholipase assay

The method was adapted from that described by Singh (2018). 2% (w/v) agar-agar (Sigma-Aldrich, Gillingham, UK), 1% (w/v) bacteriological peptone (Lab-M, Heywood, UK), 5% (w/v) NaCl and 0.0006% (w/v) $CaCl_2$ were dissolved in distilled water and autoclaved for 15 minutes at 121 ºC. The 10% (v/v) sterile egg yolk emulsion (Sigma-Aldrich, Gillingham, Germany) was prepared by centrifugation at 3000 rpm for 10 minutes in an Microcentaur (MSE, Heathfield, UK) centrifuge. The supernatant was removed and added to the molten agar together with 10% (v/v) sterile filtered synthetic urine (prepared as a 10x concentrated stock) at varying glucose concentrations. Inside a Class II Laminar Flow cabinet, the Duran (DURAN®, Mainz, Germany) bottle containing molten agar with added egg yolk and synthetic urine was placed in a beaker of hot water to keep molten until the pH was adjusted to either pH 5.2, 5.6 or 6.3 as required. The agar was then poured into sterile petri dishes and once set, stored in a refrigerator. An overnight culture was prepared as above. The cells were washed to remove MEB by adding 1 mL of the culture to the microcentrifuge tube and

centrifuging to pellet at 3000 rpm for 3 minutes. The supernatant was removed and 1 mL of sterile ¼ strength Ringer's solution (Oxoid, Hampshire, UK) was added. The cells were resuspended using a Whirlmixer vortex mixer (Fisherbrand, Loughborough, UK) and centrifuged for a further 3 minutes at 3000 rpm. The Ringer's was removed, and a fresh 1 mL of sterile Ringer's was added. The cells were resuspended using a vortex mixer. 0.1 mL of culture was added to a microcentrifuge tube containing 0.9 mL of Ringer's to make a suspension equivalent to 10^8 cfu/mL. The agar plates were divided into four, and 0.01 mL of the culture was spotted onto the centre of each quarter of the agar. The plates were left to dry for around 20 minutes inside a Class II cabinet before they were sealed with parafilm and incubated statically upside down aerobically at 37 ºC for 3 days. After three days, growth was confirmed visually at all pH and glucose levels. The phospholipase activity (P_Z) was observed qualitatively by the presence of a precipitation zone around the colony (Larkin *et al*., 2017) and given a score of:

- = no precipitation zone

+ = precipitation zone

2.5.3 Protease assay

The method was adapted from a method described by Borst & Fluit (2003) and 2% (w/v) agar-agar (Sigma-Aldrich), 0.5% (w/v) $MgSO_4$, 1% (w/v) KH_2PO_4 were dissolved in distilled water and autoclaved for 15 minutes at 121 ºC. To the molten agar, 0.16% (w/v) BSA (Sigma-Aldrich, Gillingham, UK) (dissolved in water and filter sterilised) and 10% (v/v) sterile filtered synthetic urine (prepared as a 10x concentrated stock) was added. The pH was adjusted to either pH 5.2, 5.6 or 6.3

as required and the mixture was poured into petri dishes and stored as per the method used for the phospholipase assay preparation. An OVC was prepared in MEB and the cells washed above. The plates were quartered, and each quarter was inoculated with a 0.01 mL aliquot of cells at a concentration equivalent to 10^8 cfu/mL. The plates were incubated for 5 days aerobically at 37 ºC without shaking. After 5 days, growth was confirmed visually at all pH and glucose levels. The plates were stained using an adapted method described by Vermelho *et al* (1996). The staining solution was prepared with 0.1% (w/v) Coomassie Brilliant Blue R250 (Life Science, California, United States), 10% (v/v) acetic acid and 50% (v/v) methanol in distilled water. The plates were flooded with the staining solution and left to incubate at room temperature for 20 minutes. The plates were destained by flooding. The plates were flooded three times with a destaining solution prepared with 10% (v/v) acetic acid and 40% (v/v) methanol in distilled water and incubated at 37 ºC for 20 minutes and finally with distilled water for a further 20 minutes at 37 ºC. Following destaining the protease activity (Pr_z) was observed qualitatively by the presence of a clear zone around the colony and given a score of:

- = no clear zone

+ = clear zone

2.6 Statistical analysis

Normality testing using a histogram, Q-Q plot and Shapiro-Wilks was performed to confirm the data are normally distributed. One-way ANOVA was used to compare the means followed by Tukey's post-hoc analysis where a result was significant. A significant level of $\alpha = 0.05$ was used for all tests.

3: Results

C. auris is an emerging fungal pathogen that was first identified in 2009. The pathogen is a cause for concern for world health organisations as it has shown resistance to antifungal treatments from each class of drugs and to routine cleaning regimes. Those most at risk are patients with impaired immunity, such as patients with DM, and those with an indwelling medical device, such as a urinary catheter, *in situ.* The study exposed an aggregating and non-aggregating strain of *C. auris* to conditions mimicking those found in the urine of patients with diabetes mellitus to observe if there was a difference in the production of biofilms, phospholipase and protease when the pH and glucose levels were altered.

3.1 Planktonic growth

The cells were grown over 24 hours by the method demonstrated in 2.6. Flasks were prepared (n=3) with 1-20 mg glucose/mL and a control without glucose at pH 5.2, 5.6 and 6.3. In the non-aggregating strain (NCPF 8971), at pH 5.2, the growth increases with the glucose concentration. At pH 5.6 (see Fig. 3.1.2), the growth increases with glucose, though the mean absorbance is significantly lower overall than pH 5.2. At pH 6.3 (see Fig 3.1.3), the growth appears to plateau after 5 mg glucose/mL, though there is a large variation amongst the results.

