Characterisation of Biofilm-Forming Ability and Haemolytic Activity of Clinical Group B Streptococcus (GBS) Isolates From the Urinary Tract

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Declaration of Originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

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Abstract

Urinary tract infections (UTIs) are among the most common infections caused by both Gram-negative and Gram-positive bacteria, acquired in the community and hospitals. There are three main groups of UTIs: (i) lower UTIs that affect the urethra or the bladder, (ii) upper UTIs that affect the kidneys, or (iii) asymptomatic bacteriuria (ABU).

Group B Streptococcus (GBS) is a Gram-positive bacteria known to cause a variety of infections in neonates, pregnant women, the elderly or immunocompromised individuals. GBS has been estimated to cause 1-2% of all single organism UTIs. GBS has been shown to form biofilms, on abiotic and biotic surfaces, protecting it from killing by antibiotics or host immune cells and promotes host colonisation. Various factors have been shown to affect the biofilm forming ability of GBS. Here we determined that LB supplemented with glucose was the optimal media for biofilm formation by a strong biofilm forming strain. We then investigated the biofilm forming phenotype of 292 clinical GBS isolates that presented with asymptomatic, acute, or recurrent infection. We found that there was no significant difference in the biofilm forming ability across the clinical presentations. We also showed a significant reduction in the biofilm forming ability of a strong biofilm forming strain and its isogenic *maeK* and *maeE* mutants in LB supplemented with 1% glucose. A multiplex PCR screen for genes encoding *bsaB, pil1, pil2a,* and *pil2b* found that there was no significant difference in the number of strains that had the right sized fragments for all four genes across the three clinical presentations. We also found that there was a significant difference in the proportion of strains that had the right sized fragments for the *pil* genes across the three different levels of biofilm activity under shaking conditions. High biofilm forming strains had the lowest proportion of strains that possessed all four genes, compared to low and medium biofilm formers. Lastly, we assessed the haemolytic activity of the strains by growing them on tryptic soy agar plates

supplemented with 5% horse blood and found that asymptomatic strains had a significantly higher number of strains with high haemolytic activity compared to acute and recurrent strains.

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Table of Contents

List of figures

List of tables

Abbreviations list

^{O}C – Degrees Celcius

% – Percentage

µl – Microlitre

ABU – Asymptomatic bacteriuria

ANOVA – Analysis of Variance

Bp – Base pairs

CAUTI – Catheter-associated Urinary Tract Infection

CC – Clonal complex

CV – Crystal Violet

DNA – Deoxyribonucleic acid

EPS – Extracellular polymeric substance

GBS – Group B Streptococcus

gDNA – Genomic DNA

h – Hour

H_2O – Water

IL – Interleukin

L – Litre

LB – Luria-Bertani Broth

M - Molar

min – Minute

ml – Millilitre

mM – Millimolar

MLST – Multilocus Sequencing Typing Scheme

NaCl – Sodium Chloride

NHU – Natural Human Urine

OD – Optical Density

PCR – Polymerase Chain Reaction

RO – Reverse osmosis

rpm – Revolutions per minute

RT – Room temperature

s – Seconds

SEM – Scanning Electron Microscopy

SHU – Human Synthetic Urine

SPSS – Statistical Package for Social Sciences

ST – Sequence type

TAE – Tris-acetate-EDTA

THB – Todd-Hewitt Broth

TSA – Tryptic Soy Agar

UPEC – Uropathogenic *Escherichia coli*

UTI – Urinary Tract Infection

WT – Wild-type

1. Literature Review

1.1. Urinary tract infection (UTI)

Urinary tract infections (UTIs) are among the most common bacterial infections acquired in the community and hospitals, affecting 150 million people each year worldwide (1). UTIs account for roughly three million emergency department visits and are responsible for approximately US$3.5 billion in healthcare expenditure each year (2). UTIs are a significant cause of morbidity in infant boys, older men and females of all ages (3). They are the second most common bacterial infections in children, affecting 8.4% of girls and 1.7% of boys by the age of seven (4). Thirty percent of individuals that get a UTI during their childhood will develop a second UTI, with many risk factors such as age, gender, race, and circumcision status, increasing the risk of recurrent UTI (4). Due to high prevalence, frequent recurrence, numerous associated morbidities, and rapid evolving antimicrobial resistance, UTIs are one of the most difficult challenges in clinical practice (5).

UTIs are classified as either uncomplicated or complicated (6). Uncomplicated UTIs most often occur in young, sexually active, nonpregnant, premenopausal women, with anatomically normal urinary tracts and resolve with short courses of antibiotics, with little effect on long-term renal function (7, 8). Symptoms of uncomplicated UTIs include dysuria, urinary frequency, urinary urgency, and suprapubic pain (8). On the other hand, complicated UTIs occur in patients who have functionally, metabolically, or anatomically abnormal urinary tracts, making them harder to treat (8, 9). Complicated UTIs usually occur in nosocomial or institutional settings, in individuals with alterations of the urinary tract, or underlying metabolic, renal, or immunological disorders (10). An example of complicated UTIs are catheter-associated UTIs (CAUTIs), which are associated with increased morbidity and mortality, and are collectively the most common cause of

secondary bloodstream infections (11). UTIs may be categorised into three main groups: (i) lower UTIs that affect the urethra (urethritis) or the bladder (cystitis), (ii) upper UTIs that affect the kidneys (pyelonephritis), or (iii) asymptomatic bacteriuria (ABU) which is characterised by bacteriuria in the absence of clinical symptoms (6, 12).

UTIs are caused by both Gram-negative and Gram-positive bacteria, as well as by some fungi, with uropathogenic *Escherichia coli* (UPEC) being the most common cause of both complicated and uncomplicated UTIs (3), due to the ability of the bacteria to grow in human urine. Staphylococci and enterococci are other organisms that have been reported to grow in urine (13, 14). These bacteria are able to tolerate the low pH of urine, high urea levels, nutrient limitation, nitrite in mildly acidified urine, and exposure to antimicrobial proteins and peptides (15). Uropathogens survive by invading the bladder epithelium, producing toxins and proteases to release nutrients from host cells, and synthesising siderophores to obtain iron (3). Many uropathogens initiate UTIs using pili that mediate adhesion to host surfaces, facilitating invasion into host tissues and promoting interactions to form biofilms (16, 17).

A UTI typically starts with periurethral contamination by a uropathogen inhabiting the gut, followed by colonisation of the urethra and migration to the bladder (3). Common treatment for symptomatic UTIs is antibiotics, which can lead to long-term alteration of the normal microbiota of the vaginal and gastrointestinal tracts, as well as the development of multidrug-resistant microorganisms (18). Increasing rates of antibiotic resistance and high recurrence rates enhance the burden that these infections place on the community (3).

UTIs are more common in women, with one-third of women experiencing a symptomatic UTI by the age of 24, and more than 50% of women will be affected at least once in their lifetime (5). The recurrence rate of UTIs in women ranges from 27% to 46% within one year (19). Soto *et*

al. (2006) showed that bacteria that are able to form biofilms are more often associated with recurrence due to persistence in either the vaginal reservoir, the bladder epithelial cells or both (20). Pregnant women are at an increased risk for UTIs; this is because up to 70% of pregnant women develop glycosuria encouraging bacterial growth in the urine (21). Pregnancy also leads to increases in progestins and oestrogens which could decrease the ability of the lower urinary tract to evade the bacteria (21). UTIs have been shown to be associated with up to 27% of preterm births (22).

1.2. Uropathogenic Group B *Streptococcus*

Streptococcus agalactiae (Group B *streptococcus* [GBS]) is a Gram positive β-haemolytic chain-forming coccus that is considered part of the normal human microbiota and colonises the gastrointestinal and urogenital tracts of approximately 40-50% of the adult population (23-25). GBS was originally identified as a veterinary pathogen and a frequent cause of bovine mastitis in the early 1930s (26). GBS can be life-threatening in humans, and cause infections in neonates, pregnant women, the elderly, or immunocompromised individuals(27, 28). Pregnancy-associated disease is most often manifested during labour or within the first few days of an infant's life (24). GBS can also cause severe invasive neonatal infections, such as pneumonia, septicaemia and meningitis (25). Neonatal GBS infections are divided into early-onset and late-onset infections. Early-onset infections are the most common and occur within the first week of birth, while late-onset infection occurs between one week and three months of birth (24, 29). The primary route of GBS transmission in early-onset infection is maternal colonisation, where the bacteria either spread *in utero* through ascending infection, or during birth through neonatal aspiration of contaminated amniotic or vaginal fluids (30-32). Late-onset infections may develop through

vertical transmission from mother to neonate, nosocomial transmission or contaminated breast milk (31, 33, 34). Development of GBS disease suggests either successful bacterial colonisation, penetration of placental or epithelial barriers, resistance to immune clearance, and in the case of meningitis the ability to breach the blood-brain barrier (30). Fibrinogen acts as a molecular bridge between GBS and human tissues, because it is present in plasma, tissues and on the surface of host cells, allowing GBS to participate in a number of pathogenic processes (35, 36).

Approximately 1-2% of all single organism UTIs are caused by GBS (6). GBS colonisation of the urinary tract in women most likely occurs from the vagina in an ascending manner, where the bacteria can persist asymptomatically (37). Asymptomatic GBS presence is generally harmless in healthy women; it can, however, lead to serious infections in pregnant women, causing bloodstream infections, meningitis, osteomyelitis, and endocarditis (38). Ascending spread leads to amniotic infection, which could also lead to maternal sepsis, or rarely meningitis (39). GBS colonisation status is intermittent and can be transient during pregnancy (38). Positive colonisers in early or mid-pregnancy may become negative colonisers at delivery (32, 40). Risk factors include the presence of diabetes mellitus, older age (≥ 60 years), presence of malignant neoplasms and infection with the human immunodeficiency virus (HIV) (41). The spectrum of GBS UTIs includes ABU, cystitis, pyelonephritis, urethritis, and urosepsis (27). GBS is able to cause disease in the kidneys significantly better than in the bladder, which is a common feature of other Gram-positive uropathogens (42). A study carried out by Beyer *et al.* (2001) on elderly populations with UTIs showed GBS involvement in 39% of nursing home residents over the age of 70 (43). The incidence of systemic GBS in nonpregnant adults is approximately 4.4 cases per 100,000, 14% of which are cases of urosepsis (41).

The burden of GBS UTI is a public health concern. Despite the fact that GBS only affects 1 to 2% of the general population, approximately 7% of pregnant women have high titers of GBS (44). Moreover, although GBS colonisation of the urinary tract in pregnancy is often asymptomatic, GBS bacteriuria is responsible for approximately 10% of all cases of maternal pyelonephritis (44). GBS UTI is also associated with chorioamnionitis, preterm labour and an increased risk of vertical transmission (45). Universal screening of GBS vaginal-rectal colonisation at 35-37 weeks' gestation is recommended by the Centers of Disease Control and Prevention, with antibiotic prophylaxis for culture-positive women during labour and delivery (8). A study compared the treatment of GBS bacteriuria with penicillin to a placebo, they showed a significant reduction in preterm labour in women that received the antibiotics (46). However, development of a vaccine is the most promising approach for the prevention of GBS infections due to potential adverse effects of antimicrobial prophylaxis, as well as the need to prevent both adult and late prenatal disease (47).

Uropathogenic GBS strains isolated from patients with acute cystitis and pyelonephritis adhered to urothelial cells and induced inflammatory responses, but were incapable of growing in human urine (48). A study carried out by Ipe *et al.* (2016) showed that some strains of ABU-causing GBS are able to robustly grow in human urine, and that this is related to malic acid metabolism (15). An operon comprising genes encoding a malate oxidoreductase enzyme (*maeE),* a primase/transporter *(maeP),* a transcriptional regulator (*maeR)*, and an accessory membrane-anchored sensor kinase (*maeK),* is typically associated with the metabolism of malic acid (49). In this study, they disrupted the malic enzyme (ME) pathway in ABU-causing GBS 834 by mutating the *maeE* and *maeK* genes, and compared the growth of the mutants to that of the wild-type (WT) in synthetic human urine (SHU) and pooled natural human urine (NHU), both containing 40 mM

malic acid. They found that the growth of both the ABU-causing GBS 834Δ*maeE* and ABU-causing GBS 834Δ*maeK* mutants was significantly attenuated in SHU and pooled NHU containing 40mM malic acid, compared to WT ABU-causing GBS 834. They also found that WT ABU-causing GBS 834 also had significantly better growth in NHU without supplemented malic acid compared to the *maeE* and *maeK* mutants. This data showed that functional *maeE* and *maeK* are needed for optimal growth of ABU-causing GBS in human urine in the presence of malic acid.

A seven-gene multilocus sequence typing scheme (MLST) was introduced for GBS classification in 2003 (38), which showed that GBS strains with the same MLST sequence type (ST) may have different serotypes (50). STs that share six or seven alleles are grouped into clonal complexes (CC) (51). Human GBS isolates can be grouped into six CCs, namely CC1, CC10, CC17, CC19, CC23, and CC26 (52, 53). CCs 1 and 23 have been linked to asymptomatic colonisation, while CCs 17 and 19 were found predominantly in neonates (54-56).

1.3. GBS virulence factors

GBS strains possess an array of surface-associated and secreted virulence factors which allow them to colonise the host and cause disease (Figure 1).

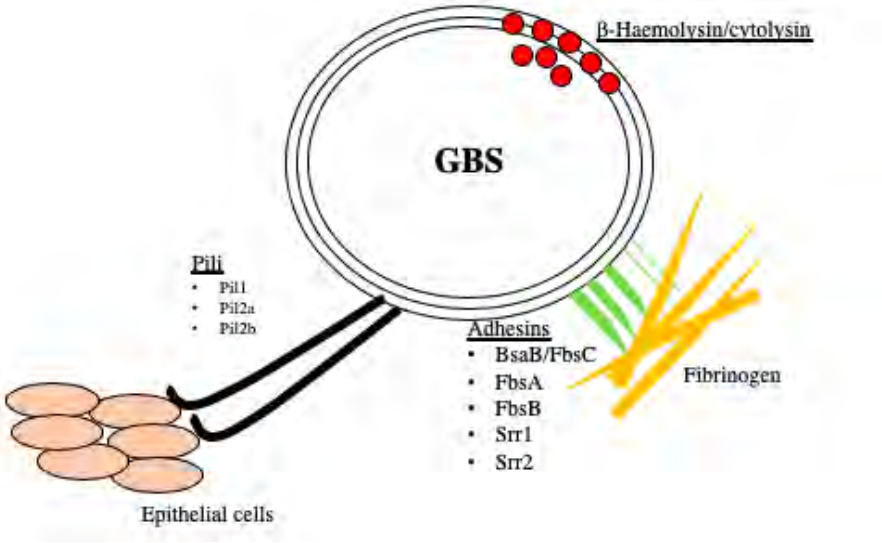


Figure 1: Virulence factors of GBS

GBS strains encode various factors which contribute to the colonisation and persistence in the host.

1.3.1. Capsule

Clinical isolates of GBS possess capsular polysaccharides with varying repeat unit structures. The variations in the antigenically distinct capsular types is thought to be due to selective pressure imposed by host immunity (57). GBS isolates can be classified into 10 different serotypes (Ia, Ib and II to IX) according to their capsular polysaccharide composition (58, 59). The different capsular polysaccharides consist of high-molecular-weight polymers with a repeating unit composed of glucose, galactose, N-acetylglucosamine and sialic acid (60). Serotype VIII contains rhamnose but not N-acetylglucosamine, while serotype VI also lacks N-

acetylglucosamine (61, 62). Serotype Ia, Ib and III have sialic acid as the terminal sugar residue of a side chain, which plays an important role in virulence (63, 64). Sialic acid residues on the glycosyl portion of polysaccharides has an important impact on modulation of cell-cell interactions and, on infectivity and immune response of the host (65). Removal of sialic acid from serotype III by sialidase treatment leads to a loss of virulence and increased phagocytosis by human neutrophils (66). Synthesis of the capsule comprises of 12 genes (*lysR, cpsR, cpsA-cpsJ*) that are conserved among all GBS strains of different serotypes (67).

Multiple studies have shown that all serotypes have the ability to colonise the vagina and perianal region of pregnant women. However, serotypes Ia, Ib, II, III and V are prevalent in the vagina and perianal region of pregnant women and are the most common in human infections (68-70). Serotype III isolates of a particular genotype cluster, ST-17 displays a conserved specific combination of the secreted and surface-exposed proteins (25, 71), and cause late-onset GBS disease (50, 51) and more frequently cause meningitis compared to other sequence types (54). Serotype III isolates have also been shown to produce biofilm in nutrient rich media that has been supplemented with glucose, with ST-17 strain producing more biofilm than non-ST-17 strains (23).

Additionally, Kline *et al* (2011) demonstrated that sialic acid residues of the GBS capsular polysaccharide are essential for GBS establishment in the urinary tract (42). Different GBS serotypes have been shown to be associated with UTI through epidemiological studies, however serotype III GBS was found to cause acute symptomatic disease more often than the other serotypes, that were more likely to cause asymptomatic bacteriuria (37).