Figure 3.1.1

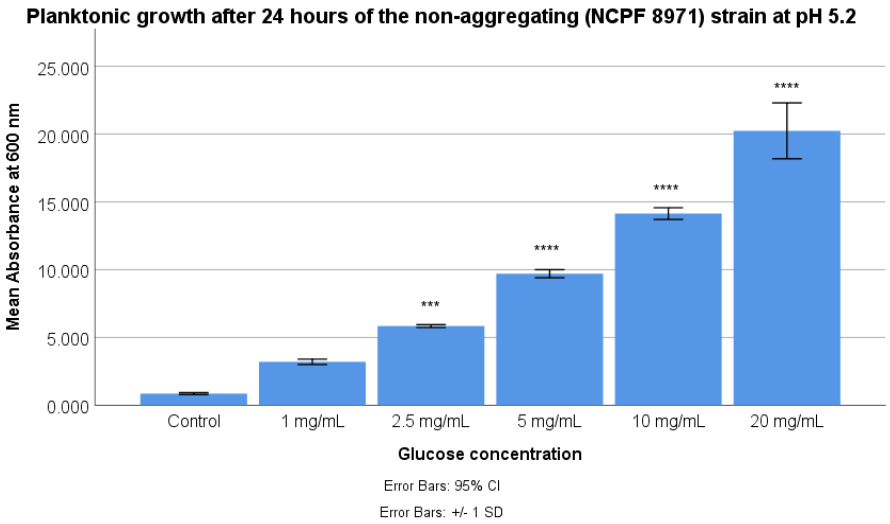


Figure 3.1.2

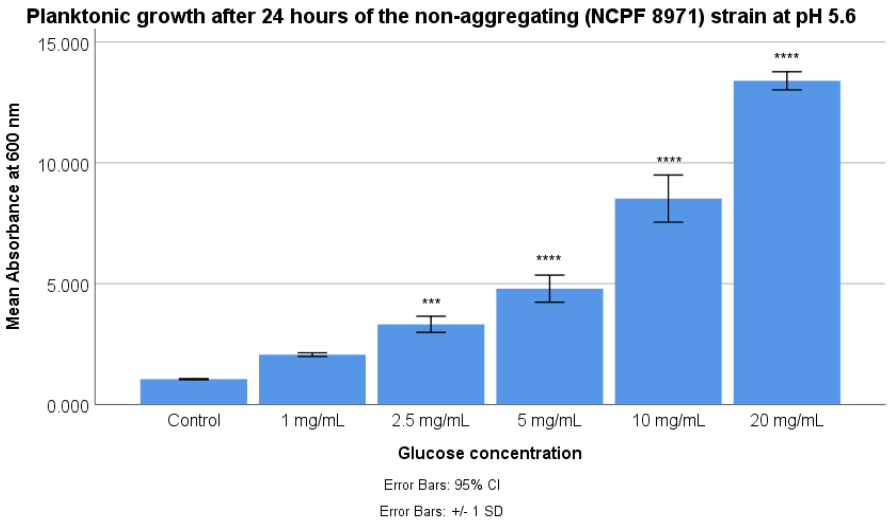
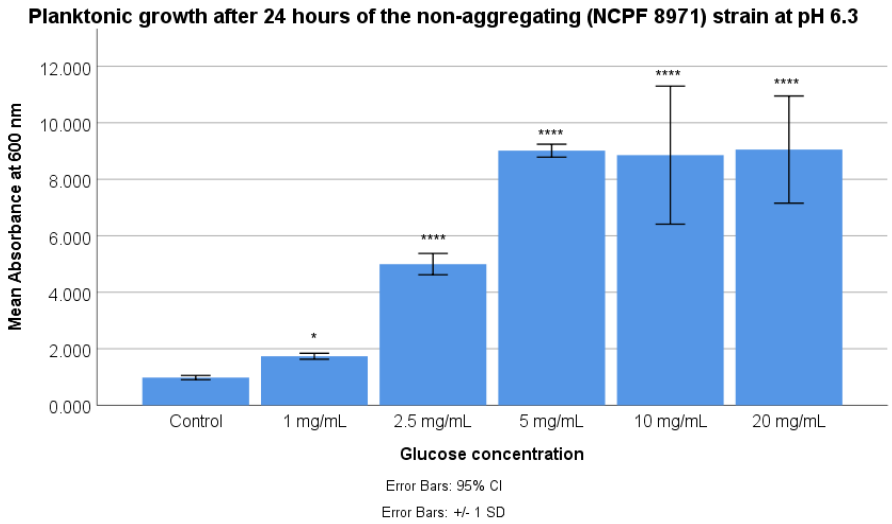


Figure 3.1.3



Flasks were prepared (n=3) for the aggregating strain (NCPF 8977) using the same method described in 2.6. At pH 5.2 (see Fig. 3;1;4), the growth is similar to the growth seen in the non-aggregating (NCPF 8971) strain. At pH 5.6 (see Fig. 3.1.5), the growth is comparable to the non-aggregating strain. At pH 6.3 (see Fig. 3.1.6), there was no measurable growth was seen in the control group. At 20 mg glucose/mL, growth is comparable to pH 5.2.

Figure 3.1.4

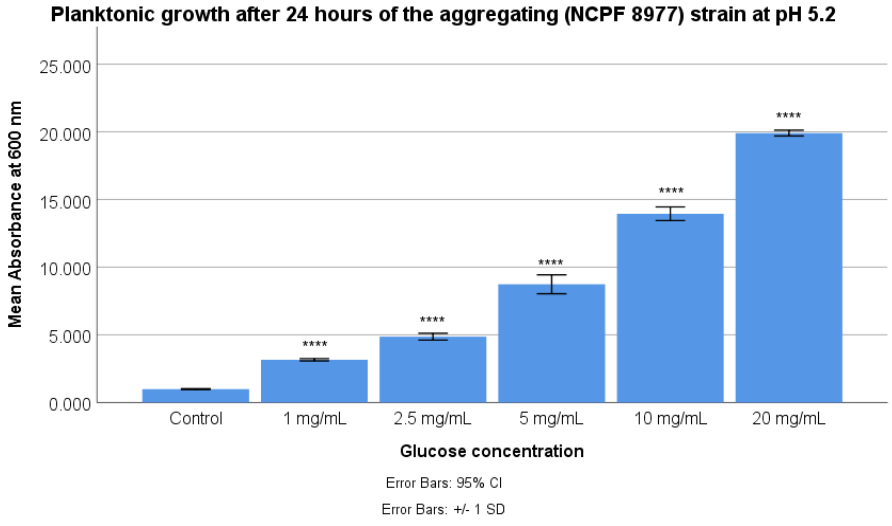


Figure 3.1.5

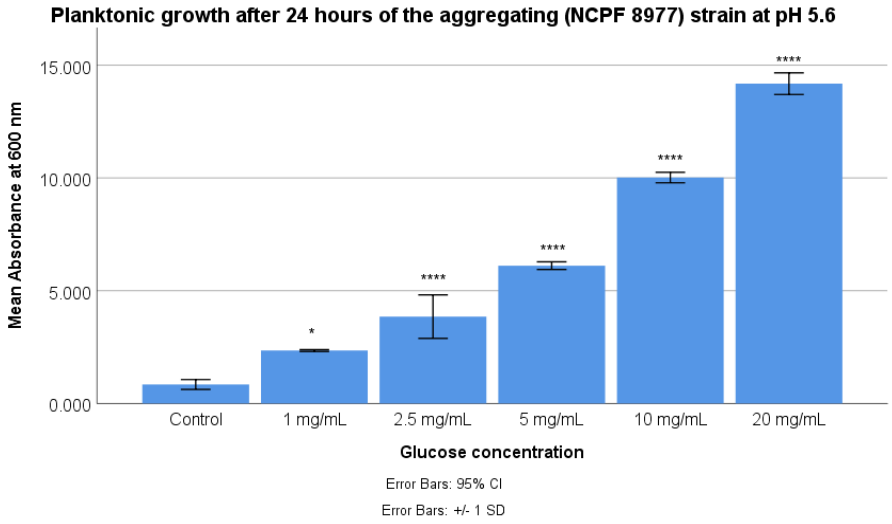
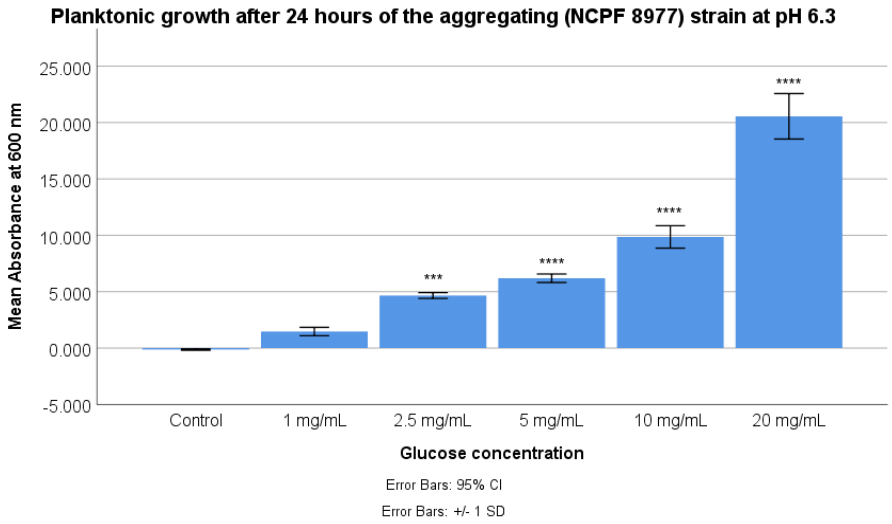