1.3.2. β-Haemolysin

Nearly all clinical GBS isolates produce β-haemolysin, an important virulence factor involved in the invasion of human epithelial cells and release of pro-inflammatory cytokines (72). β-haemolysin is an oxygen-stable, non-immunogenic, pore-forming cytolysin (60). Expression of β-haemolysin correlates with the severity of disease in murine models of intranasal and intravenous administration (42). Kulkarni *et al.* (2013) hypothesised that the β-haemolysin cytotoxin produced by GBS played a role in establishment and maintenance of GBS UTI (73). However, *in vitro* and *in vivo* assays indicate that the cytotoxin is non-essential for epithelial cell adherence, bladder colonisation and ascending infection of the kidneys (73). Nonetheless they did find that β-haemolysin promotes inflammation, which is known to drive UTI-associated symptoms. In murine models the presence of GBS induces IL-1α, macrophage inflammatory protein-1α (MIP-1α), MIP-1β, IL-9 and IL-10 (42, 74). *In vivo* studies have shown that β-haemolysin contributes to the development and severity of meningitis, pneumonia, arthritis, and sepsis (75).

In order to determine function of GBS β-haemolysin in terms of urothelial cell death Leclercq *et al.* (2016) generated an isogenic β-haemolysin-deficient uropathogenic GBS 807 mutant and analysed cytotoxicity (76). In contrast to the wild-type, the mutant was not haemolytic and was significantly less cytotoxic to urothelial cells. The study also showed that 807 induced inflammation and local neutrophil infiltration in the bladder is mediated by β-haemolysin, which contributes to bacterial survival *in vivo*.

Fettucciari *et al*. (2000), found that growing GBS in glucose-supplemented media downregulates β-haemolysin expression, and inhibits macrophage apoptosis (77). This suggests that the surface-bound β-haemolysin of GBS is the factor responsible for inducing apoptosis in

infected macrophages (77). However, a study carried out by Ulett *et al.* (2003) found that apoptosis in GBS-infected macrophages does not depend on β-haemolysin but is mediated by a factor coregulated with β-haemolysin by glucose (78). They inhibited the β-haemolytic activity of WT GBS by growing the bacteria in glucose-supplemented media, and found that the level of apoptosis in infected macrophages was significantly lower. This was consistent with the findings of Fettucciari et al. (77). However, they also constructed a β-haemolysin deficient mutant of GBS and showed similar levels of apoptosis in macrophages infected with the mutant, compared to macrophages infected with WT GBS. They also grew the mutant in high concentrations of glucose, and showed that the level of macrophage apoptosis was inhibited to a similar degree as those infected with the WT (78).

1.3.3. Adhesins

GBS produces surface proteins to mediate bacterium-host receptor interactions (58), with several proteins being identified as adhesins that are involved in attachment of the bacterium to host cells and/or the extracellular matrix (79). A study demonstrated that uropathogenic GBS binds directly to human bladder epithelial cells, facilitating colonisation *in vivo* (74). Genome sequencing of GBS strain 515, a clinical isolate from an infected neonate, identified a cell wall-anchored protein encoded by *sal0825* (80, 81), which is one of seven surface proteins conserved across GBS strains (82). The protein contains a typical N-terminal signal peptide and C-terminal LPXTG sorting signal (83), and is involved in either attachment to human cells or binding to extracellular matrix components (79, 84). This protein was renamed BsaB (bacterial surface adhesin of GBS), and functional analysis of the protein revealed that it mediates binding of GBS to human fibronectin and laminin, human vaginal cells (VK2) and cervical epithelial cells (ME-

180) cells, and biofilm production (83). However, they did not study the interaction of BsaB with fibrinogen. Deletion of the gene encoding for BsaB resulted in a decrease in GBS adherence to VK2 cells and immobilised human fibronectin. ScpB is another GBS surface protein, and can inactivate human C5a and mediate bacterial binding to fibronectin (79, 85). However, it has not been fully resolved whether BsaB contributes to GBS invasion or promotes GBS colonisation through enhancing biofilm formation (86).

FbsA (a cell wall-anchored protein) and FbsB (a secreted protein) are two structurally unrelated proteins that are capable of binding to fibrinogen *in vitro*, expression of both proteins was initially associated with GBS interactions (87, 88). FbsA may be involved in adhesion to epithelial cells, but not in cell invasion, which FbsB is required for (89). Srr1 and Srr2 are LPXTG glycoproteins that have also been reported to contribute to fibrinogen binding (90), Srr1 has also been shown to mediate invasion of brain vascular endothelial cells and translocation through the blood-brain barrier (91). Buscetta *et al* (2014) showed that BsaB is a specific fibrinogen-binding protein and renamed it FbsC, which is encoded by the *gbs0791* locus in NEM316 (92). They found that FbsC mediated fibrinogen binding, biofilm formation, and invasion of epithelial and brain endothelial cells by GBS.

1.3.4. Pili

Bacterial pili are multi-subunit protein assemblies that extend from the bacterial cell surface and mediate adhesion to host cells, bacterial motility, and other critical aspects of colonisation (93). Pili in Gram-positive bacteria are usually shorter than those in Gram-negative bacteria (94). Pili play an important role in adhesion and attachment of pathogens to host cells by promoting initial association with host cells, followed by more intimate attachment mediated by

other adhesins (95). Pili in GBS were discovered during screening of multiple genomes for surface-exposed protein antigens as possible vaccine targets (96). GBS pili are multimeric structures consisting of three pilin proteins, a PilA subunit at the terminus of each pilus, PilB subunits that make up the majority of the backbone of the pilus, and PilC protein at the base (97-99). Three types of pili have been identified in GBS, namely pilus type 1, 2a and 2b, with each strain carrying one or two types (100). The genes involved in the synthesis and assembly of these three GBS pili are clustered in characteristic genomic loci (termed PI-1, PI-2a and PI-2b). PI-1 is coded in a pathogenicity island, while the PI-2 locus is part of the stable bacterial genome (97). PI-2a and PI-2b represent two variants of the pilus island 2, since they are alternatively present in the same genomic locus (97). Each locus encodes three structural proteins, containing a LPXTG motif and two dedicated subfamily C sortase (SrtC) proteins involved in covalent polymerisation of the subunits (97). While all GBS isolates have one of the PI-2 loci, only 72% of GBS isolates have PI-1, explaining why the PI-1 pilus is less well studied than the PI-2a or PI-2b pili (101). Martins *et al.* (2013) studied 898 GBS isolates recovered from humans and found that PI-2a was the most widespread locus, found in 79% of the isolates, while PI-2b was only found in 21% of the isolates (102). PI-2a mediates biofilm formation by GBS on abiotic and biotic surfaces (100). The same study also showed that antibodies against pilus 2a proteins are effective in interfering with the initial steps of biofilm formation. Therefore, identification of these pilus structures and their importance in GBS colonisation and pathogenicity is important in developing a vaccine (102).

A study carried out by Alvim *et al.* (2019) looked at 134 GBS strains, and found that they all had at least one of the pilus gene variants, with significantly different distribution between isolates from human and animal sources (103). They found that the most common variant in GBS from human isolates was PI-2a (76%), while PI-2b (74%) was frequently seen in animal isolates.

These results were similar to previous studies that all showed a strong correlation between PI-2a and human-derived isolates, and PI-2b and animal-derived isolates (102, 104, 105).

1.4. Biofilm formation

Microbial biofilms are populations of microorganisms that stick to both biological and non-biological surfaces, encased in an extracellular polymeric substance (EPS) matrix (106). The matrix is a complex mixture of macromolecules including exopolysaccharides, proteins, and DNA (107). Biofilms can contain single microbial species or multiple microbial species and can form on both biotic or abiotic surfaces (108). Organisms within biofilms can withstand nutrient depravation, pH changes, oxygen radicals, disinfectants and antibiotics (109). Bacterial cells use quorum sensing to regulate gene expression in response to fluctuations in cell-population density (110). Bacteria in biofilms coordinate behaviour by cell-cell communication using secreted chemical signals, which allow the bacteria to sense and phenotypically respond to the environment (111). Quorum sensing systems in Gram-positive bacteria typically use secreted peptides as signal molecules and a two-component regulatory system to detect the peptide and trigger the required changes in gene expression (112). Bacteria in biofilms exhibit resistance to antimicrobial compounds and persistence in spite of host defences, making biofilm infections clinically important (111). Antibiotic penetration into the biofilm may not be inhibited by the matrix, it can however hinder the rate of penetration enough to induce expression of resistance genes within the biofilm (113).

Biofilm formation can protect GBS from killing by antibiotics or host immune cells and promote host colonisation. GBS can form biofilms on abiotic and biotic structures, with little known about the environmental factors that regulate biofilm development (114). The adherence of

GBS to vaginal epithelial cells is strongly enhanced at acidic pH, through increased expression of potential vaccine antigens such as pilus components and some surface proteins (115, 116). A study carried out by Donlan and Costerton (2002) found GBS on intrauterine devices in association with other well-known biofilm formers such as *Streptococcus aureus* and *Staphylococcus epidermidis* (117).

A study carried out by Ho *et al.* (2012) showed a striking effect of pH and nutrient-limited medium (M9YE) on biofilm production of GBS strains. Biofilm production of 80 GBS strains was determined in M9YE medium at pH4.5 and pH 7.0. Analysis showed that 96% of the strains produced a biofilm at pH4.5; in contrast 90% of strains did not form a biofilm at pH7.0 (114). The biofilm production at the two pH conditions was further confirmed using scanning electron microscopy (SEM), where multilayer cell aggregates encased in a thick adhesive matrix were observed at an acidic pH, whereas small monolayered bacterial aggregates were observed at neutral pH. In addition to analysing the effects of pH on biofilm production, the study also performed a PCR analysis using primers against genes found in the PI-1, PI-2a and PI-2b islands to investigate pili distribution among the 80 GBS strains. This revealed that each strain had at least one or two islands. The authors then examined the correlation between pilus types and biofilm production, and found that at pH 4.5 all of the PI-2b positive strains and 96% of PI-2a positive strains produced a biofilm. However only 10% of PI-2a positive strains produced biofilm at pH7.0. These findings suggest that GBS is able to produce a biofilm preferentially under acidic conditions and under nutrient-limited conditions, implying the ability of the bacteria to colonise its natural habitats, such as the vagina of pregnant women (pH4.0-4.5). Nevertheless, it does not rule out the possibility of strain-specific or other environmental factors such as salt, osmolarity and metal ions.

The data regarding pH and media composition in biofilm production is controversial. D'Urzo *et al.* (2014) found that a low pH induces biofilm formation in nutrient rich media (THB) but not nutrient limited media (RPMI) (23). The study showed that addition of 1% glucose to THB caused a drop in pH to below 5.0, which induces GBS biofilm production. However, addition of 1% glucose RPMI did not cause a drop in pH below 5.0, resulting in no significant biofilm production. The authors went on to show that the metabolism of glucose by the bacteria in nutrient rich media forms organic acids that reduce the pH of the media and therefore initiate biofilm formation. Additionally, other studies have shown that GBS produces a better biofilm at a pH of 6.5 rather than at pH 4.2 (118, 119). However, those studies did not incubate the biofilm assays under shaking conditions and therefore did not account for *in vitro* fluid circulation, which could be the reason for the contradicting results.

Alvim *et al.* (2019) found a significant difference in strong biofilm formation in isolates from human and animal sources according to the pilus variants present. Human isolates that had a combination of PI-1 and PI-2b represented the lowest percentage of strong biofilm producers, while the same combination in animal isolates represented the highest percentage of strong biofilm producers (103). They also found that although human GBS isolates with PI-1 alone, PI-2a alone, and a combination of PI-1and PI-2a, had similar numbers of strong biofilm formers, there was a significant difference in the absorbance values. Isolates that had PI-2a alone had a higher absorbance reading compared to the others, which further suggests that the type of pilus present determines the ability of GBS to colonise host cells and cause disease. A study performed by Parker *et al* (2016) showed similar results, where strains containing a PI-2 variant only were significantly more likely to produce a higher biofilm compared to strains containing both a PI-2

variant and PI-1 (104). Therefore, identification of these pilus structures and their importance in GBS colonisation and pathogenicity is important in developing a vaccine (102).

2. Significance

UTIs are a major public health concern affecting approximately three million people and accounting for approximately $3.5 billion in healthcare expenditure each year (2). UTIs occur more frequently in women than in men; 50% of women being affecting during their lifetime, with a recurrence rate of 30% (5). GBS is known to colonise vaginal and cervical epithelial cells, in an ascending manner where the bacteria can persist asymptomatically, and may cause UTIs in women that have risk factors that reduce their immune functioning. The urinary tract of approximately 7% of pregnant women are colonised by GBS, but many may not show clinical symptoms (44). GBS can be passed on from the mother to infant during childbirth or through placental penetration, leading to neonatal meningitis, pneumonia or sepsis.

GBS possess many virulence factors that contribute to biofilm formation. Biofilm formation by GBS allows recurrence of infection due to persistence of the bacteria in vaginocervical cells. Bacterial cells within the biofilms also develop increased antimicrobial resistance and evade host immunity. Studies have shown that pH plays a role in biofilm formation by GBS, with most studies stating that an acidic pH facilitates biofilm formation (23, 120). Although there is contradicting information regarding the ideal media for GBS biofilm formation, all the studies that have used media supplemented with glucose show that the presence of glucose enhances biofilm formation.

Due to the contradicting information about the ideal media for GBS biofilm formation, this study will provide a better understanding about the role of environmental factors (e.g. pH) in

causing infection. The ability of GBS to survive and produce biofilms under acidic conditions plays an essential role in infection of human vaginocervical cells. Previous research has found that GBS possess various virulence factors allowing the bacteria to adhere and invade host cells. The data obtained from this project will provide more information about the optimal conditions for GBS biofilm formation, as well as the virulence profiles of a large collection of GBS strains isolated from the urogenital tract of women. This will provide better understandings into how GBS persists and evades the host immune system, and may identify potential targets for future therapeutic development.

3. Aims

There are different kinds of GBS isolates that have different clinical presentations, we have an in-house collection of 292 clinical GBS strains isolated from the genitourinary tract of patients with different clinical presentations (asymptomatic, acute, or recurrent infections). We hypothesised that asymptomatic strains would form weaker biofilms and express less haemolysin as compared to the other clinical presentations.

The main aim for this project was to characterise the biofilm forming ability and virulence profiles of the collection of 292 clinical GBS isolates. This was further broken down in to five specific aims.

Aim 1: Determine the optimal conditions to promote GBS biofilm formation and whether it affects growth.

Aim 2: Determine the biofilm phenotype of 292 clinical GBS isolates.

Aim 3: Assess if malic acid affects biofilm formation using isogenic maeK and maeE mutants.

Aim 4: Determine the haemolytic activity and presence of four adhesins in 292 clinical GBS isolates.

Aim 5: Assess if there is any correlation between phenotypes and clinical metadata.

4. Materials and methods

4.1. Bacterial strains and culture conditions

The GBS strains used in this study are listed in Appendix 1. Cells were routinely cultured either under static or shaking (250 rpm) conditions at 37^{O}C in Todd-Hewitt broth (THB). The composition of media used in this study is listed in Table 1.

Table 1: Composition of media used

Media	Composition
Todd-Hewitt broth (THB)	36.4 g Todd-Hewitt powder and reverse osmosis (RO) water to make 1 L.
Luria-Bertani (LB) broth	10 g tryptone, 10 g NaCl, 5 g yeast extract, and RO water to make 1 L.
10% glucose	50 g of glucose in 500ml RO water, and filter sterilised.
THB + 1% glucose	Same as THB but RO water added to make 900 ml, and 100 ml 10% glucose.
LB + 1% glucose	Same as LB but RO water added to make 900 ml, and 100 ml 10% glucose.
Tryptic soy agar + 5% horse blood	30 g tryptic soy, 15 g agar, 1 L RO water, and 50 ml horse blood.
Malic acid (1M) pH7	5.363 g in 40 ml RO water
LB + 1% glucose + Malic acid	1:25 dilution of 1 M malic acid in LB + 1% glucose

4.2. Biofilm assays

Polystyrene 96-well U-bottom microtiter plates (Corning) were used to monitor biofilm formation. An overnight culture grown in THB was subcultured 1:100 into the appropriate media (THB, LB, THB + 1% glucose, or LB + 1% glucose), and 200 µl was transferred into each well of the plate. The plates were then wrapped with parafilm to prevent the media from drying out and incubated at 37^{O}C under either static or shaking (150rpm) condition for 24 h. After incubation, the plates were rinsed with RO water, dried, and 150 µl of a 0.1% solution of crystal violet (CV) was added to each well. After further incubation at 4^{O}C for 30 min, the plates were rinsed, and the CV was solubilised by the addition of 150 µl of a 80:20 ethanol/acetone mix. The plates incubated on a shaker for 30 min at room temperature (RT) and the OD 595 determined. Results were presented as the mean of eight replicated wells in three independent experiments.

4.3. Growth curve analysis

Polystyrene 96-well flat-bottom microtiter plates were used for growth curve analysis. The appropriate media was inoculated with a 1:100 dilution of an overnight culture in THB media. The plates were covered with a 'Breath-Easy' membrane to allow for gas exchange, and incubated in a plate reader at 37^{O}C for 18 h under shaking conditions. Results were presented as the mean of four replicated wells.

4.4. Haemolytic activity of the clinical GBS strains

Tryptic soy agar plates supplemented with 5% horse blood were used to assess the haemolytic activity of each strain. Each strain was streaked out onto the blood agar plates, and

incubated at 37^{O}C overnight to obtain single colonies. Each plate had 2 µl spots of GBS 874391, 874391Δ*covR* (positive), and 874391Δ*cylE* (negative), as controls. The haemolytic activity of each strain was identified by observing the zone of clearance formed around single colonies after 24 h of incubation.

4.5. Polymerase chain reaction (PCR)

The prevalence of the genes encoding for PI-1, PI-2a, PI-2b and BsaB were assessed by a multiplex PCR screening approach. PCRs were done in a 25 µl reaction, containing 1µl of each primer (P1-P8), 1 µl genomic DNA, 12.5 µl MyTaq Mix, and 3.5 µl sterile H_2O. The PCRs were carried out in 96-well PCR plates using the cycle program shown in Table 1. The genome of some commonly studied GBS strains (874391, NEM316, A909, and COH1) were used to help design the primers that bound to and amplified regions of these genes. Table 2 shows the sequence of the forward and reverse primers used. Each PCR plate also contained gDNA of 874391 and NEM316 as positive controls, and a no gDNA negative control.

Table 2: PCR cycle conditions

Step	**Temperature**	**Time**	**Cycles**
Initial denaturation	95 ^{O}C	1 min	1
Denaturation	95 ^{O}C	15 s	30
Annealing	60 ^{O}C	15 s	
Extension	70 ^{O}C	20 s	
Final Extension	72 ^{O}C	1 min	1
Hold	10 ^{O}C	∞	

Table 3: PCR primers used in this study

Primers			**Sequence (5'→3')**
P1	Pil1-scrn-F	209 bp	ATTGTTTTCGGCTGCTGTTT
P2	Pil1-scrn-R		CTTCGCCGTCTTTATTCTCG
P3	Pil2a-scrn-F	382 bp	CCAATCAACCCATCAGAACC
P4	Pil2a-scrn-R		CACCGGCGTTAGAGATCAAT
P5	Pil2b-scrn-F	579 bp	GGGCAGCTACCAATACTCCA
P6	Pil2b-scrn-R		CACCTGTTGAAGGCAACTCA
P7	bsaB-scrn-F	797 bp	TTGGTTTCTGGTGACAATGG
P8	bsaB-scrn-R		GGTGCTGTTGGTTTCGAAGT

4.6. Gel electrophoresis of PCR products

A 1% Tris-acetate-EDTA (TAE) gel stained with SYBR Safe (Thermo Fisher Scientific) was used to resolve the PCR products. PCR products were mixed with 5 µl loading dye, loaded onto the gel, and ran for 50 min at 120V. The 1kb plus DNA ladder (Invitrogen) was used to estimate the size of the DNA fragments. DNA was visualised on a Gel Doc XR+ Imager (Bio-Rad).

4.7. Genomic DNA extraction.

The GenElute Bacterial Genomic DNA extraction kit (Sigma-Aldrich) was used to extract the genomic DNA for 874391 and NEM316. Three to four colonies of the strains were inoculated in 10mL of THB and incubated overnight at 37^{O}C. The following day, a stock solution of lysozyme was prepared using the included Gram-positive lysis solution as the diluent (45mg of lysozyme in 1 ml of Gram-positive lysis solution). The mixture was pipetted up and down to mix it. The overnight cultures were pelleted by centrifugation for 10 min at 10,000 x g and discarding the supernatant. The pellets were resuspended thoroughly in 200 µl of the lysozyme solution. 10 µl of 10U mutanolysin and 2 µl of 20 mg/ml RNase A were added, and mixed by pipetting up and down. After 1 h incubation at 37^{O}C, 20 µl of proteinase K solution was added, followed by 200 µl of lysis solution C (B8803). The tubes were vortexed for 15 s and incubated at 55 ^{O}C for a further 10 min to form the lysate. During this incubation, 500 µl of the column preparation solution was added to two pre-assembled GenElute Miniprep Binding Columns seated in a 2 mL collection tube, and centrifuged at 12,000 x g for 1 min. After a 10 min incubation at 55 ^{O}C, 200 µL of ethanol (95-100%) was added to the lysate and mixed thoroughly by vortexing for 5-10 s. The lysate was loaded into the columns and centrifuged at 12,000 x g for 1 min. The collection tubes containing

the eluate was discarded and the columns were placed in new 2 mL collection tubes. For the first wash, 500 µL of wash solution 1 (W0263) was added to the columns and centrifuged at 12,000 x g for 1 min. The collection tubes containing the eluate was discarded and the columns were placed in new 2 mL collection tubes. The second wash step was performed by adding 500 µL of Wash Solution (10 mL of 95-100% ethanol added to Wash Solution Concentrate) to the columns and centrifuged for 3 min at maximum speed. The eluate was discarded, and the columns were centrifuged for an additional 1 min. The collection tubes containing the eluate were discarded and the columns placed in new 2 mL collection tubes. To elute the gDNA, 200 µL of sterile water was added directly to the center of the column and incubated for 5 min at RT. The columns were then centrifuged for 1 min at 12,000 x g to elute the DNA.

The Qiagen Ultraclean gDNA extraction kit was used to extract the gDNA of 29 clinical GBS isolates. Overnight cultures of the isolates grown in THB were pelleted by centrifuging for 10 min at 10,000 x g. The pellet was resuspended in 300 µl of the PowerBead solution (supplemented with 15 mg/ml lysozyme) and vortexed to resuspend the cells. 10 µl of mutanolysin (10 U/µl stock) and 2µl of RNase A (20 mg/µl stock) was added to the resuspended cells and incubated for 90 min at 37 ^{O}C. The resuspension was then transferred to a PowerBead tube, 50 µl of solution SL was added, and the tubes were heated at 65 ^{O}C for 5 min. The tubes were vortexed and put in a TissueLyser for 1 min at 30 beats/sec, and then centrifuged for 5 min at 15,000 x g. The supernatant was transferred to a clean tube, 100 µl of solution IRS was added and immediately vortexed for 10 s, and incubated on ice for 10 min. The tube was centrifuged for 5 min at 15,000 x g, and the supernatant transferred to a new tube. 900 µl of solution SB was added to each tube and vortexed to mix. 680 µl was loaded into a spin column and centrifuged for 1 min at 10,000 x g, this was repeated until all the supernatant was used. 300 µl of solution CB was added to each

tube and centrifuged for 1 min at 10,000 x g. The flowthrough was discarded, and the column centrifuged for a further 2 min at 10,000 x g. The spin column was transferred to a new tube, 50 µl of sterile water was added to the middle of the filter membrane and incubated at RT for 5 min. DNA was eluted by centrifuging the tubes at 10,000 x g for 30 s. The concentration of the gDNA was quantified using a BioSpectometer (Eppendorf).

4.8. Statistical analysis

The Statistical Package for Social Sciences (SPSS) version 26 (IBM) was used to carry out normality tests for all the data obtained. The skewness and kurtosis (range 3 to -3) of the data was analysed in order to determine whether or not the data was normally distributed. The GraphPad prism software was used to perform further statistical analysis such as ANOVA, area under the curve (AUC), Chi-square analysis and paired t-tests. A significance value of $p \leq 0.05$ was used for all statistical analyses performed.

5. Results

5.1. GBS forms a strong biofilm when cultured in LB + 1% glucose.

Two GBS strains (874391, a weak biofilm-forming strain and negative control, and NEM316, a strong biofilm-forming and positive control) were used to determine the optimal media required for biofilm formation (Figure 2). The strains were tested for biofilm formation in a microtiter plate biofilm assay, where each strain was grown at 37 oC in either LB, LB + 1% glucose, THB, THB + 1% glucose, under shaking (150rpm) and static conditions. NEM316 cultured in LB + 1% glucose produced the highest OD595 reading under both shaking (OD595= 0.63) and static (OD595 = 0.51) conditions, whereas 874391 was unable to form a strong biofilm under any of the conditions tested (Figure 2).

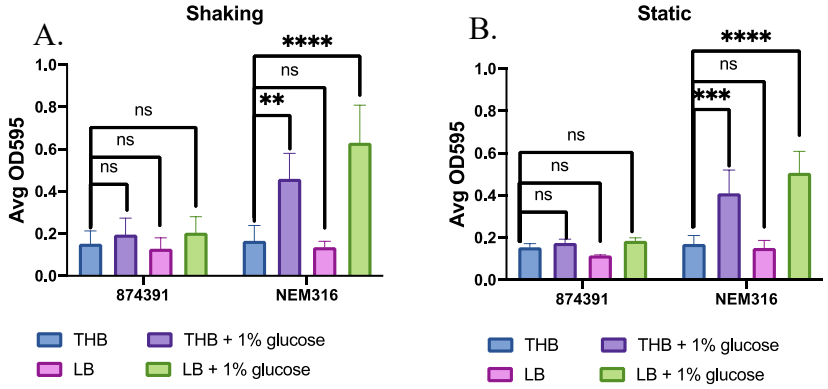


Figure 2: Biofilm formation by GBS 874391 and NEM316.

*Biofilm formation by GBS 874391 and NEM316 in microtiter plates under different conditions at 37 ^{O}C. Strains were grown shaking (**A**) or static (**B**) in either THB, THB + 1% glucose, LB, or LB + 1% glucose. Bar charts represent the average absorbance values at OD595 and error bars show the standard deviation calculated from three independent experiments. The average absorbance values were compared using a two-way ANOVA; ns – not significant, ** $p<0.005$, *** $p<0.001$**** $p < 0.0001$.*

The data were normally distributed under both shaking and static conditions. A two-way ANOVA analysis was used to compare average biofilm formation by the two strains in THB + 1% glucose, LB, and LB + 1% glucose to average biofilm formation in THB. There was no significant difference in the biofilm formation of 874391 in all four media under both shaking and static conditions. NEM316 produced a significantly higher biofilm in THB + 1% glucose (shaking: $p = 0.0072$; static: $p < 0.0001$) and LB + 1% glucose (shaking: $p = 0.0006$; static: $p < 0.0001$) compared to THB in both growth conditions.

In order to investigate whether the differences in biofilm formation by the two strains was due to differences in growth, growth curve analyses for both strains in the four different media were performed under aerobic conditions at 37 oC. The results were presented as the means of four replicated wells in three independent experiments (Figure 3). Both 874391 and NEM316 grew better and reached a higher OD600 in THB and THB + 1% glucose compared to in LB and LB + 1% glucose. Additionally, there was no significant difference in growth between the two strains in either THB ($p = 0.833$) or LB + 1% glucose ($p = 0.975$).

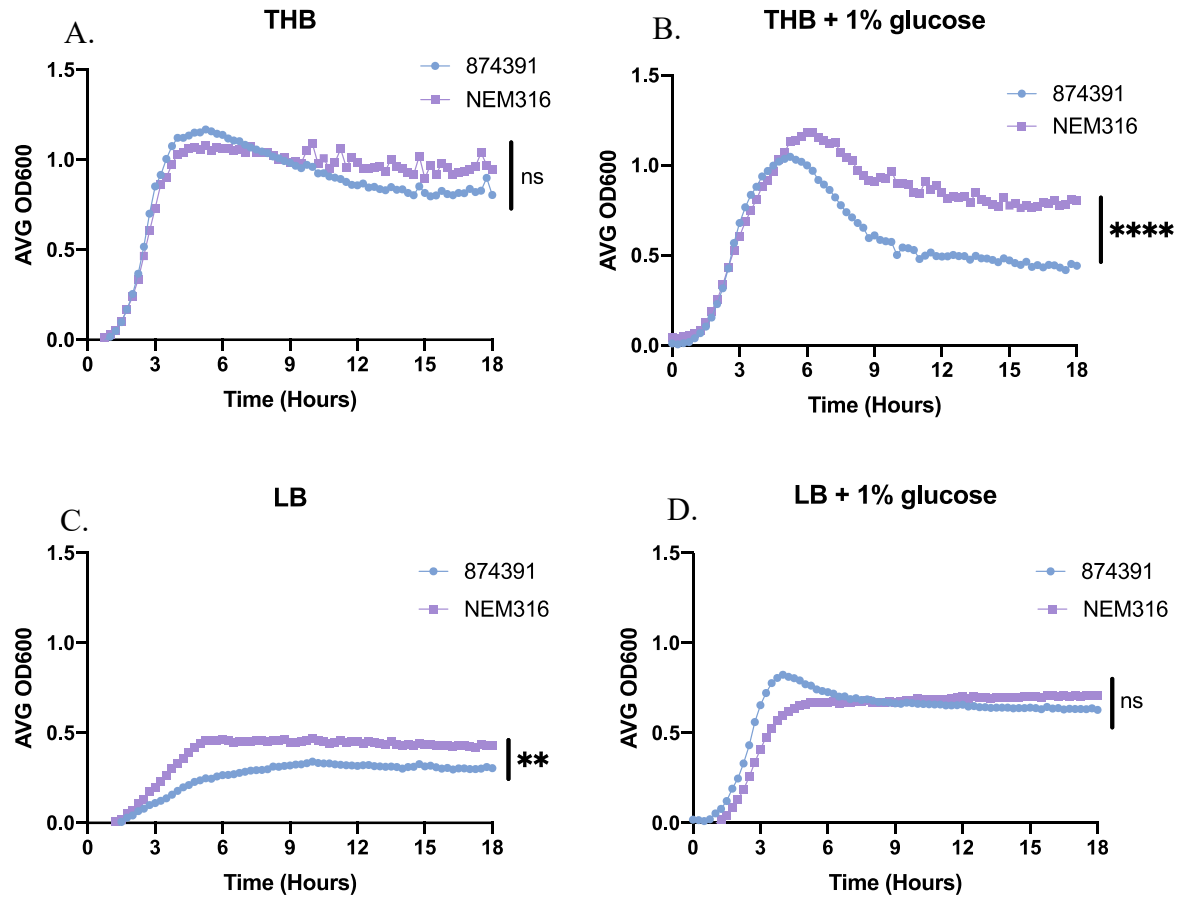


Figure 3: Growth curve analysis of GBS 874391 and NEM316.

*Growth curve analysis of GBS 874391 and NEM316 under aerobic conditions at 37 ^{O}C for 18h. Strains were grown in either THB (**A**), THB + 1% glucose (**B**), LB (**C**), or LB + 1% glucose (**D**). Graphs show the average absorbance values at OD600 from three independent experiments. Average absorbance values were compared using a one-way ANOVA; ns - not significant, ** $p < 0.005$, **** $p < 0.001$.*

Taken together, these results demonstrate that LB + 1% glucose is the optimal media for biofilm formation in GBS, and that the higher biofilm formation by NEM316 compared to 874391 in LB + 1% glucose was not due to a difference in growth.

5.2. Clinical GBS isolates from the urinary tract exhibit a range of biofilm phenotypes.

The biofilm forming ability of a collection of 292 clinical GBS isolates in LB + 1% glucose, was assessed under both shaking and static conditions. These strains were isolated from the urine of patients that had clinical presentations of infection (asymptomatic [n = 184], acute [n = 61], or recurrent [n = 47]), or through routine screening during pregnancy. The biofilm forming ability of the strains was compared according to the clinical presentation. There was no significant difference in the biofilm forming ability of the 292 clinical GBS isolates across the three different clinical presentations, under both shaking ($p = 0.129$) and static ($p = 0.612$) conditions (Figure 4). This can also be seen in Figure 5B which shows the percentage of strains that formed either low, medium or high biofilms across the three clinical presentations.