Figure 3.1.6



3.2 Statistical analysis for planktonic growth in SU

Analysis for the non-aggregating (NCPF 8971) strain and aggregating (NCPF 8977) strain. A one-way ANOVA was performed and demonstrated a significant difference and Tukey's post-hoc analysis revealed significant differences. Table 3.2.1 shows growth of the non-aggregating (NCPF 8971) strain (n=3) and shows

a significant difference from the control in 2.5-20 mg glucose/mL at all pH levels. Table 3.2.2 shows growth of the aggregating (NCPF 8977) strain (n=3) and shows growth was significantly different from the control in all glucose concentrations at pH 5.2 and pH 5.6, and in 2.5-20 mg/mL at pH 6.3.

Table 3.2.1 Statistical analysis for planktonic growth in the non-aggregating (NCPF 8971) strain

	pH 5.2		pH 5.6		pH 6.3	
Glucose (mg/mL)	Mean absorbance at 600 nm	p value	Mean absorbance at 600 nm	p value	Mean absorbance at 600 nm	p value
Control	0.860	-	1.046	-	0.980	-
1	3.200	0.057	2.062	0.209	1.730	0.975
2.5	5.840	< 0.0001	3.316	0.001	5.000	0.022
5	9.700	< 0.0001	4.792	< 0.0001	9.010	< 0.0001
10	14.130	< 0.0001	8.519	< 0.0001	8.850	< 0.0001
20	20.240	< 0.0001	13.393	< 0.0001	9.050	< 0.0001

Table 3.2.2 Statistical analysis for planktonic growth in the aggregating (NCPF 8977) strain

	pH 5.2		pH 5.6		pH 6.3	
Glucose (mg/mL)	Mean absorbance at 600 nm	p value	Mean absorbance at 600 nm	p value	Mean absorbance at 600 nm	p value
Control	0.980	-	0.841	-	0.000	-
1	3.150	< 0.0001	2.346	0.018	1.470	0.448
2.5	4.860	< 0.0001	3.850	< 0.0001	4.660	0.001
5	8.730	< 0.0001	6.109	< 0.0001	6.190	< 0.0001
10	13.940	< 0.0001	10.015	< 0.0001	9.860	< 0.0001
20	19.910	< 0.0001	14.180	< 0.0001	20.550	< 0.0001

The SU media was shown to promote growth of planktonic cells (cells which remain in suspension and do not adhere to a surface) in both strains of *C. auris*. An increase in growth was generally seen in line with increasing glucose concentrations, although the absorbance showed some variation with respect to

a change in pH. The non-aggregating strain (NCPF 8971) saw the highest mean absorbance at pH 5.2 with the growth increasing significantly (p=<0.0001) as the glucose concentration increased. The same pattern of growth could be seen at pH 5.6, though the absorbance was much lower with a mean absorbance of 13.393 at 20 mg glucose/mL at pH 5.6 compared with an absorbance of 20.240 at pH 5.2 at the same glucose concentration. At pH 6.3, the mean absorbance was almost double that with 2.5 mg glucose/mL supplementation than at pH 5.2 and 5.6 and growth at 5 mg glucose/mL was similar to pH 5.2, though almost double the growth seen at pH 5.6. The non-aggregating strain (NCPF 8971), showed comparable growth to the aggregating strain (NCPF 8977) at pH 5.2 and pH 5.6; however, some differences can be observed at pH 6.3 which showed no measurable growth in the control group but showed growth in the 20 mg/mL comparable to the growth seen at pH 5.2. The key findings showed that there was no significant difference in the control groups at pH 5.2 or pH 5.6, though SU inoculated with the tested strains at pH 5.6 produced significantly (p= <0.0001-0.05) less growth than pH 5.2 once glucose was added at all concentrations (except 2.5 mg/mL at pH 5.6 which showed wide variability amongst the results).

3.3 Determination of biofilm formation

Biofilm formation was determined by the method described in section 2.5.1. The tested strains were grown in SU media (n=6) with varying glucose and pH and analysed after 24 hours. Fig. 3.3.1 and Fig. 3.3.2 show that though there was a difference in biofilm formation between the two tested strains, there was no significant difference with respect to a change in glucose concentration or pH.