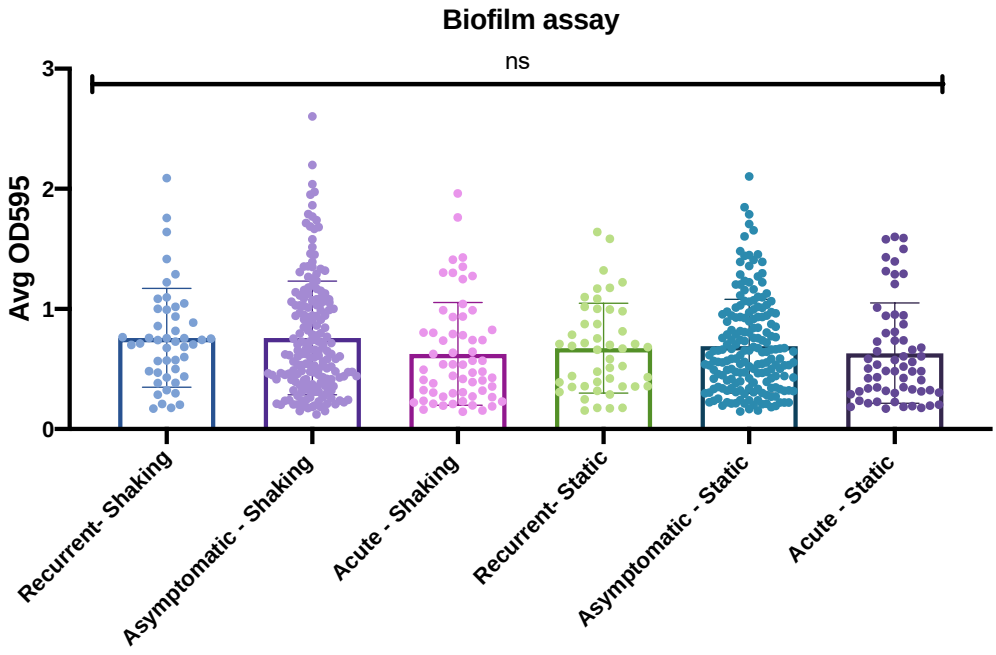


Figure 4: Biofilm formation by 292 clinical GBS isolates

Biofilm formation by 292 clinical GBS isolates according to clinical presentation (recurrent, asymptomatic, or acute), grown in LB + 1% glucose under either shaking or static conditions at 37 ^{O}C. Each point on the bar graph represents average absorbance values of a single strain at OD595 calculated from three independent experiments, and error bars show the standard deviation of the OD595 of the strains within the clinical presentation. A one-way ANOVA was used to compare the differences in biofilm formation across asymptomatic, acute and recurrent strains under shaking and static conditions; ns – not significant

The strains were classified into either low-, medium-, or high- biofilm formers, based on the average OD595 readings. OD readings below 0.5 were classified as low, between 0.5-1 were classified as medium, and above 1 were high biofilm formers. Under shaking conditions 113 (38.7%) isolates were low biofilm formers, 105 (36.0%) were medium biofilm formers, and 74 (25.3%) were high biofilm formers. On the other hand, under static conditions 116 (39.7%) isolates

were low biofilm formers, 118 (40.4%) were medium biofilm formers, and 58 (19.9%) were high biofilm formers (Figure 5A). A Chi-square analysis showed that there was no significant difference in the percentage of strains that had either low, medium, or high biofilm forming activity across the different clinical presentation, under both shaking ($p = 0.053$) and static ($p = 0.493$) conditions.

Together these results demonstrate that there was no significant difference in the ability of asymptomatic strains to form a biofilm when compared to acute or recurrent strains.

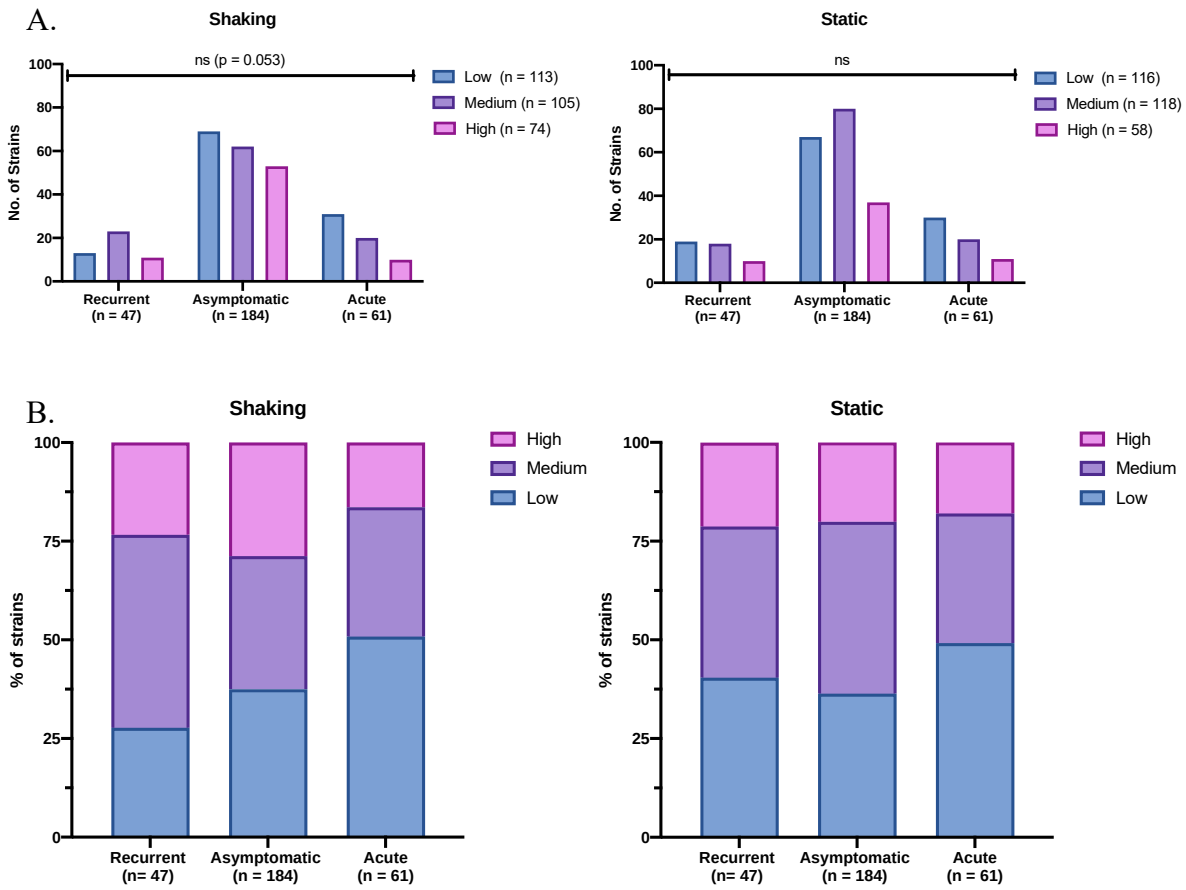


Figure 5: Classification of 292 clinical GBS isolates according to biofilm forming ability.

292 clinical GBS isolates classified according to either low, medium or high biofilm forming strains. ***(A)*** *The bar graphs show the number of strains that were low, medium and high biofilm formers across the three clinical presentations, under both shaking and static conditions. Chi-square analysis was used to compare the number of strains that had low, medium or high biofilm forming ability across all three presentations; ns – not significant.* ***(B)*** *Percentage of strains that had low, medium or high biofilm forming ability, according to either recurrent, asymptomatic or acute clinical presentation.*

5.3. Recurrent strains have a slower initial growth rate compared to asymptomatic and acute strains.

To investigate if the differences in biofilm formation were due differences in growth of the strains, we performed a growth curve analysis for all 292 clinical GBS isolates using LB + 1% glucose under aerobic conditions at 37 ^{O}C. The results were presented as the means of four replicated wells in one independent experiment (Figure 6). The data were divided in two parts, the early to mid growth phase including the exponential phase (0-3 h) and the late to stationary growth phase including the decline phase(3-12 h). The AUC was analysed using a one-way ANOVA. Recurrent strains grew significantly slower than both asymptomatic ($p = 0.0001$) and acute ($p = 0.0003$) strains, and there was no significant difference in the growth rate of asymptomatic and acute strains ($p = 0.9285$). There was no significant difference in the ability of the strains to survive, in all the three clinical presentation categories ($p = 0.0563$). Taken together these results demonstrate recurrent strains grew at a slower rate compared to asymptomatic and acute strains, but were able to survive at the same rate as the other two. These results do not provide evidence that the difference in biofilm formation was due to differences in growth of the strains.

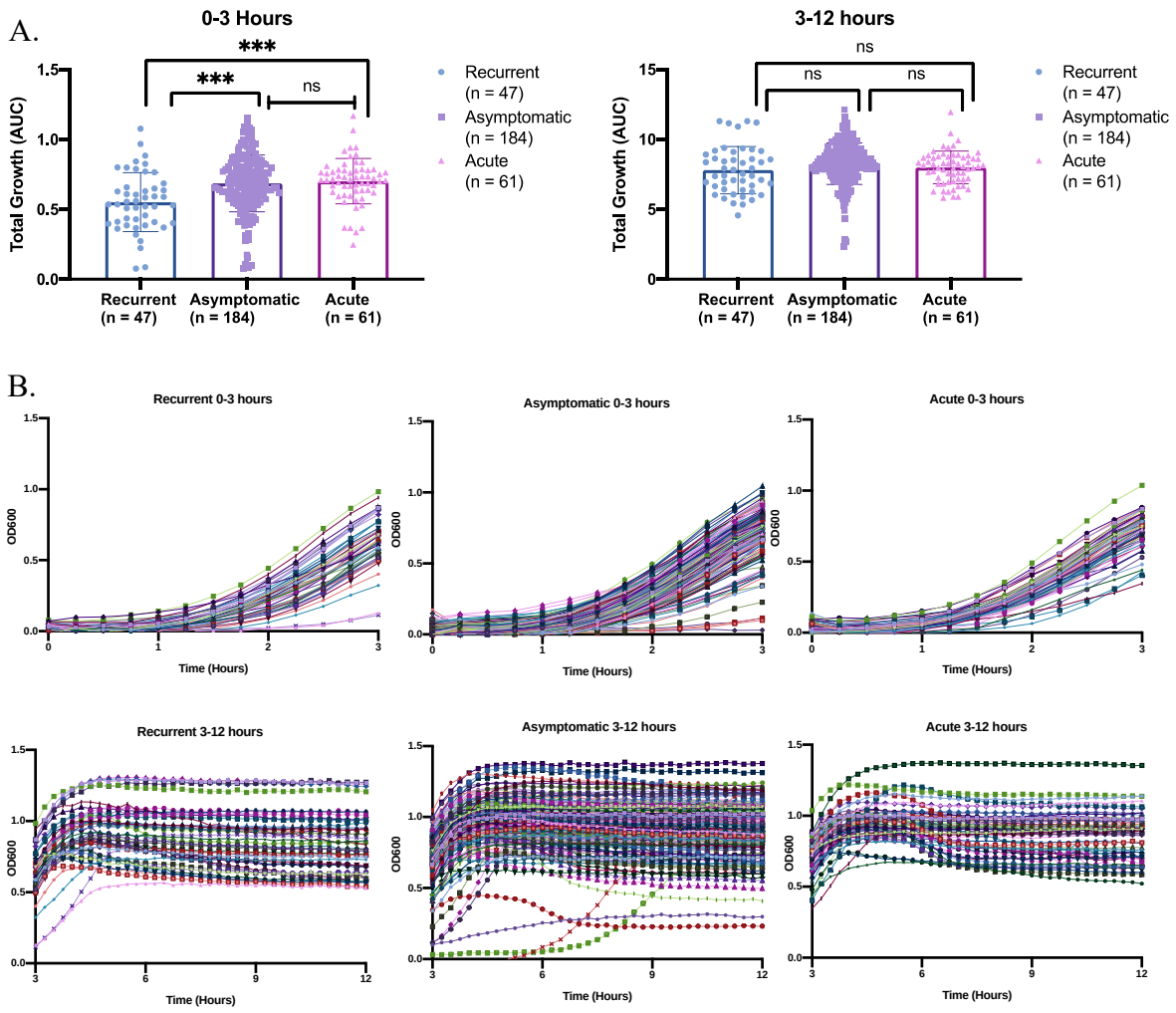


Figure 6: Growth curves of 292 clinical GBS isolates.

Growth (0-3 h) and survival (3-12 h) curves of the 292 clinical isolates in LB + 1% glucose according to clinical presentation under aerobic conditions at 37 ^{O}C. ***A:*** *The bar graphs represent total growth in terms of area under the curve (AUC), each point represents the AUC of a single strain, and error bars show the standard error of the total area. An ordinary one-way ANOVA was used to compare AUC among the three clinical presentations; ns – not significant, *** $p < 0.001$.* ***B:*** *The graphs represent the absorbance values at OD600 from one independent experiment.*

5.4. Malic acid inhibits the biofilm forming ability of GBS.

Malic acid metabolism has been previously shown to play a role in the growth of 834 in urine (15). As the strain is also a strong biofilm former, we wanted to investigate if the presence of malic acid would contribute to biofilm formation as well. Hence, biofilm assays were performed using 834 and its respective isogenic *maeK* and *maeE* mutants. The *maeK* gene encodes an accessory membrane-anchored sensor kinase that forms part of a two-component system with *maeR* (encoding a transcriptional regulator), and plays a role in the induction mechanism of *maePE* in the presence of malic acid. The *maeE* gene encodes for a malate oxidoreductase enzyme which is essential for growth in L-malic acid (49).

Biofilm assays were performed in LB + 1% glucose with or without 40mM malic acid, for all the strains under both shaking and static conditions. The *maeK* mutant formed the highest biofilm under both shaking (OD595 = 1.67) and static (OD595 = 2.03) conditions when cultured in LB + 1% glucose, however when cultured in LB + 1% glucose + malic acid the biofilm forming ability of the mutant decreased under both shaking (OD595 = 0.90) and static (OD595 = 0.84) conditions. Addition of malic acid to the media significantly inhibits the biofilm forming ability of all strains tested in this assay (Figure 7A). The data for all the strains under the different conditions were normally distributed. A two-way ANOVA was used to compare the average OD595 values within each strain in the two media, under shaking or static conditions. Under shaking conditions NEM316 ($p = 0.0149$), 834 ($p = 0.0002$), 834*ΔmaeK* ($p < 0.0001$) and 834*ΔmaeE* ($p < 0.001$) produced a significantly lower biofilm in LB + 1% glucose supplemented with malic acid. However, under static conditions the reduction in biofilm formation by 834 ($p = 0.0452$) and 834*ΔmaeE* ($p = 0.0015$) was significantly lower than under shaking conditions.

The biofilm forming ability of the mutants was then compared to that of the WT in both media with and without malic acid, under shaking and static conditions. A one-way ANOVA was used to analyse the data, there was no significant difference in the biofilm forming ability of either mutant compared to the WT in all the conditions (Figure 7B).

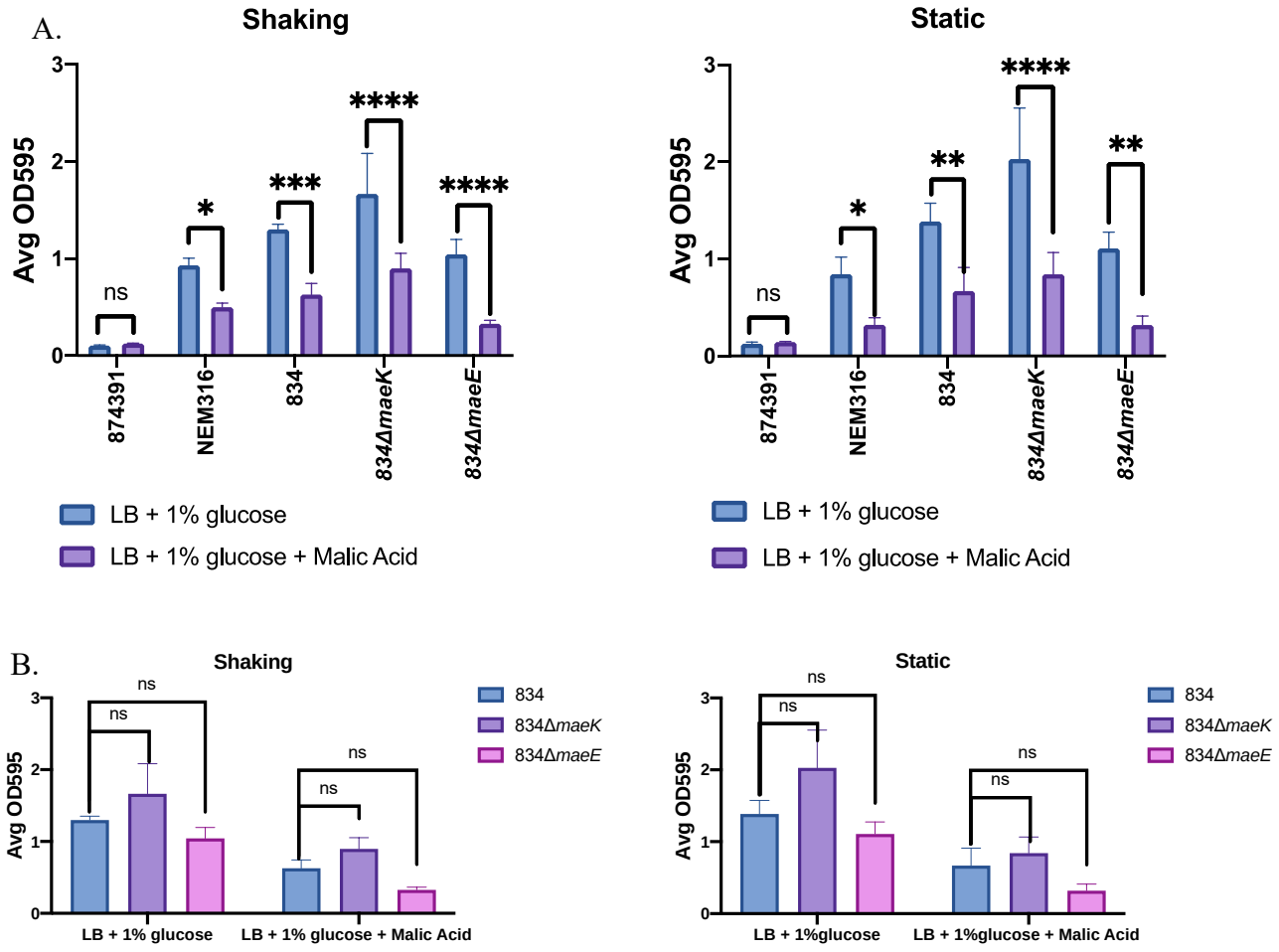
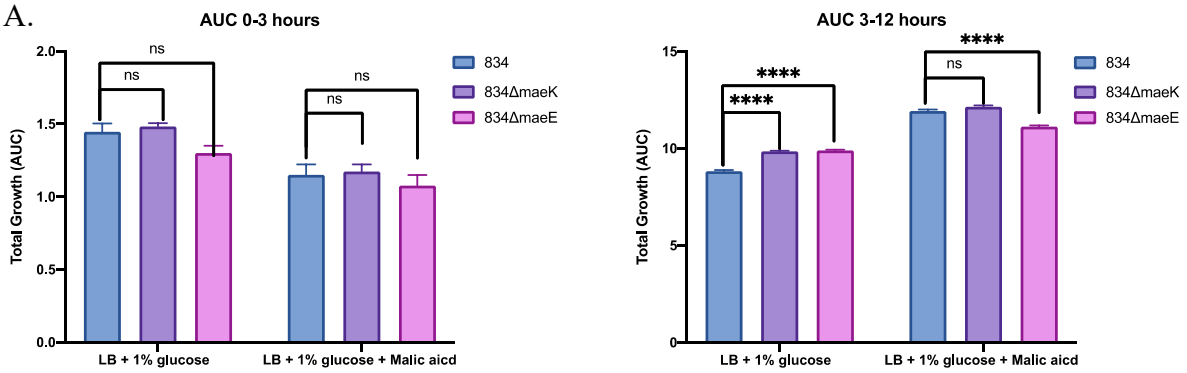


Figure 7: Biofilm formation in media with and without malic acid.

Biofilm formation of 874391, NEM316, 834, 834ΔmaeK and 834ΔmaeE in microtiter plates under different conditions. Strains were grown shaking or static in either LB + 1% glucose or LB + 1% glucose + malic acid. The results were presented as the mean of eight replicate wells in three independent experiments. Bar charts represent the average absorbance values at OD595, and error bars show the standard deviation calculated from the three independent experiments. ***A:*** *Average absorbance values were compared for each strain in the two media was compared using a two-way ANOVA;* ***B:*** *a one-way ANOVA was used to compare the average absorbance of the mutants to the WT; ns – not significant, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, **** $p < 0.0001$.*

In order to investigate whether addition of malic acid to LB + 1% glucose affected growth of 834 and its mutants, and in turn affected biofilm formation by the three strains, growth curve analysis was performed under aerobic conditions at 37 oC. The results were presented as the total growth (AUC) and mean OD600 of three independent experiments in two parts, the early to mid growth phase including the exponential phase(0-3 h) and the late to stationary phase including the decline phase (3-12 h) (Figure 8). A two-way ANOVA showed that there was no significant difference in the growth rate of the mutants compared to the WT strain in both LB + 1% glucose and LB + 1% glucose + malic acid. However the strains did have a lower total growth in the media supplemented with malic acid (Figure 8A). Both mutants were able to survive significantly more than the WT in LB + 1% glucose, but the *maeE* mutant had a significantly lower survival rate than the WT in LB + 1% glucose + malic acid. A one-way ANOVA used to analyse the difference in growth of each strain in the two media found, all three strains had a significant reduction in growth rate when grown in LB + 1% glucose supplemented with malic acid compared to media without malic acid (834: $p = 0.0009$, 834*ΔmaeK:* $p = 0.0005$, 834*ΔmaeE*: $p = 0.0175$) (Figure 8B). The one-way ANOVA also showed that all three strains were able to survive significantly more in malic acid supplemented media ($p < 0.0001$). Overall the three strains were able survive better in malic acid supplemented media.

A.


AUC 0-3 hours
Total Growth (AUC)
2.0
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1.0
0.5
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ns
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ns
LB + 1% glucose
LB + 1% glucose + Malic aicd
834
834ΔmaeK
834ΔmaeE
AUC 3-12 hours
Total Growth (AUC)
15
10
5
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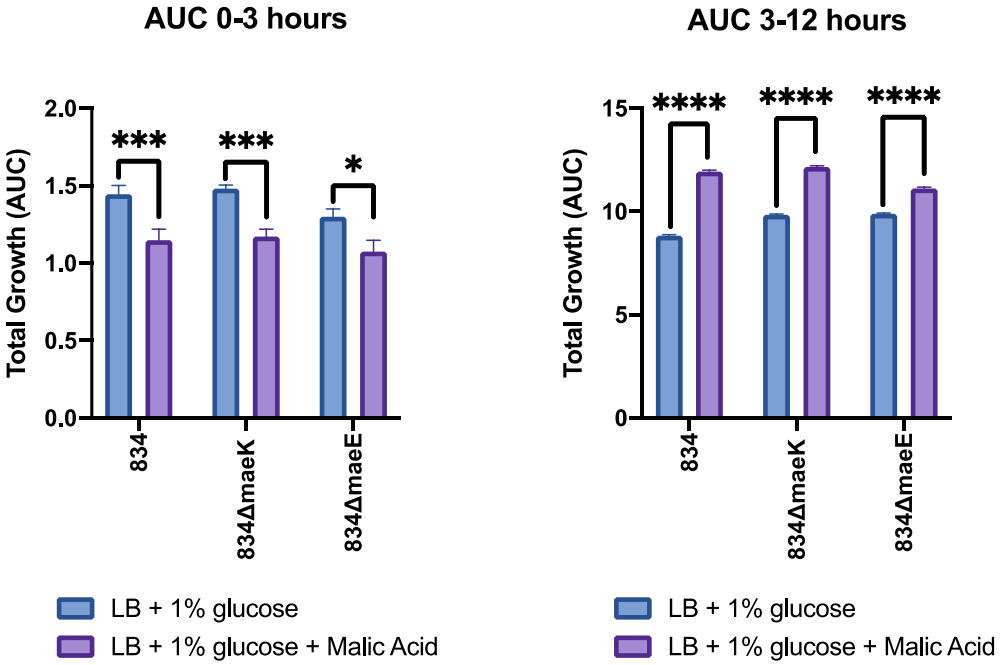
LB + 1% glucose
LB + 1% glucose + Malic aicd
834
834ΔmaeK
834ΔmaeE

B.


AUC 0-3 hours
Total Growth (AUC)
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1.5
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834
834ΔmaeK
834ΔmaeE
LB + 1% glucose
LB + 1% glucose + Malic Acid
AUC 3-12 hours
Total Growth (AUC)
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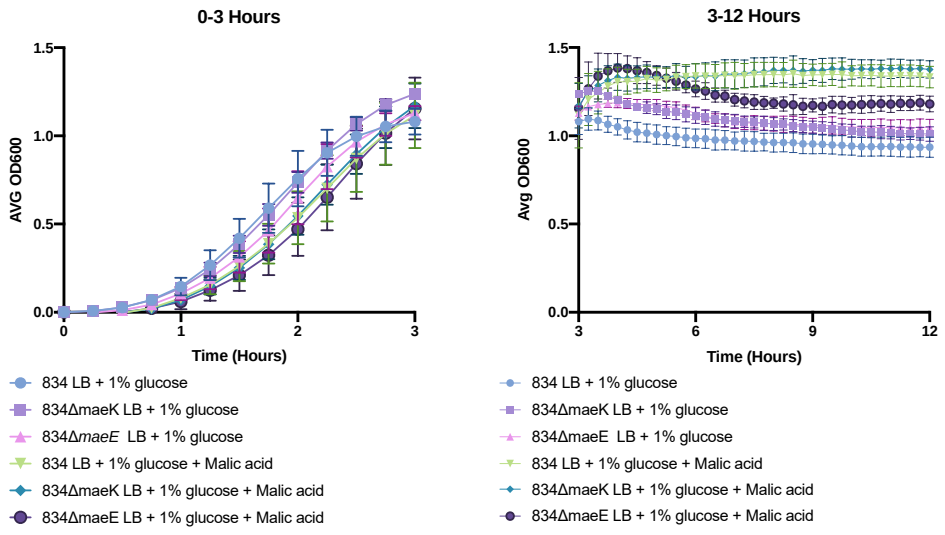
834
834ΔmaeK
834ΔmaeE
LB + 1% glucose
LB + 1% glucose + Malic Acid

C.


0-3 Hours
AVG OD600
1.5
1.0
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1
2
3
Time (Hours)
834 LB + 1% glucose
834ΔmaeK LB + 1% glucose
834ΔmaeE LB + 1% glucose
834 LB + 1% glucose + Malic acid
834ΔmaeK LB + 1% glucose + Malic acid
834ΔmaeE LB + 1% glucose + Malic acid
3-12 Hours
Avg OD600
1.5
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0.5
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12
Time (Hours)
834 LB + 1% glucose
834ΔmaeK LB + 1% glucose
834ΔmaeE LB + 1% glucose
834 LB + 1% glucose + Malic acid
834ΔmaeK LB + 1% glucose + Malic acid
834ΔmaeE LB + 1% glucose + Malic acid

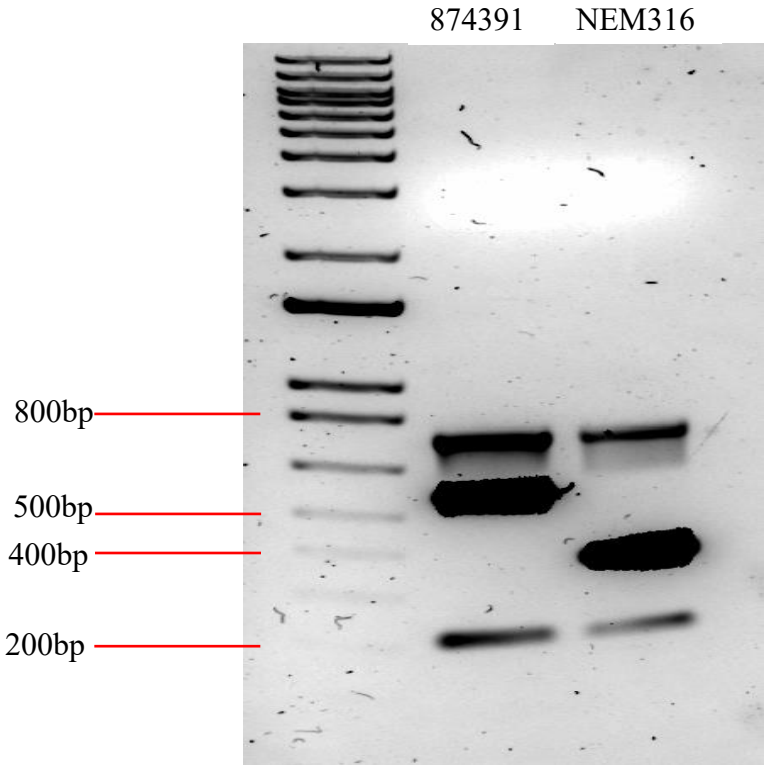
Figure 8: Growth curves of 834 and its isogenic mutants in media with and without malic acid.

Growth (0-3 h) and survival (3-12 h) curves of 834 and its isogenic mutants maeK and maeE under aerobic conditions at 37^{O}C. The results were presented as the means of eight wells in three independent experiments. ***A and B*** *The bar graphs represent total growth in terms of area under the curve (AUC), and error bars show the standard error of the total area. A two-way ANOVA was used to analyse the difference in AUC of the mutants compared to the WT in* ***A****, a one way ANOVA was used to analyse the difference in AUC of each strain in LB + 1% glucose and LB + 1% glucose + malic acid in* ***B****; ns – not significant, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, **** $p < 0.0001$.* ***C*** *The graphs represent the average absorbance values at OD600, and error bars show the standard deviation calculated from the three independent experiments.*

Taken together, this suggests that although there is a reduction in biofilm formation by 834 and its isogenic mutants in LB + 1% glucose + malic acid, this phenotype is due to the reduced ability of each strain to grow in malic acid supplemented media.

5.5. PCR screening for genes that contribute to biofilm formation

A multiplex PCR screening approach was used to assess the prevalence of the genes encoding the adhesins PI-1, PI-2a, PI-2b and BsaB. GBS 874391 possesses *pil1, pil2b,* and *bsaB*, while NEM316 possesses *pil1, pil2a* and *bsaB*. These two strains were used as positive controls in all the PCR screens performed, while a no template sample was used as a negative control. The primers were first assessed for their specificity in a PCR (Appendix 6). A multiplex PCR was also performed to assess specificity (Figure 9).



Gene	*pil1*	*pil2a*	*pil2b*	*bsaB*
Length	209 bp	382 bp	529 bp	797 bp

Figure 9: Multiplex PCR of GBS 874391 and NEM316.

Multiplex PCR screening to assess the prevalence of pil1, pil2a, pil2b and bsaB in GBS 874391 and NEM316. The table shows the length of each gene.

The right sized fragment for *pil1* was amplified from 204 isolates (70.2%), 116 isolates (39.7%) for *pil2a*, 35 isolates (12.0%) for *pil2b*, and 266 isolates (91.8%) for *bsaB*. There is no significant difference in the proportion of strains that had the right sized fragments for all four genetic loci. The PCR screen did not work for two strains (GU0205 and GU0927) even after extracting the gDNA again, both these strains presented with asymptomatic infection.

Table 4: Contingency table comparing the proportion of asymptomatic, acute and recurrent strains with *bsaB, pil1, pil2a and pil2b.*

Genetic loci	Asymptomatic (n = 182)	Acute (n = 61)	Recurrent (n = 47)	Total (n = 290)	P-value
bsaB	167 (91.8%)	55 (90.2%)	44(93.6%)	266 (91.7%)	0.812
pil1	134 (73.6%)	43 (70.5%)	27(57.4%)	204 (70.3%)	0.096
pil2a	71 (39.0%)	26 (42.6%)	19 (40.4%)	116 (40.0%)	0.881
pil2b	19 (10.4%)	11 (18.0%)	6 (12.8%)	36 (12.4%)	0.297

To determine the correlation between the four genetic loci and biofilm forming abilities of the strains, the four genetic loci of the strains were analysed according to the biofilm forming abilities under shaking and static conditions. Under shaking conditions there was a significant difference in the presence of *pil1, pil2a* and *pil2b,* across the three different levels of biofilm activity, while under static conditions there was only a significant difference in the presence of *pil1* and *pil2b.*

Table 5: Contingency table proportion of low medium and high biofilm forming strains with *bsaB, pil1, pil2a and pil2b* under shaking conditions.

Genetic loci	Low (n = 112)	Medium (n = 105)	High (n = 73)	Total (n = 290)	P-value
bsaB	103 (91.2%)	98 (93.3%)	65 (87.8%)	266 (91.7%)	0.440
pil1	91 (80.5%)	72 (68.6%)	41 (55.4%)	204 (70.3%)	< 0.0001 ****
pil2a	46 (40.7%)	48 (45.7%)	22 (29.7%)	116 (40.0%)	0.010 *
pil2b	21 (18.6%)	8 (7.6%)	7 (9.5%)	36 (12.4%)	0.002 **

The chi-square analysis showed that there was a significant difference in the proportion of strains that had the right size fragments for *pil1, pil2a* and *pil2b* across the three different levels of biofilm activity under shaking conditions. Further analysis of these three genetic loci found that the proportion of strains that had the right sized fragment for *pil1* was significantly different between all the biofilm activity levels. The most significance being between the low and high biofilm forming strains ($p < 0.0001$), followed by low and medium ($p = 0.006$), and medium and high biofilm forming strains ($p = 0.004$). The proportion of strains that had the right sized fragment for *pil2a* was only significantly different between low and high biofilm forming strains (0.019), and medium and high biofilm forming strains ($p = 0.003$). Low biofilm forming strains had a significantly different proportion of strains that possessed the right sized fragment for *pil2b* compared to medium ($p = 0.002$) and high ($p = 0.010$) biofilm forming strains.

Table 6: Contingency table proportion of low medium and high biofilm forming strains with *bsaB, pil1, pil2a and pil2b* under static conditions.

Genetic loci	Low (n = 115)	Medium (n = 117)	High (n = 58)	Total (n = 290)	P-value
bsaB	105 (90.5%)	109 (92.4%)	52 (89.7%)	266 (91.7%)	0.714
pil1	92 (79.3%)	82 (69.5%)	30 (51.7%)	204 (70.3%)	0.001 *
pil2a	48 (41.4%)	52 (44.1%)	16 (27.6%)	116 (40.0%)	0.089
pil2b	21 (18.1%)	11 (9.3%)	4 (6.9%)	36 (12.4%)	0.045 *

The chi-square analysis showed that there was a significant difference in the proportion of strains that had the right size fragments for *pil1* and *pil2b* across the three different levels of biofilm activity under static conditions. Further analysis of these two genetic loci found that high biofilm forming strains had a significantly different proportion of strains that possessed the right sized fragment for *pil1* compared to low ($p < 0.0001$) and medium ($p = 0.017$) biofilm forming strains. While the proportion of strains that had the right sized fragments for *pil2b* was only significantly different between the low and high biofilm forming strain ($p = 0.045$).

Overall the results from the PCR screen showed that there is no difference between the presence of *bsaB* and biofilm forming ability. All the strains had a larger proportion of strains with *pil1* followed by *pil2a* and then *pil2b*, regardless of their biofilm forming abilities. High biofilm forming strains had the lowest proportion of strains that possessed all four genes, compared to low and medium biofilm formers.