Figure 3.3.1 Biofilm growth of tested strains at varying glucose concentrations

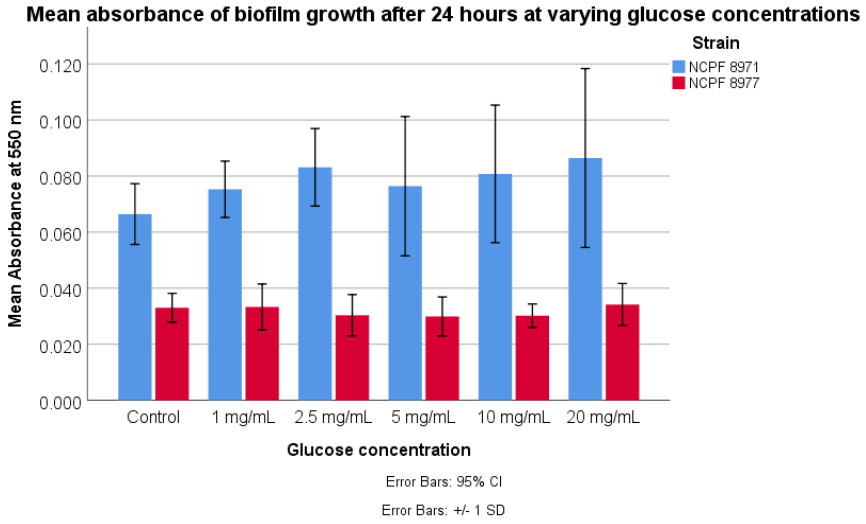
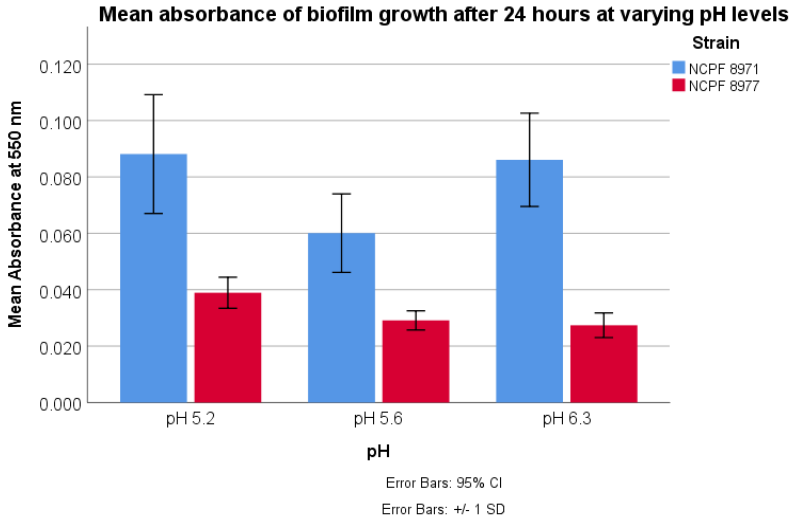
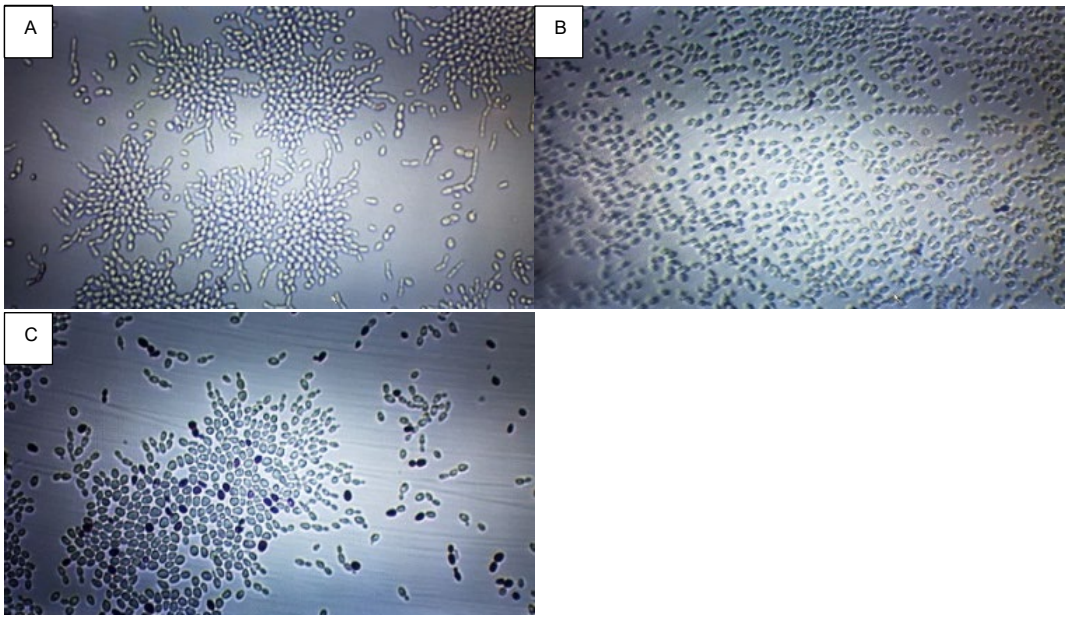


Figure 3.3.2 Biofilm growth of tested strains at varying pH levels



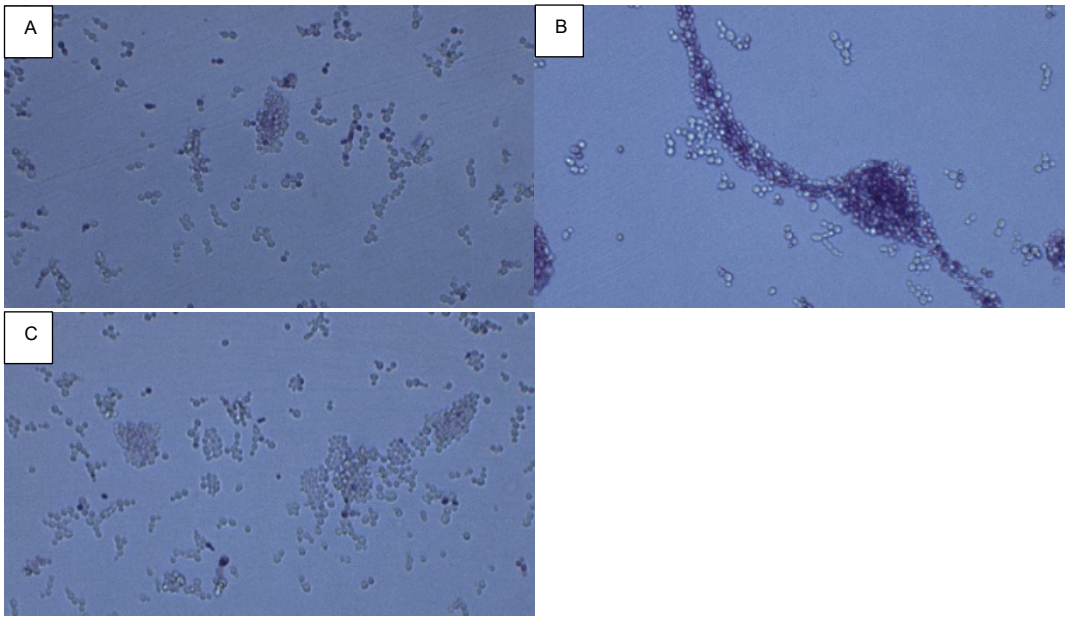
When viewed under an inverted light microscope at x400, the biofilm cells stained with 0.1% crystal violet (as per the method described in section 2.5.1) appeared to vary in morphology with the alterations in pH and glucose.

Figure 3.3.3 Images of non-aggregating (NCPF 8971) biofilm



A. Control without glucose. Cells appear elongated or oval shaped and are joined in pairs or short chains. **B**. With 1 mg glucose/mL, cells appear larger and rounder than the control group and contains mostly single cells. **C**. With 2.5 mg glucose/mL, many budding cells can be seen. All cells were observed using an inverted light microscope at x400.

Figure 3.3.4 Images of aggregating (NCPF 8977) biofilm



A. Control without glucose, very few single cells can be seen with most cells seen in pairs or groups. Budding cells can be seen. **B.** with 1 mg glucose/mL, few single cells can be seen. **C.** with 2.5 mg glucose/mL, some single cells can be observed in addition to large groups. All cells were observed using an inverted light microscope at x400.

The crystal violet assay has shown that, overall, the glucose concentration and pH level did not appear to have a great effect upon biofilm production after 24 hours (Figure 1). Some differences could be seen when in the non-aggregating strain (NCPF 8971), particularly at pH 5.6 where the mean biomass was lower at some glucose concentrations (5, 10 and 20 mg/mL) than in pH 5.2 and pH 6.3. There are some notable differences in the morphology of the biofilm cells in response to the glucose concentration; in the control group, the non-aggregating strain (NCPF 8971) appeared to form chains (Fig. 3.3.3. A), though there are mostly single cells present at 1 mg glucose/mL (Fig 3.3.3. B). Very few single cells can be seen in the aggregating strain (NCPF 8977) in the presence or absence of glucose (Fig. 3.3.4).

3.4 Determination of phospholipase activity

The phospholipase assay was prepared using the method described in section 2.5.2. The tested strains were inoculated onto the SU agar supplemented with egg yolk at varying glucose concentrations and pH levels (n=4). Qualitative analysis was used to determine if phospholipase activity was positive or negative (see Table 3.4.1) by a visually examining the colonies for the presence or absence of a precipitation zone (see Fig. 3.4.2).

Table 3.4.1 The phospholipase activity of the tested strains

	pH 5.2		pH 5.6		pH 6.3	
Glucose (mg/mL)	Non-aggregating (NCPF 8971)	Aggregating (NCPF 8977)	Non-aggregating (NCPF 8971)	Aggregating (NCPF 8977)	Non-aggregating (NCPF 8971)	Aggregating (NCPF 8977)
Control	+	+	+	+	+	-
1	-	+	+	+	+	-
2.5	-	+	+	+	+	-
5	-	-	+	-	+	-
10	-	-	+	-	+	-
20	-	-	+	-	+	-

Figure 3.4.2 Image of a colony positive for phospholipase

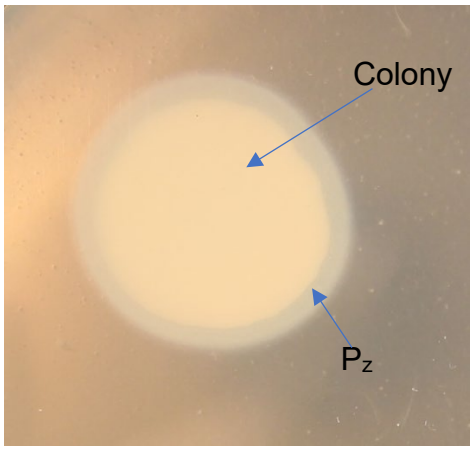


Image shows a colony of *C. auris* NCPF 8971 positive for phospholipase activity (Pz). The colony was grown on an SU agar plate without glucose and supplemented with egg yolk at pH 5.6.

At pH 5.6, the aggregating strain (NCPF 8977) showed similar phospholipase activity to pH 5.2, whereas the non-aggregating strain (NCPF 8971) showed activity at all concentrations of glucose including the control. At pH 6.3, the non-aggregating strain (NCPF 8971) again showed phospholipase activity at each glucose concentration, though there was no significant difference between the

control and the groups with glucose. The aggregating strain (NCPF 8977) showed no activity at pH 6.3.

3.5 Determination of protease activity

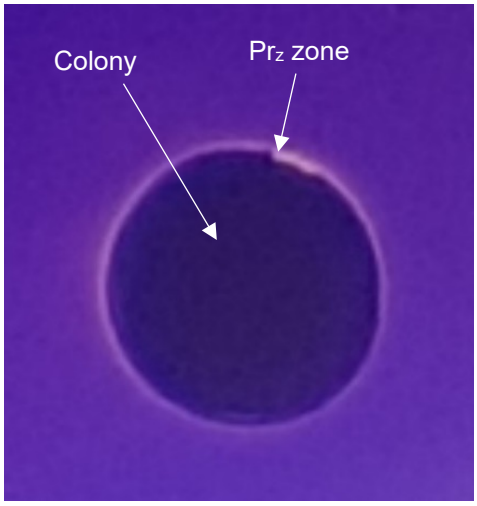
The protease assay was prepared using the method described in section 2.5.3. The tested strains were inoculated onto the SU agar supplemented with BSA at varying glucose concentrations and pH levels (n=4). As per the phospholipase assay, qualitative analysis was used to determine if protease activity was positive or negative (see Table 3.5.1) by a visually examining the colonies for the presence or absence of a clear zone (see Fig. 3.5.2) following staining as per the method described in section 2.5.3.

Table 3.5.1 The protease activity of the tested strains

	pH 5.2		pH 5.6		pH 6.3	
Glucose (mg/mL)	Non-aggregating (NCPF 8971)	Aggregating (NCPF 8977)	Non-aggregating (NCPF 8971)	Aggregating (NCPF 8977)	Non-aggregating (NCPF 8971)	Aggregating (NCPF 8977)
Control	-	-	-	+	-	+
1	-	-	+	+	+	+
2.5	-	+	+	+	+	-
5	-	+	+	+	+	-
10	-	+	-	-	+	-
20	+	-	-	-	+	-

The protease activity of non-aggregating (NCPF 8971) and aggregating (NCPF 8977) strains of *C. auris*. The cells were grown on SU agar supplemented with BSA at varying pH levels and glucose concentrations and incubated aerobically at 37 ºC for 5 days. A negative or positive result shows whether protease activity was observed.

Figure 3.5.2 Image of a colony positive for protease



The image shows a colony of *C. auris* NCPF 8971 positive for proteinase activity (Prz). The colony was grown on an SU agar plate without glucose and supplemented with BSA at pH 5.6. The colony has been stained with Coomassie Brilliant Blue R250 and a destaining solution was used to reveal a clear zone around the colony.

At pH 5.2, the non-aggregating strain (NCPF 8971) only showed proteolytic activity at the highest glucose concentration whereas the aggregating strain (NCPF 8977) showed proteolytic activity between 2.5 and 10 mg/mL. At pH 5.6, the non-aggregating strain (NCPF 8971) showed proteolytic activity between 1 and 5 mg/mL and the aggregating strain (NCPF 8977) demonstrated proteolytic activity between the control group and 5 mg/mL. At pH 6.3, the non-aggregating strain (NCPF 8971) showed proteolytic activity in all glucose concentrations except the control group and the aggregating strain (NCPF 8977) showed activity in only the control group and 1 mg/mL. The non-aggregating strain (NCPF 8971) showed no proteolytic activity in the groups without glucose at any pH, whereas the aggregating strain (NCPF 8977) showed proteolytic activity in the control group at pH 5.6 and pH 6.3, though the activity was arrested as the glucose concentration increased.