5.6. Haemolytic activity of the clinical GBS strains

To assess haemolytic activity, each clinical GBS isolate was streaked out onto tryptic soy agar plates containing 5% horse blood. Each plate also had 2µl spots of GBS 874391, 874391Δ*covR* (positive), and 874391Δ*cylE* (negative), as controls. The *covR* mutant contains a mutation in a repressor of β-haemolysin expression, and hence overexpresses the protein (121). On the other hand, the 874391Δ*cylE* mutant is not able to produce β-haemolysin, as the *cylE* gene is part of a cluster of genes required for β-haemolysin expression in GBS (122). The strains were ranked in terms of zone of clearance, 0 – none, 1- low, 2 – medium and 3 – high (Figure 10).

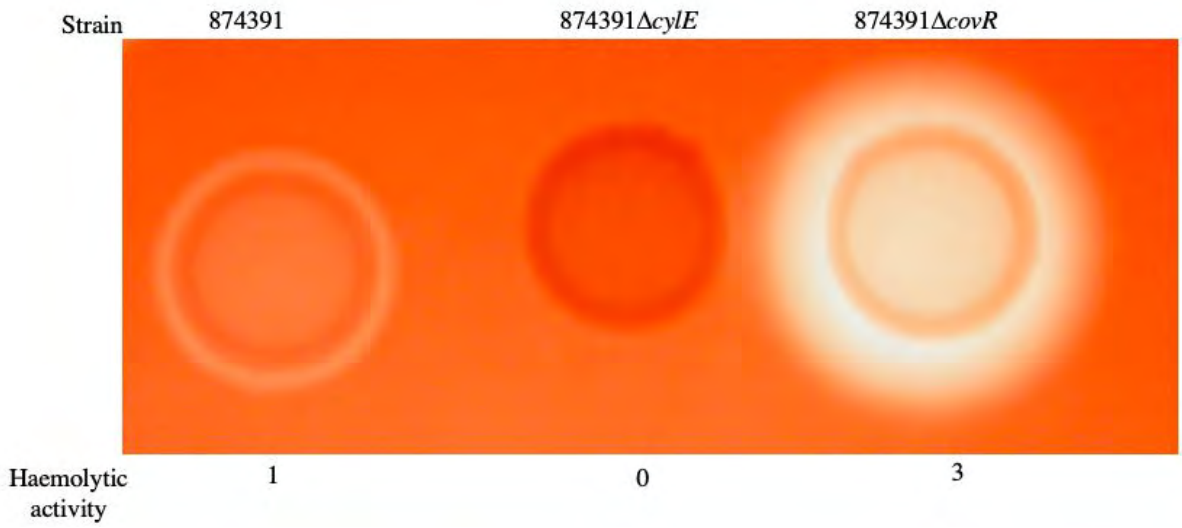


Figure 10: Haemolytic activity of controls.

Representative image of the controls that were present on every plate. 1 represents GBS 874391 which has low haemolytic activity, 0 represents the 874391ΔcylE mutant that is not able to produce β-haemolysin, and 3 represents the 874391ΔcovR that over expresses β-haemolysin.

180 (61.6%) of the strains had low haemolytic activity, 72 (24.7%) had medium haemolytic activity, and 34 (11.6%) had high haemolytic activity. Only 6 (2.1%) of the clinical isolates had no zone of clearance; all of them cause asymptomatic infection (Figure 11A). The data were analysed using a Chi-square analysis, which showed that there was a significant difference in the number of strains that had no, low, medium, or high haemolytic activity across the different clinical presentations ($p = 0.022$).

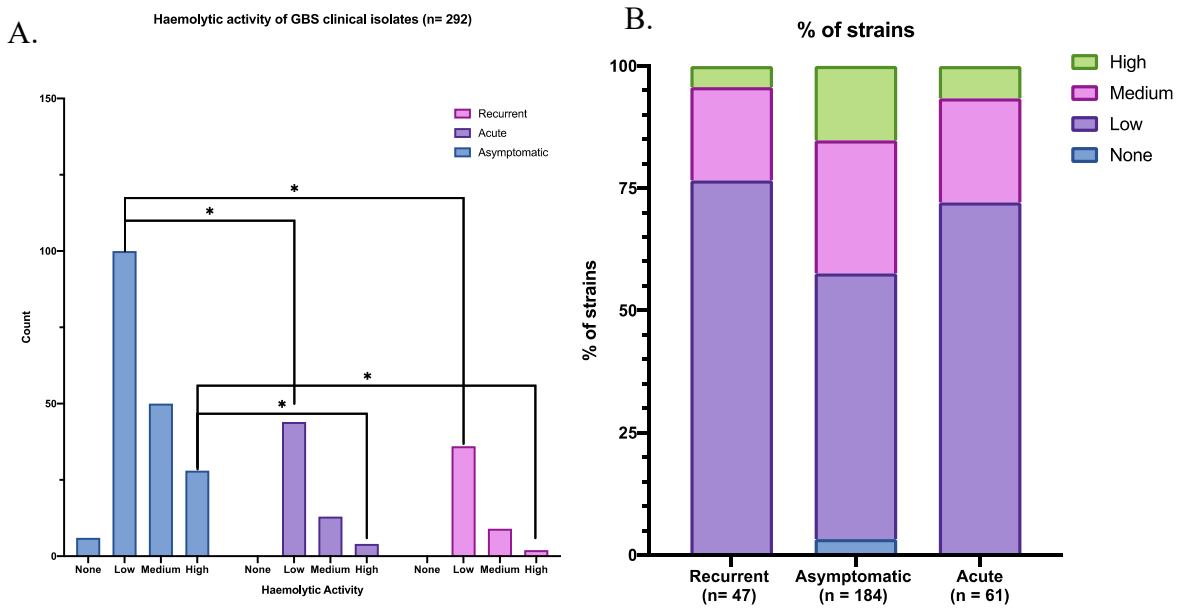


Figure 11: Haemolytic activity of 292 clinical GBS isolates.

Haemolytic activity of 292 clinical GBS isolates ranked in terms of zone of clearance (none, low, medium and high). Strains were further separated by clinical presentation (recurrent, acute and asymptomatic). ***A*** *The proportion of strains that had either no, low, medium or high haemolytic activity was analysed using Chi-square analysis; * $p < 0.05$.* ***B*** *Percentage of strains that had no, low, medium, or high haemolytic activity, according to either recurrent, asymptomatic or acute clinical presentation.*

Further analysis found that asymptomatic strains had a significantly different proportion of strains that had low and high haemolytic activity compared to recurrent ($p = 0.017$) and acute ($p = 0.047$) strains (Table 7, Figure 11B). Taken together, these results show that asymptomatic strains are more haemolytic compared to recurrent and acute strains.

Table 7: Pairwise comparison of haemolytic activity and clinical presentations. Bolded and underlined cells represent comparisons that are significantly different.

Clinical presentation	**Haemolytic activity**		**Fisher's Exact p-value**
Asymptomatic	Low	Medium	0.098
Recurrent			
Asymptomatic	**Low**	**High**	**0.017**
Recurrent			
Asymptomatic	Medium	High	0.322
Recurrent			
Asymptomatic	Low	Medium	0.176
Acute			
Asymptomatic	**Low**	**High**	**0.047**
Acute			
Asymptomatic	Medium	High	0.405
Acute			
Recurrent	Low	Medium	0.811
Acute			
Recurrent	Low	High	0.690
Acute			
Recurrent	Medium	High	1.000
Acute			

6. Discussion

GBS is known to cause a variety of infections in neonates, pregnant women, the elderly, or immunocompromised individuals (27, 28). GBS is estimated to cause approximately 1-2% of all single organism UTIs (6), with approximately 7% of pregnant women having high titers of GBS (44). Asymptomatic GBS infection in pregnant women can cause bloodstream infections, meningitis, osteomyelitis, and endocarditis (37). Uropathogenic GBS strains isolated from patients with acute cystitis and pyelonephritis adhered to urothelial cells and induce inflammatory responses, but were incapable of growing in human urine (47). In this study we sought to determine the optimal media for biofilm formation by GBS, the biofilm forming ability of 292 clinical GBS isolates, the presence of four previously studied genes that have been shown to play a role in GBS virulence, and the haemolytic activity of the clinical isolates.

6.1. GBS forms a strong biofilm when cultured in LB + 1% glucose.

There have been conflicting reports about the optimal media and pH for GBS biofilm formation. Some studies have shown that nutrient rich media promotes biofilm formation (23), while others have shown that nutrient-limited media is the best (120). However, all previous studies have indicated that supplementing the media with glucose enhances biofilm formation.

The first hypothesis for this study was that supplementing media with glucose enhances the biofilm forming ability of a strong GBS biofilm forming strain. Hence, aim 1 set out to determine the optimal conditions to promote GBS biofilm formation and whether it affects growth. This was done by using two previously described GBS strains in a biofilm experiment, using four different media (LB, LB + 1% glucose, THB, and THB + 1% glucose) under shaking and static conditions, to quantify the amount of biofilm formed in the wells of microtiter plates. GBS 874391

(a weak biofilm-former), and NEM316 (a strong biofilm-former), were used as the negative and positive controls respectively. NEM316 cultured in LB + 1% glucose produced the highest OD595 reading under both conditions. Additionally, growth curve analyses for both the strains in the four media was performed to demonstrate that higher biofilm formation by NEM316 in LB + 1% glucose was not due to a difference in growth of the two strains. The results from these two experiments demonstrated that LB supplemented with glucose was the optimal media for biofilm formation by GBS. This is consistent with findings by Konto-Ghiorghi et al (2009) that showed uniform biofilm production only in LB and RPMI supplemented with 1% glucose and not in THB (123). These results support the hypothesis.

Metabolism of glucose by bacteria in nutrient rich media forms organic acids that reduce the pH of the media and therefore initiate biofilm formation (23). Santi *et al.* (2009) performed a comparative global gene expression analysis of GBS at acidic and neutral pHs to identify factors involved in the response of GBS to environmental pH. They found that transcription of 317 genes was increased at an acidic pH compared to at neutral pH, while 61 genes were downregulated (124). The global response to acidic environments included modulation of genes involved in GBS adaptation and vaginal persistence, as well as virulence-related genes which are under the control of the CovRS two-component regulatory system. These findings suggest that GBS translocation from acidic to neutral niches switches on virulence-related genes, favouring the transition from commensal to invasive bacterial pathogen (38). Based on these observations, it can be explained that the reason why NEM316 formed a higher biofilm in LB + 1% glucose, could be due to the increased expression of adhesins under the acidic conditions.

6.2. Clinical GBS isolates from the urinary tract exhibit a range of biofilm phenotypes.

Aim 2 set out to determine the biofilm forming ability of an in-house collection of 292 clinical GBS isolates based on the optimal conditions identified in aim 1. The GBS isolates were cultured in LB + 1% glucose in microtiter plates, with each plate containing ten strains, and the first two rows containing the negative (874391) and positive (NEM316) controls to ensure reproducibility of the assay across different plates. The experiments were repeated in triplicate under both shaking and static conditions. The isolates were then categorised into either low, medium, or high biofilm formers based on the average OD595 readings from all three experiments. There was no significant difference in the number of strains that were able for form either low, medium or high biofilms across the different clinical presentations under both shaking and static conditions. However, a p-value of 0.053 for the shaking data trends towards significant, and if more isolates were studied then the value may have been significant.

Growth curve analysis for the strains using AUC found that recurrent strains grew at a significantly lower rate than asymptomatic or acute strains, when grown in LB + 1% glucose. The survival rate of the strains across the three presentations was not significantly different. Overall, since the growth of the strains was similar it explains the lack of difference in the biofilm formation across the three clinical presentations. However a limitation of using the OD values to analyse the growth of the strains is that they are not a measure of viability, and are only a surrogate measure of growth and survival.

6.3. Malic acid inhibits the biofilm forming ability of GBS.

L-Malic acid is a naturally occurring organic dicarboxylic acid that plays a role in sour foods. In humans malic acid is derived from food as well as through synthesis through the citric acid cycle, and can be present in urine (125, 126). Malic acid can be degraded under aerobic and anaerobic conditions by different Gram-positive bacteria (127-129). Ipe *et al*. (2016) showed that some strains of ABU-causing GBS are able to grow in human urine, and that this is related to malic acid metabolism (15).

The hypothesis for this section was that supplementing LB + 1% glucose with malic acid would enhance biofilm formation of GBS strains that are able to metabolise malic acid through increased growth.

Aim 3 was to assess whether adding malic acid to the media affects biofilm formation by a high biofilm forming ABU-causing strain. Strain 834 was used together with its respective *maeK* and *maeE* mutants to investigate the effect malic acid had on biofilm formation. NEM316 and GBS 874391 were also used as controls for comparison. There was a significant reduction in biofilm formation when the strains were cultured in LB + 1% glucose + malic acid compared to LB + 1% glucose, under both shaking ($p = 0.0242$) and static ($p = 0.0312$) conditions. 834 and its isogenic mutants all had reduced biofilm forming abilities in LB + 1% glucose + malic acid under both shaking and static conditions. The biofilm forming ability of the mutants was then compared to that of the WT, there was no significant difference found. These results do not support the hypothesis that supplementing media with malic acid enhances the biofilm forming ability of a strong biofilm forming strain that is able to metabolise malic acid.

The growth curve assays for these were analysed in two ways, the first was by comparing the growth of the mutants to that of the WT strains, there was no significant difference in the

growth rates in both media with and without malic acid. All three strains did however have a higher survival rate in LB + 1% glucose + malic acid, and only the *maeE* mutant had a lower survival rate compared to the WT. These findings were inconsistent with that of Ipe *et al.* (2016), who showed significantly attenuated growth of both the *maeK* and *maeE* mutants in SHU and NHU containing malic acid. A reason for this is due to the difference in composition of NHU and SHU compared to LB + 1% glucose. LB is a very rich media that has been formulated to enable quick and robust growth of bacteria (130), additionally the 1% glucose is another energy rich source. On the other hand, NHU and SHU are considered are nutritionally deplete and contain high concentrations of nitrites and urea inhibit most bacteria (131). For example it takes 834 72 hours to reach an OD of about 1 in SHU, while it barely reached an OD of 0.8 in NHU (15). The likely reason why we did not see as much of a difference as compared to Ipe *et al.* is because the media used in this study was much richer, so the bacteria might not have needed to use the malic acid to grow, so being able to metabolise malic acid or not would be irrelevant. Conversely, Landete *et al.* (2010) found that the *maeE* mutant of *Lactobacillus casei* was able to grow in media supplemented with glucose or glucose and L-malic acid, at growth rates similar to the wild-type strain but were not able to grow with L-malic acid alone (129). The second analysis compared the growth of each strain in LB + 1% glucose with and without malic acid, all three strains grew significantly less in the media supplemented with glucose, but were able to survive at a significantly higher rate than when grown in just LB + 1% glucose.

These results suggest that malic acid supplemented in the media plays two roles, the first is that of a substrate for metabolic utilisation, which would have caused the reduced growth in the malic acid supplemented media. On the other had it could act as a supplement for growth by causing a slight acidification of the media helping the mutant strains to grow at the same rate as

the WT, and therefore increasing the ability of the strains to survive significantly more than when grown in LB + 1% glucose.

6.4. PCR screening for genes that contribute to biofilm formation.

Several adhesins have been shown to play a role in GBS biofilm formation, including *bsaB* (132) and pili namely pilus type 1, 2a and 2b (97). The evidence of pili involvement in biofilm formation was first identified in *S. pyogenes* (133). A correlation between the high surface exposure of pilus 2a and the biofilm formation phenotype of 289 GBS clinical isolates has previously been observed (134). D'Urzo *et al*. studied the biofilm forming ability of 389 GBS isolates, they also found variability among strains both in pilus expression and biofilm forming ability of the strains even when they belonged to the same serotype (23). The CovRS two-component regulatory system has been shown to control the expression of multiple virulence factors (135-137). Park *et al*. showed that a CovRS two-component regulatory system knockout mutant had increased adherence to host cells and ability to form biofilm-like structures (138). The CovRS system has also been shown to regulate the expression of *bsaB* (92, 132, 138), which is slightly downregulated in acidic environments compared to at neutral pH.

The first part of aim 4 was to determine the presence of *bsaB, pil1, pil2a* and *pil2b* in the 292 clinical GBS isolates using multiplex PCR screening. There was no significant difference in the proportion of strains that had the right sized fragments for all four genetic loci. However, a p-value of 0.096 for *pil1* shows that the presence of *pil1* trended towards significance. Further analysis showed that the proportion of asymptomatic strains that had the right sized fragment for *pil1* was significantly more than recurrent strains ($p = 0.030$). This result is unexpected because pili are involved in promoting initial association with host cells, and therefore mediating adhesion

and attachment of pathogens to host cells. It would therefore be expected that strains that possess *pil1* would cause either acute or recurrent infections rather than asymptomatic infections. Previous studies have also found that *pil2a* was the most common variant found in human rectovaginal, UTI, and oropharynx GBS isolates, while the current study found that *pil1* was the most common variant found in the isolates studied. In the current study *pil2a* was only found in 40% of the strains, which is much lower than previously seen in other studies.

Low biofilm forming strains had a significantly higher proportion of strains that had the right sized fragment for *pil1* under both shaking and static conditions. This can be explained by the findings of Park *et al.* which showed the regulatory effect of CovRS on adherence and biofilm formation correlated with the expression of several adhesins but not of pilus type 1, discounting the role of this pilus variant in biofilm formation by the isolate studied (138). High biofilm forming strains had a significantly lower proportion of strains that had *pil2a* compared to low and medium biofilm forming strains under shaking conditions. This result was unexpected since PI-2a has previously been shown to mediate biofilm formation by GBS. Lastly, a larger proportion of strains that possessed *pil2b* were able to form low biofilms significantly more than medium and high biofilms.

The PCR screen did not work for all four genes in two asymptomatic strains even after the gDNA was reextracted. This could be due to unspecific binding of the primers to the DNA so the regions of the genes could not be amplified.

The benefit of performing a PCR to screen for genes is that PCR has a high specificity, provided that the primers are well designed (139). PCR screens are also very quick and easy to do and provide results within a few hours. Performing a multiplex PCR produces results even faster since all the primers for all the genes that are being screened can be added in one reaction, rather

than performing separate screens for each gene. However, one of the limitations of using PCR is cross-contamination of samples which could lead to false positive results. In this study the risk of cross-contamination was high because the PCR reactions were carried out in 96-well PCR plates, which could have led to a few false positives for some strains. In order to reduce the chances of this happening the MyTaq Mix, all the primers, and sterile H_2O were all mixed together in a master mix solution from which 24µl was added to each well, followed by 1µl of gDNA of a strain. Another limitation is that point mutations, insertions and/or deletions in the nucleotide sequence my inhibit the primers from binding to the DNA, so those genes cannot be amplified giving rise to false negatives.

Although the correct sized PCR fragments were amplified, there is no way of telling if the genes encode for the full length proteins by looking at these results only. Another method could be used to sequence the genomes of the strains and perform bioinformatic analyses (like BLAST) to determine if they have the gene. This will overcome the issues with the primers, and identify any gene variants.

6.5. Haemolytic activity of the clinical GBS strains.

β-haemolysin is an oxygen-stable, non-immunogenic, pore-forming cytolysin (60), involved in the invasion of human epithelial cells and release of pro-inflammatory cytokines (72). Studies have found that β-haemolysin is not required for establishment of UTIs in a murine model (42), however GBS induces proinflammatory cytokine production in a β-haemolytic dependent manner both *in vitro* and *in vivo* (73). Most human GBS strains produce β-haemolysin which plays a key role in GBS pathogenesis (140). Leclercq *et al.* (2019) found that β–haemolysin produced by a uropathogenic GBS strain induced inflammation and local neutrophil infiltration in the

bladder, which contributed to bacterial survival *in vivo* (141). The cytotoxin is non-essential for epithelial cell adherence, bladder colonisation and ascending infection of the kidneys (73)

We hypothesised that strains that caused asymptomatic infection express less haemolysin compared to strains that caused acute or recurrent infections. The second half of aim 4 was to determine the haemolytic activity of the 292 clinical GBS isolates using blood agar plates. We found a significant difference in the number of strains that had no, low, medium, or high haemolytic activity across the different clinical presentations. Asymptomatic strains were shown to have a significantly smaller proportion of strains that had low haemolytic activity compared to acute and recurrent strains. They also had significantly more strains with a high haemolytic activity compared to acute and recurrent strains. Overall all but six strains produced β-haemolysin. These results do not support our hypothesis.

The results obtained from this study were unexpected because previous studies have shown that β-haemolysin production contributes to bacterial survival, and that β-haemolysin promoted inflammation which drives UTI-associated symptoms. The CovRS two-component regulatory system tightly controls transcription of the *cyl* operon required for haemolysin expression (140). β-haemolysin drives expression of IL-10 an anti-inflammatory cytokine and inhibits expression of both IL-2 and NOS2 expression in GBS-infected macrophages, which are essential factors in host defense (142). Expression levels of β-haemolysin therefore appear to determine whether GBS stabilised its niche allowing for colonisation, or whether it becomes invasive (86). These findings suggest that strains that have higher haemolytic activity would most likely cause either acute or recurrent infection rather than asymptomatic infection. Under certain circumstances the pore-forming toxin and co-haemolysin CAMP factor may also contribute to GBS pathogenesis (143).

7. Conclusion

In summary the results from this study have shown that supplementing media with 1% glucose enhances the biofilm forming ability of a strong biofilm forming strain. Using 292 clinical urinary GBS isolates from patients that had either asymptomatic, acute or recurrent infection, we showed that there is no significant difference in the biofilm forming abilities of strains within the three different clinical presentations. We showed that supplementing the media with malic acid significantly inhibits the biofilm forming ability of a strain, which could be due to reduced ability of the strain to metabolise malic acid. We performed growth curve analyses to assess whether that was the case. Supplementing the media with malic acid seemed to have decreased the ability of the 834 and its mutants to grow, but enhanced their ability to survive compared to when they were grown in LB + 1% glucose, suggesting a dual role of malic acid. The decreased growth in LB + 1% glucose + malic acid provides an explanation as to why the strains had a decreased ability to form strong biofilms in the media. We carried out a PCR screening for genes encoding the adhesins PI-1, PI-2a, PI-2b and BsaB, all of which have previously been shown to contribute to GBS virulence. The prevalence of these genes was first analysed in terms of the clinical presentations of the strains, there was no significant difference in the presence of the genes and the clinical presentations. However the prevalence of *pil1* trended towards significant further analysis showed that asymptomatic strains had a significantly larger proportion with this gene compared to recurrent strains. The prevalence of the genes were then analysed in terms of the biofilm forming ability of the strains (low, medium and high), the results were unexpected since the high biofilm forming strains had a lower proportion of strains that possessed all four genes compared to low or medium biofilm forming strains. We found that the presence of BsaB did not have a significant difference in the biofilm forming ability of the strains, and that the pilus genes were responsible

for the differences in biofilm forming ability. Lastly the haemolytic activity of each strain was analysed in terms of the clinical presentations, and we found that asymptomatic strains had a larger proportion of strains that had high haemolytic activity compared to strains that caused acute or recurrent infections.

Overall, we concluded that there was no difference in the biofilm forming phenotype and presence of adhesins among the strains that were tested in this study. However, asymptomatic strains produce more β-haemolysin compared to acute and recurrent strains. Further work is now required to test out other aspects that may contribute to a strains ability to be able to cause UTIs.

7.1. Future work

Future work could include:

- Assessing the biofilm forming ability of strong biofilm formers from each clinical presentation in LB + 1% glucose of different pH values, to see if pH affects the biofilm forming phenotypes of the strains.
- Assess whether the biofilm forming ability of strong biofilm forming strains changes when they are cultured in urine rather than in LB + 1% glucose.
- Use latex agglutination to determine the serotype and/or sequence type of the clinical GBS isolates, and assess if there is an association between them and the strain phenotypes.
- Assess the biofilm forming ability of 834 in SHU with malic acid.
- Assess the growth of recurrent and acute strains in human urine.
- Examine the expression of the genes encoding BsaB, PI-1, PI-2a and PI-2b in the different strains to see if the difference in biofilm formation is due to differential expression of the genes.

- PCR screen for other GBS virulence factors that have been shown to play a role in biofilm formation, such as biofilm regulatory protein A, encoded by *brpA* and the LCP family of proteins.
- Make a NEM316 knockout for all four genes to assess whether there is an increase or decrease in biofilm forming ability.

8. References

1. Stamm WE, Norrby SR. Urinary tract infections: disease panorama and challenges. J Infect Dis. 2001;183 Suppl 1:S1-4.
2. Foxman B. Urinary tract infection syndromes: occurrence, recurrence, bacteriology, risk factors, and disease burden. Infect Dis Clin North Am. 2014;28(1):1-13.
3. Flores-Mireles AL, Walker JN, Caparon M, Hultgren SJ. Urinary tract infections: epidemiology, mechanisms of infection and treatment options. Nat Rev Microbiol. 2015;13(5):269-84.
4. Keren R, Shaikh N, Pohl H, Gravens-Mueller L, Ivanova A, Zaoutis L, et al. Risk Factors for Recurrent Urinary Tract Infection and Renal Scarring. Pediatrics. 2015;136(1):e13-21.
5. Dielubanza EJ, Schaeffer AJ. Urinary tract infections in women. Med Clin North Am. 2011;95(1):27-41.
6. Foxman B. The epidemiology of urinary tract infection. Nat Rev Urol. 2010;7(12):653-60.
7. Barnett BJ, Stephens DS. Urinary Tract Infection: An Overview. The American Journal of the Medical Sciences 1997;314(4):245-9.
8. Kline KA, Lewis AL. Gram-Positive Uropathogens, Polymicrobial Urinary Tract Infection, and the Emerging Microbiota of the Urinary Tract. Microbiol Spectr. 2016;4(2).
9. Desforges JF. Management of Urinary Tract Infections in Adults The New England Journal of Medicine. 1993;329(18):1328-34.
10. Wagenlehner FM, Naber KG. Current challenges in the treatment of complicated urinary tract infections and prostatitis. Clin Microbiol Infect. 2006;12 Suppl 3:67-80.
11. Chenoweth CE, Gould CV, Saint S. Diagnosis, management, and prevention of catheter-associated urinary tract infections. Infect Dis Clin North Am. 2014;28(1):105-19.
12. Nicolle LE, Committee* ACG. Complicated urinary tract infection in adults. Can J Infect Dis Med Microbiol. 2005;16(6):349-60.
13. Vebo HC, Solheim M, Snipen L, Nes IF, Brede DA. Comparative genomic analysis of pathogenic and probiotic Enterococcus faecalis isolates, and their transcriptional responses to growth in human urine. PLoS One. 2010;5(8).
14. Sakinc T, Michalski N, Kleine B, Gatermann SG. The uropathogenic species Staphylococcus saprophyticus tolerates a high concentration of D-serine. FEMS Microbiol Lett. 2009;299(1):60-4.
15. Ipe DS, Ben Zakour NL, Sullivan MJ, Beatson SA, Ulett KB, Benjamin WH, Jr., et al. Discovery and Characterization of Human-Urine Utilization by Asymptomatic-Bacteriuria-Causing Streptococcus agalactiae. Infect Immun. 2016;84(1):307-19.
16. Vallet I, Olson JW, Lory S, Lazdunski A, Filloux A. The chaperone/usher pathways of Pseudomonas aeruginosa: identification of fimbrial gene clusters (cup) and their involvement in biofilm formation. Proc Natl Acad Sci U S A. 2001;98(12):6911-6.
17. Kline KA, Dodson KW, Caparon MG, Hultgren SJ. A tale of two pili: assembly and function of pili in bacteria. Trends Microbiol. 2010;18(5):224-32.
18. Kostakioti M, Hultgren SJ, Hadjifrangiskou M. Molecular blueprint of uropathogenic Escherichia coli virulence provides clues toward the development of anti-virulence therapeutics. Virulence. 2012;3(7):592-4.

19. Nicolle LE, Ronald AR. Recurrent urinary tract infection in adult women: diagnosis and treatment. Infect Dis Clin North Am. 1987;1(4):793-806.
20. Soto SM, Smithson A, Horcajada JP, Martinez JA, Mensa JP, Vila J. Implication of biofilm formation in the presence of urinary tract infection caused by uropathogenic *Escherichia coli*. Clinical Microbiology and Infection. 2006;12(10):1034-36
21. Delzell JE, Jr., Lefevre ML. Urinary tract infections during pregnancy. Am Fam Physician. 2000;61(3):713-21.
22. Kaul AK, Khan S, Martens MG, Crosson JT, Lupo VR, Kaul R. Experimental gestational pyelonephritis induces preterm births and low birth weight in C3H/HeJ mice. Infect Immun. 1999;67(11):5958-66.
23. D'Urzo N, Martinelli M, Pezzicoli A, De Cesare V, Pinto V, Margarit I, et al. Acidic pH Strongly Enhances In Vitro Biofilm Formation by a Subset of Hypervirulent ST-17 Streptococcus agalactiae Strains. Applied and Environmental Microbiology. 2014;80:2176-85.
24. Schuchat A. Group B streptococcus. The Lancet. 1999;353(9146):51-6.
25. Brochet M, Couve E, Zouine M, Vallaeys T, Rusniok C, Lamy MC, et al. Genomic diversity and evolution within the species Streptococcus agalactiae. Microbes Infect. 2006;8(5):1227-43.
26. Keefe GP. Streptococcus agalactiae mastitis: a review. Can Vet J. 1997;38(7):429-37.
27. Edwards MS, Baker CJ. Group B streptococcal infections in elderly adults. Clin Infect Dis. 2005;41(6):839-47.
28. Dermer P, Lee C, Eggert J, Few B. A history of neonatal group B streptococcus with its related morbidity and mortality rates in the United States. J Pediatr Nurs. 2004;19(5):357-63.
29. Edwards M, Baker CJ. Group B streptococcal infections. Infectious diseases of the fetus and the newborn infant 2001.
30. Masiey HC, Doran KS, Nizet V. Recent advances in understanding the molecular basis of group B *Streptococcus* virulence. Expert Rev Mol Med Author manuscript. 2008;10(27).
31. Rajagopal L. Understanding the regulation of Group B Streptococcal virulence factors. Future Microbiol. 2009;4(2):201-21.
32. Verani JR, McGee L, Schrag SJ, Division of Bacterial Diseases NCfI, Respiratory Diseases CfDC, Prevention. Prevention of perinatal group B streptococcal disease--revised guidelines from CDC, 2010. MMWR Recomm Rep. 2010;59(RR-10):1-36.
33. Le Doare K, Kampmann B. Breast milk and Group B streptococcal infection: vector of transmission or vehicle for protection? Vaccine. 2014;32(26):3128-32.
34. Zimmermann P, Gwee A, Curtis N. The controversial role of breast milk in GBS late-onset disease. J Infect. 2017;74 Suppl 1:S34-S40.
35. Adams RA, Schachtrup C, Davalos D, Tsigelny I, Akassoglou K. Fibrinogen signal transduction as a mediator and therapeutic target in inflammation: lessons from multiple sclerosis. Curr Med Chem. 2007;14(27):2925-36.
36. Rivera J, Vannakambadi G, Hook M, Speziale P. Fibrinogen-binding proteins of Gram-positive bacteria. Thromb Haemost. 2007;98(3):503-11.
37. Ulett KB, Benjamin WH, Jr., Zhuo F, Xiao M, Kong F, Gilbert GL, et al. Diversity of group B streptococcus serotypes causing urinary tract infection in adults. J Clin Microbiol. 2009;47(7):2055-60.
38. Shabayek S, Spellerberg B. Group B Streptococcal Colonization, Molecular Characteristics, and Epidemiology. Front Microbiol. 2018;9:437.
39. Schuchat A. Group B streptococcus. Lancet. 1999;353(9146):51-6.

40. Hansen SM, Uldbjerg N, Kilian M, Sorensen UB. Dynamics of Streptococcus agalactiae colonization in women during and after pregnancy and in their infants. J Clin Microbiol. 2004;42(1):83-9.
41. Farley MM, Harvey RC, Stull T, Smith JD, Schuchat A, Wenger JD, et al. A population-based assessment of invasive disease due to group B Streptococcus in nonpregnant adults. N Engl J Med. 1993;328(25):1807-11.
42. Kline KA, Schwartz DJ, Lewis WG, Hultgren SJ, Lewis AL. Immune activation and suppression by group B streptococcus in a murine model of urinary tract infection. Infect Immun. 2011;79(9):3588-95.
43. Beyer I, Mergam A, Benoit F, Theunissen C, Pepersack T. Managment of urinary tract infections in the elderly. Zeitschrift für Gerontologie und Geriatrie. 2001;34(2):153-7.
44. Muller AE, Oostvogel PM, Steegers EA, Dorr PJ. Morbidity related to maternal group B streptococcal infections. Acta Obstet Gynecol Scand. 2006;85(9):1027-37.
45. Kessous R, Weintraub AY, Sergienko R, Lazer T, Press F, Wiznitzer A, et al. Bacteruria with group-B streptococcus: is it a risk factor for adverse pregnancy outcomes? J Matern Fetal Neonatal Med. 2012;25(10):1983-6.
46. Thomsen AC, Morup L, Hansen KB. Antibiotic elimination of group-B streptococci in urine in prevention of preterm labour. Lancet. 1987;1(8533):591-3.
47. Edwards MS. Group B streptococcal conjugate vaccine: a timely concept for which the time has come. Hum Vaccin. 2008;4(6):444-8.
48. Stamey TA, Mihara G. Observations on the Growth of Urethral and Vaginal Bacteria in Sterile Urine. Journal of Urology. 1980;124(4):461-3.
49. Mortera P, Espariz M, Suarez C, Repizo G, Deutscher J, Alarcon S, et al. Fine-tuned transcriptional regulation of malate operons in Enterococcus faecalis. Appl Environ Microbiol. 2012;78(6):1936-45.
50. Jones N, Bohnsack JF, Takahashi S, Oliver KA, Chan M-S, Kunst F, et al. Multilocus Sequence Typing System for Group B Streptococcus. Journal of Clinical Microbiology. 2003;41(6):2530-6.
51. Luan S-L, Granlund M, Sellin M, Lagergård T, Spratt BG, Norgren M. Multilocus Sequence Typing of Swedish Invasive Group B Streptococcus Isolates Indicates a Neonatally Associated Genetic Lineage and Capsule Switching. Journal of Clinical Microbiology. 2005;43(3727-3733):3727-33.
52. Sorensen UB, Poulsen K, Ghezzo C, Margarit I, Kilian M. Emergence and global dissemination of host-specific Streptococcus agalactiae clones. mBio. 2010;1(3).
53. Da Cunha V, Davies MR, Douarre PE, Rosinski-Chupin I, Margarit I, Spinali S, et al. Streptococcus agalactiae clones infecting humans were selected and fixed through the extensive use of tetracycline. Nat Commun. 2014;5:4544.
54. Manning SD, Springman AC, Lehotzky E, Lewis MA, Whittam TS, Davies HD. Multilocus Sequence Types Associated with Neonatal Group B Streptococcal Sepsis and Meningitis in Canada. Journal of Clinical Microbiology. 2009;47:1143-8.
55. Jones N, Bohnsack JF, Takahashi S, Oliver KA, Chan MS, Kunst F, et al. Multilocus sequence typing system for group B streptococcus. J Clin Microbiol. 2003;41(6):2530-6.
56. Bisharat N, Crook DW, Leigh J, Harding RM, Ward PN, Coffey TJ, et al. Hypersensitive neonatal group B Streptococcus has arisen from a bovine ancestor. J Clin Microbiol. 2004;42(5):2161-7.