4: Discussion

This study aimed to investigate the role of pH and glucose in biofilm, phospholipase and protease production in *C. auris. C. auris* is an emerging species of *Candida* that was first isolated in 2009. It has been identified as a growing concern amongst health organisations worldwide because it is highly resistant to multiple drugs from all three antifungal categories and routine cleaning protocols. Those most at risk are severely immunocompromised patients with indwelling medical devices such as an *in situ* urinary catheter. Patients with diabetes have been identified as at risk due to their poor immunity and associated co-morbidities. This study aimed to determine whether a high glucose concentration and low pH associated with the urine of patients with diabetes increased the virulence of *C. auris,* thus increasing the risk of invasive infections. The study used a synthetic urine medium to mimic the conditions found in the urine of DM patients. The SU medium was shown to promote growth of planktonic cells of the two strains of *C. auris* with a low pH and high glucose concentration which provided good growth conditions. According to Ries *et al*., 2018, glucose is an ideal carbon source as pathogenic fungi have a demonstrable preference for carbon sources that can be metabolised quickly and provide the energy required for growth and colonization in a host niche.

4.1 Planktonic growth

Larkin *et al*., (2017) found that *C. auris* had a similar planktonic growth profile to *C. albicans* when grown in RPMI (Roswell Park Memorial Institute) 1640 medium. In this study, there was some difference observed in the behaviour of the two strains with respect to both glucose and pH. After 5 mg glucose/mL, growth appeared to plateau in pH 6.3, though the variability amongst the 10 and 20

mg/mL groups questions the confidence in this result. The findings by Larkin *et al*., (2017) suggest that the non-aggregating strain (NCPF 8971) should have a similar pattern of growth to the aggregating strain (NCPF 8977) and the plateau seen in pH 6.3 may be an experimental error as indicated by the variation of results within the group.

4.2 Biofilm production

In this study, though not significant, a decline was also seen in the biofilm assay where there appeared to be a decline in biomass after 5 mg glucose/mL at pH 6.3; however, again, the variability amongst the results in this study means that this cannot be commented upon with any certainty. It is worth noting that Larkin *et al*., (2017) did not alter the glucose concentration of the RPMI 1640 medium they used, which had a concentration of 2 mg glucose/mL which is lower than most of the concentrations used in this study. Larkin *et al*., (2017) also did not investigate the effect of pH on growth and if no adjustments were made to RPMI 1640, when prepared according to the manufacturer's instructions, the media would have a pH of 7-7.6 which is higher than the pH levels used in this study. Studies into the *C. albicans* have found that it has adapted mechanisms to thrive at a range of acidic pH levels such as those found in the vagina (pH 4-5) and oral mucosa (pH 6), it has also been observed to grow in pH levels as low as 2 such as those found in the stomach and as high as pH 10 (Sherrington *et al*., 2017).

4.2.1 Biofilm production in response to environment

Yeast cells like *C. albicans* prefer a slightly acidic pH which is largely driven by nutrient uptake. According to Polke *et al*., (2015) micronutrients such as iron are soluble at an acidic pH, and the stability and functionality of fungal proteins are

negatively affected by a raised pH. However, *C. albicans* has adapted mechanisms to respond to environmental pH where signalling pathways drive downstream responses allowing the species to survive at an alkaline pH (Polke *et al*., 2015). This study used 3 acidic pH levels which provide a favourable environment for *C. albicans*, though the ranges are much narrower than those seen in similar studies which have compared much more acidic and alkaline pH levels. This has allowed the opportunity to see alterations in growth a slight change in the pH can cause such as the difference seen between pH 5.2 and pH 5.6. A normal urine pH is considered to be around 6.0 to 7.5 (Maalouf *et al*., 2010). The results have shown that, overall, glucose and pH did not appear to have a great effect upon biofilm production after 24 hours. Some differences could be seen in the non-aggregating strain (NCPF 8971), particularly at pH 5.6 where the mean biomass was significantly lower at some glucose concentrations (5- 20 mg/mL) than in pH 5.2 and pH 6.3. The low variation in the biofilm formation in the tested strains across the range of pH and glucose levels when compared to growth of planktonic cells could indicate that *C. auris* biofilms have an ability to tolerate and thrive in a wide range of conditions. Indeed, Modrzewska *et al* (2016) found that protease enzymes, for example, had optimum activity in varying conditions. Whilst the results did not show a statistically significant difference between the biofilm formation in the two strains, the consistently higher mean across all results may indicate that the non-aggregating strain (NCPF 8971) had a greater propensity for biofilm formation than the aggregating strain (NCPF 8977) as demonstrated by Sherry *et al* (2017). This contradicts Oh *et al* (2011) who tested 15 strains of *C. auris* and found they did not produce a biofilm. However, Oh *et al* (2011) used Sabouraud Dextrose Broth (SDB) with 8% glucose

which is four-fold greater than the highest glucose concentration used in this study. The study by Oh *et al* (2011) does not indicate whether the pH was adjusted from a pH of 5.6 when prepared according to the manufacturer's instructions. In this study, biofilm formation at pH 5.6 has the lowest biofilm formation overall, and the lowest for the non-aggregating strain (see Fig. 3.3.2). This observation is more pronounced as the glucose concentration was increased which indicates that high glucose and a pH of 5.6 may not provide ideal conditions for biofilm formation. In planktonic growth at pH 5.6, *C. auris* showed the lowest growth after 24 hours. Sherry *et al* (2017) also investigated NCPF 8971 and NCPF 8977 and used RPMI-1640 medium which has 2 mg/mL of glucose and found that both strains produced a biofilm. The non-aggregating strain (NCPF 8971) produced more biomass than the aggregating strain (NCPF 8977), which is consistent with the observations of this study.

4.2.3 Biofilm production in response to glucose

In a study on biofilm formation of *Candida* species other than *C. albicans,* Ng *et al* (2016) found that low glucose environments (0.1-2 mg/mL) promoted *Candida glabrata* biofilm formation significantly more ($p = <0.05$) than high glucose environments (10-20 mg/mL). They found that the optimal glucose concentration for biofilm production in *C. glabrata* was 2 mg/mL. A study of *Candida parapsilosis* by Pereira *et al* (2015) found that glucose enhanced biofilm formation, particularly at high glucose concentrations of 10% (100 mg/mL), though this was strain dependent with one of the tested strains showing no significant difference in biofilm formation across all tested glucose concentrations. Further studies of biofilms in *C. albicans* and *C. glabrata* by Hosida *et al* (2018) found that biofilm formation varied dependent on the strain, media, glucose concentration and

whether biofilms were composed of single or mixed species. As a single biofilm, Hosida *et al* (2018) saw that *C. glabrata* demonstrated no significant change in biofilm formation in RPMI 1640 regardless of the glucose concentration, though there was a significant difference when they used SDB supplemented with 1 mg glucose/mL rather than no glucose or 5 mg glucose/mL. This study is limited as it only used synthetic urine as a culture media and differences in biofilm formation may have been seen if other types of media had been tested.

4.2.4 Biofilm production in synthetic urine

A study by Uppuluri et al (2009) compared *C. albicans* biofilms grown in a SU media to those grown in RPMI 1640 and found that after 24 hours there was significantly less biomass in the SU group and that the SU biofilms were less mature than those grown in RPMI 1640. Studies of *C. auris* by Larkin *et al* (2017) and Borman *et al* (2016) have defined a clear difference between strains and identified them as aggregating and non-aggregating strains.

4.3 *C. auris* virulence

Larkin *et al* (2017) have noted that the aggregating strain (NCPF 8977) appears to produce less enzymatic activity and biofilm formation than the non-aggregating strain. Overall, *C. auris* appeared less virulent than *C. albicans* by producing around 50% less biomass in its biofilm and only 1 of the 16 isolates they tested had phospholipase activity comparable to *C. albicans*. They advise that *C. auris'* multi-resistance to antifungal treatments has come at a cost to its virulence which is demonstrable by *C. auris* causing infection in severely immunocompromised patients rather than immunocompetent patients like *C. albicans* (Larkin *et al*., 2017). However, its multi-resistance to antifungal drugs is concerning as it makes

infections difficult to treat and its resistance to routine cleaning means that outbreaks are more likely to spread (ECDC., 2018). In terms of planktonic and biofilm growth, this study has found no clear differences between strains. However, clear differences can be seen when viewing biofilm images (see Fig. 3.3.3 and Fig. 3.3.4). The non-aggregating strain (NCPF 8971) consists of mostly single cells when in the presence of glucose, though it formed short chains in the control group. Its biofilm appeared to grow across the surface of the substrate rather upwards in a 3D structure. The aggregating strain (NCPF 8977) consists of few single cells, with most cells in pairs or groups. It appeared to have a 3D structure to its biofilm. It could also be noted that many cells in the images had not taken up the CV stain which could have impacted the measurement of the biomass. Larkin *et al* (2017) also observed the presence of oval, budding cells either as single cells or aggregates in a strain dependent manner.

4.4 Aggregating vs non-aggregating

Though not empirically tested, it was observed in this study that the aggregating strain (NCPF 8977) required continuous shaking to keep the cells suspended indicating that cells are settling as seen in species like *Saccharomyces cerevisiae* as discussed above. However, Borman *et al* (2016) suggests that the aggregation seen in the aggregating strain (NCPF 8977) is likely to be the result of budding cells not relinquishing their daughter cells rather than flocculation. When examining the images taken in this study, few single cells can be seen in the aggregating strain (NCPF 8977), though there are many pairs and groups (see Fig. 3.3.4).

A study by Verstrepen and Klis (2006) investigated flocculation of *Saccharomyces cerevisiae* and found that flocculating strains settled at the bottom of a liquid medium after 5 minutes, non-flocculating strains remained suspended, whereas a third strain formed microclumps of 2-10 cells resulting in some sedimentation. Though not as dramatic as the flocculating strain seen in the study by Verstrepen and Klis (2006), the cells of the aggregating strain (NCPF 8977) used in this study did not remain suspended like that of the non-aggregating strain (NCPF 8971) indicating that it could be flocculation contrary to the findings of Borman *et al* (2016). Borman *et al* (2016) also stated that they were unable to disrupt the aggregates despite intense vortex mixing. This was also an observation noticed in this study as a difference between the two strains when attempting to resuspend cells with a vortex mixer following centrifugation which took much longer to achieve in the aggregating strain.

4.5 Phospholipase activity

Generally low phospholipase activity was seen in both strains though this appeared to vary according to pH and glucose concentration. This is consistent with the findings by Larkin *et al* (2017) who tested 16 *C. auris* isolates and found they had limited or no phospholipase activity. Kumar *et al* (2015) tested a clinical isolate from a 28-year-old female and found that it had high phospholipase activity. However, Kumar *et al* (2015) gives no indication of the pH used in the case study and Larkin *et al* (2017) did not investigate multiple glucose concentrations and pH levels as seen in this study. Samaranayake *et al* (1984) studied the effect of pH and glucose on *C. albicans* isolates and found that the lower pH of 3.6 produced more phospholipase activity in the P_Z positive strains than at pH 4.4, and they found no phospholipase activity at pH 5.1 or 6.3. They

also found that increasing concentrations of glucose, sucrose and galactose appeared to suppress phospholipase activity. Interestingly, Samaranayake *et al* (1984) found sucrose and galactose showed reduced phospholipase activity as the concentration increased, whereas glucose saw similar results at 50 and 100 mM (same concentrations as 10 and 20 mg/mL), and activity diminished once further glucose was added. In this study, at pH 5.2, the non-aggregating strain (NCPF 8971) showed phospholipase activity in the control group, though it showed no activity once glucose was added. The aggregating (NCPF 8977) strain showed phospholipase activity in the presence of up to 2.5 mg/mL of glucose, but no activity at higher concentrations. In line with the findings by Samaranayake *et al* (1984) for *C. albicans*, the aggregating strain (NCPF 8977) also showed no phospholipase activity at pH 6.3, though activity was seen at all glucose concentrations in the non-aggregating strain (NCPF 8971) at the same pH. There was variability within the groups and phospholipase activity did not appear to increase in line with increased glucose concentration. However, Samaranayake *et al* (1984) reported that increasing the glucose concentration diminished the 'halo' or zone of activity around the colony, which may have affected their ability to accurately measure the P_z zones. Sanita *et al* (2013) tested the phospholipase and protease activity of *C. albicans* isolates from patients with oral candidiasis and healthy patients from groups of patients with or without diabetes. Although they found that phospholipase production was greater in isolates taken from patients with candidiasis, they found no significant difference between phospholipase activity in diabetic and non-diabetic patients. However, all strains were tested on the same egg yolk medium with 30 mg/ml of glucose to determine if patients with candidiasis harboured more virulent strains of *C. albicans*.

However, it is worth noting that in this study, the strains vary their enzymatic activity according to the pH and glucose levels of the culture media regardless of their source environment. The 30 mg/mL used in the study by Sanita *et al* (2013) is much higher than is typically seen *in vivo* (normal fasting blood glucose is typically 0.6 – 1.0 mg/mL (Güemes *et al*., 2015)), and as demonstrated by Samaranayake *et al* (1983), may have suppressed the phospholipase activity. Importantly, Sanita *et al* (2013) investigated whether the diminished phospholipase halo in the presence of glucose was due to inactivity of phospholipase or due to poor visualisation of the halo and they added potassium tellurite to the egg yolk medium to improve visualisation by staining the colonies black. Future studies investigating phospholipase in the presence of glucose might consider this as a method to improve visualisation. In this study, a qualitative measure was used to record enzymatic activity due to the relatively small zones around the colonies which were difficult to measure accurately, and a quantitative measure could be employed if visualisation was improved. Sanita *et al* (2013) also reported that visualisation of enzymatic activity was improved when colonies were incubated for 7 days, and this study only incubated phospholipase and proteinase plates for 3 days and 5 days respectively.

4.6 Protease activity

The aggregating strain (NCPF 8977) appeared to have less proteolytic activity in the presence of glucose as the pH increased. The opposite appeared to be the case for the non-aggregating strain (NCPF 8971) which showed more activity as the pH increased (see Fig. 3.5.1). In all cases, only weak protease activity was observed and there was no significant difference between the groups where activity was shown. Kumar *et al* (2015) found that their *C. auris* isolates produced

phospholipase and protease with zones that could be measured quantitively. This study produced very small precipitation and clear zones around the colony which were difficult to measure accurately and could be explained by the fact that this study substituted a typical rich media for SU media which has been shown to produce less growth and biofilms (Uppuluri *et al.,* 2009). Kumar *et al* (2015) state they used a BSA agar in their experiment though the study does not specify the glucose concentration or the pH which has been shown to have some impact on the production of phospholipases and proteases. The media components can also impact on activity; Karkowska-Kuleta *et al* (2019) looked at the surface proteins of three non-albicans *Candida spp.* grown in six different media and found the lowest presence of surface proteins in *C. tropicalis* grown in a SU media. Interestingly, they found a wide variation of proteins across all media for all species used in the study. Though Sanita *et al* (2013) compared strains from diabetic and non-diabetic patients when they looked at phospholipase and protease activity, they did not consider the availability of a carbon source *in vivo* as a parameter and the diabetic patients included in the study had well-controlled type II diabetes. This study has considered glucose as a potential factor in the proteolytic activity of the two strains. The study by Sanita *et al* (2013) also investigated *C. albicans* which is used as a model for comparison in this study due to the lack of data available on *C. auris*, though it may offer a distinct virulence profile. Buu *et al* (2014) also studied *C. albicans* and found that lower rather than higher glucose concentrations were more potent in activating some SAP proteins and found higher concentrations of the protein Sap5 in the media containing the equivalent of 1 mg/mL of glucose than in the media containing the equivalent of 20 mg glucose/mL. They also found that the higher concentration of 20 mg/mL

appeared to delay or have a suppressive effect on Sap5 production. In the aggregating strain, activity was generally seen at the lower glucose concentrations, though this varied according to pH. The study by Buu *et al* (2014) did not consider the pH of the culture media. As discussed above, there may have been some discrepancy when visualising protease activity. Sanita *et al* (2013) found visualisation was improved when the protease assay plates were incubated for 7 days rather than the shorter periods in other literature such as the 5 days used in this study, which could be considered in future studies.

4.7 Limitations and future improvements

There were a number of key areas highlighted by the study that could have clinical relevance. In terms of biofilm, the was no real difference in biofilm formation with respect to the concentration of glucose or the pH, though variability in the results indicate that more repetition is required to provide a more confident conclusion. However, the results could also suggest that *C. auris* biofilms can tolerate a range of pH and glucose conditions. With respect to phospholipase and protease, glucose concentration and pH did appear to be relevant. A normal urine pH of 6.3 may offer some protection from the aggregating strain (NCPF 8977) which showed no activity from either phospholipase or protease, though a reduction in the pH could mean the patient is at risk even if there is little to no glucose present. A high glucose concentration could provide some protection from the aggregating strain (NCPF 8977), though there are accompanying co-morbidities associated with high glucosuria. A low urine pH may provide some measure of protection from this particular non-aggregating strain (NCPF 8971) in terms of protease, though a mid-normal pH could put a patient at risk. The wide variation of activity across pH levels and glucose concentrations indicate that

there is some mechanism by which *C. auris* can adapt to respond to its environment and invade the host niche by responding to pH and nutritional requirements. Certainly, Modrzewska *et al* (2016), have determined that there are SAP proteins which function at a range of pH levels. In terms of treatment, these findings can give no indication if a patient with poorly controlled DMII is at greater risk of infection by *C. auris* than a patient with well-controlled DMII as the response to pH and glucose concentration appear to be strain dependent. However, as discussed above, there is evidence to suggest that high blood glucose has a detrimental effect upon immune response and patients with poorly controlled DMII may be at greater risk of infection regardless of the level of glucose excreted or the pH of the urine (Zaccardi *et al* 2015). By providing nutrients and conditions similar to those found *in vivo*, SU is a useful media to mimic the environments that pathogenic yeast isolates are found in (Uppuluri *et al*., 2009). However, there were some difficulties ensuring an even mixture in the SU due to the number of insoluble components, such as calcium carbonate in the SU, and this was particularly evident in the highest pH of 6.3. Goss *et al* (2007) studied the role of pH in the solubility of metals including calcium carbonate and found that it became soluble below pH 4.5. The pH levels used in this study were higher, meaning that some components were insoluble. It was necessary to separate the mixture so that the correct amount of glucose could be added before it was filter sterilised. If the mixture was not properly mixed, then this resulted in the remaining stock SU having a higher proportion of insoluble precipitates which also raised the pH. This was managed by using a magnetic stirrer to mix the solution thoroughly while a syringe was used to draw the required amount. Despite this, the pH could differ between the solutions by as much as +/- 0.1,

though any change in pH was corrected, the difference suggests there could be variation in the components of the SU media. Variation in the components could also arise from using syringe filtration as a method to sterilise the media. According to Pillai *et al* (2016) syringe filters provide a means to remove bacteria and any undissolved particles. Removing undissolved particles could alter the biochemical profile of the formulation. Autoclaving was not explored as a method of sterilisation because other studies using synthetic urine only used filtration, furthermore insoluble particles would need to be removed to ensure that an accurate absorbance was achieved. Measures to overcome this could include using a commercially available SU or by using pooled clinical samples, though there would still need to be adjustments made to the pH and glucose concentration. Though not tested empirically, several observations have been made regarding the difference in behaviour between the two strains which, are of interest. This has also meant that some adjustments have been made to account for them. There have been some observations made with respect to the longevity and robustness of the two strains. It has been noticed that an OVC could not be prepared from an agar plate of the aggregating strain (NCPF 8977) that has been stored in the refrigerator for more than three weeks, whereas cells can be cultured from an agar plate of the non-aggregating strain (NCPF 8971) of the same age. It has also been observed that there was no measurable growth in the control group at pH 6.3 after 24 hours when cultured in SU broth. Though this broth lacked the addition of glucose as the main carbon source, minimal growth was seen at pH 5.2 and pH 5.6 and a biofilm grew in the control group at pH 6.3. As discussed, the aggregating strain (NCPF 8977) cells appeared to settle, and cells were required to be vortex mixed regularly throughout the experiment to keep the

cells suspended. Despite regular mixing, there could have been minor differences in the concentration of cells used in the experiments.

5: Conclusion

In conclusion, biofilm formation was not significantly impacted by changes in pH and glucose. There were interactions between pH and glucose where phospholipase and protease activity varied depending on the pH level and/or the glucose concentration, though more studies need to be conducted to determine the extent of these interactions.

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