57. Marques MB, Kasper DL, Pangburn MK, Wessels MR. Prevention of C3 deposition by capsular polysaccharide is a virulence mechanism of type III group B streptococci. Infection and Immunity. 1992;60(10):3986-93.
58. Lindahl G, Stålhammar-Carlemalm M, Areschoug T. Surface Proteins of *Streprococcus agalactiae* and Related Proteins in Other Bacterial Pathogens Clinical Microbiology Reviews 2005;18(1):102-27.
59. Slotved H-C, Kong F, Lambertsen L, Sauer S, Gilbert GL. Serotype IX, a Proposed New Streptococcus agalactiae Serotype. Journal of Clinical Microbiology. 2007 45(9):2929-36.
60. Spellerberg B. Pathogenesis of neonatal *Streptococcus agalactiae* infections. Microbes and Infection. 2000;2(14):1733-42.
61. Kogan G, Uhrin D, Brisson JR, Paoletti L, Boldgett AE, Kasper DL, et al. Structural and immunochemical characterisation of the type VIII group B streptococcus polysaccharide Journal of Biological Chemistry 1996;271:8786-90.
62. Hunolstein Cv, D'Ascenzi S, Wagner B, Jelínková J, Alfarone G, Recchia S, et al. Immunochemistry of capsular type polysaccharide and virulence properties of type VI streptococcus agalactiae (group B streptococci). Infection and Immunity. 1993;61(4):1272-80.
63. Shigeoka AO, Rote NS, Santos JI, Hill HR. Assessment of the virulence factors of Group G Streptococci: Correlation with sialic acid content The Journal of Infectious Disease. 1983;147(5):857-63.
64. Holm SE, Bergholm AM, Wagner B, Wagner M. A sialic-acid-specific lectin from *Cepaea hortensis* that promotes phagocytosis of a group-b, type-Ia, streptococcal strain. Journal of Medical Microbiology. 1985;19(3):317-23.
65. Wessels MR, Rubens CE, Benedi VJ, Kasper DL. Definition of a bacterial virulence factor: sialylation of the group B streptococcal capsule. Proc Natl Acad Sci U S A. 1989;86(22):8983-7.
66. Wessels MR, Haft RF, Heggen LM, Rubens CE. Identification of a genetic locus essential for capsule sialylation in type III group B streptococcus Infection and Immunity. 1992;60:392-400.
67. Kuypers JM, Heggen LM, Rubens CE. Molecular analysis of a region of the group B streptococcus chromosome involved in type III capsule expression. Infect Immun. 1989;57(10):3058-65.
68. Barcaite E, Bartusevivius A, Tameliene R, Kliucinskas M, Maleckiene L, Nadisauskiene R. Prevalence of maternal group B streptococcal colonisation in European countries. Acta Obstetricia et Gynecologica Scandinavica. 2008;87(3):260-71.
69. Diedrick MJ, Flores AE, Hillier SL, Creti R, Ferrieri P. Clonal Analysis of Colonizing Group B Streptococcus, Serotype IV, an Emerging Pathogen in the United States. Journal of Clinical Microbiology. 2010;48(9):3100-4.
70. Madzivhandila M, Adrian PV, Cutland CL, Kuwanda L, Schrag SJ, Madhi SA. Serotype Distribution and Invasive Potential of Group B Streptococcus Isolates Causing Disease in Infants and Colonizing Maternal-Newborn Dyads. PLOSone 2011;6(3).
71. Tazi A, Disson O, Bellais S, Bouaboud A, Dmytruk N, Dramsi S, et al. The surface protein HvgA mediates group B streptococcus hypervirulence and meningeal tropism in neonates. Journal of Experimental Medicine. 2010;207(11):2313-22.
72. Doran KS, Chang JC, Benoit VM, Eckmann L, Nizet V. Group B streptococcal beta-hemolysin/cytolysin promotes invasion of human lung epithelial cells and the release of interleukin-8. J Infect Dis. 2002;185(2):196-203.

73. Kulkarni R, Randis TM, Antala S, Wang A, Amaral FE, Ratner AJ. beta-Hemolysin/cytolysin of Group B Streptococcus enhances host inflammation but is dispensable for establishment of urinary tract infection. PLoS One. 2013;8(3):e59091.
74. Ulett GC, Webb RI, Ulett KB, Cui X, Benjamin WH, Crowley M, et al. Group B Streptococcus (GBS) urinary tract infection involves binding of GBS to bladder uroepithelium and potent but GBS-specific induction of interleukin 1alpha. J Infect Dis. 2010;201(6):866-70.
75. Patras KA, Wang NY, Fletcher EM, Cavaco CK, Jimenez A, Garg M, et al. Group B Streptococcus CovR regulation modulates host immune signalling pathways to promote vaginal colonization. Cell Microbiol. 2013;15(7):1154-67.
76. Leclercq SY, Sullivan MJ, Ipe DS, Smith JP, Cripps AW, Ulett GC. Pathogenesis of Streptococcus urinary tract infection depends on bacterial strain and β-hemolysin/cytolysin that mediates cytotoxicity, cytokine synthesis, inflammation and virulence Scientific reports 2016.
77. Fettucciari K, Rosati E, Scaringi L, Cornacchione P, Migliorati G, Sabatini R, et al. Group B Streptococcus induces apoptosis in macrophages. J Immunol. 2000;165(7):3923-33.
78. Ulett GC, Bohnsack JF, Armstrong J, Adderson EE. Beta-hemolysin-independent induction of apoptosis of macrophages infected with serotype III group B streptococcus. J Infect Dis. 2003;188(7):1049-53.
79. Cheng Q, Stafslien D, Purushothaman SS, Cleary P. The group B streptococcal C5a peptidase is both a specific protease and an invasin. Infection and Immunity. 2002;70(5):2480-13.
80. Tettelin H, Masignani V, Cieslewicz MJ, Donati C, Ward NL, Angiuoli SV, et al. Genome analysis of multiple pathogenic isolates of Streptococcus agalactiae: implications for the microbial "pan-genome". Proceedings of the National Academy of Science of the United States of America 2005;102(39):13950-5.
81. Wessels MR, Paoletti L, Rodewald A, Michon F, DiFablo J, Jennings H, et al. Stimulation of protective antibodies against type Ia and Ib group B streptococci by a type Ia polysaccharide-tetanus toxoid conjugate vaccine. Infection and Immunity. 1993;61(11):4760-6.
82. Rosinski-Chupin I, Sauvage E, Mairey B, Mangenot S, Ma L, Da Cunha V, et al. Reductive evolution in Streptococcus agalactiae and the emergence of a host adapted lineage. BMC Genomics 2012;14(1):252.
83. Jiang S, Wessels MR. BsaB, a Novel Adherence Factor of Group B Streptococcus Infection and Immunity. 2014;82(3):1007-16.
84. Santi I, Scarselli M, Mariani M, Pezzicoli A, Masignani V, Taddei A, et al. BibA: a novel immunogenic bacterial adhesin contributing to group B Streptococcus survival in human blood. Molecular Microbiology. 2007;63(3):754-67.
85. Beckmann C. Identification of Novel Adhesins from Group B Streptococci by Use of Phage Display Reveals that C5a Peptidase Mediates Fibronectin Binding. Infection and Immunity. 2002;70(6):2869-76.
86. Landwehr-Kenzel S, Henneke P. Interaction of Streptococcus agalactiae and Cellular Innate Immunity in Colonization and Disease. Front Immunol. 2014;5:519.
87. Al Safadi R, Mereghetti L, Salloum M, Lartigue MF, Virlogeux-Payant I, Quentin R, et al. Two-component system RgfA/C activates the fbsB gene encoding major fibrinogen-binding protein in highly virulent CC17 clone group B Streptococcus. PLoS One. 2011;6(2):e14658.
88. Schubert A, Zakikhany K, Schreiner M, Frank R, Spellerberg B, Eikmanns BJ, et al. A fibrinogen receptor from group B Streptococcus interacts with fibrinogen by repetitive units with novel ligand binding sites. Mol Microbiol. 2002;46(2):557-69.

89. Gutekunst H, Eikmanns BJ, Reinscheid DJ. The novel fibrinogen-binding protein FbsB promotes Streptococcus agalactiae invasion into epithelial cells. Infect Immun. 2004;72(6):3495-504.
90. Seo HS, Minasov G, Seepersaud R, Doran KS, Dubrovska I, Shuvalova L, et al. Characterization of fibrinogen binding by glycoproteins Srr1 and Srr2 of Streptococcus agalactiae. J Biol Chem. 2013;288(50):35982-96.
91. Seo HS, Mu R, Kim BJ, Doran KS, Sullam PM. Binding of glycoprotein Srr1 of Streptococcus agalactiae to fibrinogen promotes attachment to brain endothelium and the development of meningitis. PLoS Pathog. 2012;8(10):e1002947.
92. Buscetta M, Papasergi S, Firon A, Pietrocola G, Biondo C, Mancuso G, et al. FbsC, a novel fibrinogen-binding protein, promotes Streptococcus agalactiae-host cell interactions. J Biol Chem. 2014;289(30):21003-15.
93. Kang HJ, Coulibaly F, Clow F, Proft T, Baker EN. Stabilizing isopeptide bonds revealed in gram-positive bacterial pilus structure. Science. 2007;318(5856):1625-8.
94. Wu H, Fives-Taylor PM. Molecular strategies for fimbrial expression and assembly. Crit Rev Oral Biol Med. 2001;12(2):101-15.
95. Telford JL, Barocchi MA, Margarit I, Rappuoli R, Grandi G. Pili in gram-positive pathogens. Nat Rev Microbiol. 2006;4(7):509-19.
96. Lauer P, Rinaudo CD, Soriani M, Margarit I, Maione D, Rosini R, et al. Genome analysis reveals pili in Group B Streptococcus. Science. 2005;309(5731):105.
97. Rosini R, Rinaudo CD, Soriani M, Lauer P, Mora M, Maione D, et al. Identification of novel genomic islands coding for antigenic pilus-like structures in Streptococcus agalactiae. Mol Microbiol. 2006;61(1):126-41.
98. Maisey HC, Quach D, Hensler ME, Liu GY, Gallo RL, Nizet V, et al. A group B streptococcal pilus protein promotes phagocyte resistance and systemic virulence. FASEB J. 2008;22(6):1715-24.
99. Dramsi S, Caliot E, Bonne I, Guadagnini S, Prevost MC, Kojadinovic M, et al. Assembly and role of pili in group B streptococci. Mol Microbiol. 2006;60(6):1401-13.
100. Rinaudo C, Rosini R, Galeotti C, Berti F, Necchi F, Reguzzi V, et al. Specific involvement of pilus type 2a in biofilm formation in group B streptococcus PLOSone. 2010;5(2):e9216.
101. Margarit I, Rinaudo CD, Galeotti CL, Maione D, Ghezzo C, Buttazzoni E, et al. Preventing bacterial infections with pilus-based vaccines: the group B streptococcus paradigm. J Infect Dis. 2009;199(1):108-15.
102. Martins ER, Andreu A, Melo-Cristino J, Ramirez M. Distribution of pilus islands in Streptococcus agalactiae that cause human infections: insights into evolution and implication for vaccine development. Clin Vaccine Immunol. 2013;20(2):313-6.
103. Alvim D, Ferreira AFM, Leal MA, Oliveira LMA, Botelho AMN, Botelho ACN, et al. Biofilm production and distribution of pilus variants among Streptococcus agalactiae isolated from human and animal sources. Biofouling. 2019;35(8):938-44.
104. Parker RE, Laut C, Gaddy JA, Zadoks RN, Davies HD, Manning SD. Association between genotypic diversity and biofilm production in group B Streptococcus. BMC Microbiol. 2016;16(86):86.
105. Pang M, Sun L, He T, Bao H, Zhang L, Zhou Y, et al. Molecular and virulence characterization of highly prevalent Streptococcus agalactiae circulated in bovine dairy herds. Vet Res. 2017;48(1):65.

106. Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: from the natural environment to infectious diseases. Nat Rev Microbiol. 2004;2(2):95-108.
107. Sutherland IW. The biofilm matrix--an immobilized but dynamic microbial environment. Trends Microbiol. 2001;9(5):222-7.
108. Adal KA, Farr BM. Central venous catheter-related infections: A review Nutrition 1996;12(3):208-13.
109. Jefferson KK. What drives bacteria to produce a biofilm? FEMS Microbiology Letters. 2004;236(2):163-73.
110. Miller MB, Bassler BL. Quorum sensing in bacteria. Annu Rev Microbiol. 2001;55:165-99.
111. Hall-Stoodley L, Stoodley P. Evolving concepts in biofilm infections. Cell Microbiol. 2009;11(7):1034-43.
112. Kleerebezem M, Quadri LE, Kuipers OP, de Vos WM. Quorum sensing by peptide pheromones and two-component signal-transduction systems in Gram-positive bacteria. Mol Microbiol. 1997;24(5):895-904.
113. Jefferson KK, Goldmann DA, Pier GB. Use of confocal microscopy to analyze the rate of vancomycin penetration through Staphylococcus *aureus* biofilms. Antimicrob Agents Chemother. 2005;49(6):2467-73.
114. Ho Y-R, Li C-M, Yu C-H, Lin Y-J, Wu C-M, Harn I-C, et al. The enhancement of biofilm formation in Group B streptococcal isolates at vaginal pH. Medical Microbiology anf Immunology 2012;202(2):105-15.
115. Pazzicoli A, Santi I, Lauer P, Rosini R, Rinaudo D, Grandi G, et al. Pilus Backbone Contributes to Group B *Streptococcus* Paracellular Translocation through Epithelial cells The Journal of Infectious Disease. 2008;198(6):890-8.
116. Tamura GS, Kuypers JM, Smith S, Raff H, Rubens CE. Adherence of group B streptococci to cultured epithelial cells: roles of environmental factos and bacterial surface components Infection and Immunity. 1994;62(6):2450-8.
117. Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev. 2002;15(2):167-93.
118. Borges S, Silva J, Teixeira P. Survival and biofilm formation by Group B streptococci in simulated vaginal fluid at different pHs. Antonie Van Leeuwenhoek. 2012;101(3):677-82.
119. Yang Q, Porter AJ, Zhang M, Harrington DJ, Black GW, Sutcliffe IC. The impact of pH and nutrient stress on the growth and survival of Streptococcus agalactiae Antonie van Leeuwenhoek. 2012;102(2):277-87.
120. Ho Y-R, Li C-M, Yu C-H, Lin Y-J, Wu C-M, Harn I-C, et al. The enhancement of biofilm formation in Group B streptococcal isolates at vaginal pH. Medical Microbiology anf Immunology. 2012;202(2):105-15.
121. Rajagopal L, Vo A, Silvestroni A, Rubens CE. Regulation of cytotoxin expression by converging eukaryotic-type and two-component signalling mechanisms in *Streptococcus agalactiae*. Mol Microbiol. 2006;62(4):941-57.
122. Spellerberg B, Pohl B, Haase G, Martin S, Weber-Heynemann J, Lutticken R. Identification of genetic determinants for the hemolytic activity of Streptococcus agalactiae by ISS1 transposition. J Bacteriol. 1999;181(10):3212-9.
123. Konto-Ghiorghi Y, Mairey E, Mallet A, Dumenil G, Caliot E, Trieu-Cuot P, et al. Dual role for pilus in adherence to epithelial cells and biofilm formation in Streptococcus agalactiae. PLoS Pathog. 2009;5(5):e1000422.

124. Santi I, Grifantini R, Jiang SM, Brettoni C, Grandi G, Wessels MR, et al. CsrRS regulates group B Streptococcus virulence gene expression in response to environmental pH: a new perspective on vaccine development. J Bacteriol. 2009;191(17):5387-97.
125. Battat E, Peleg Y, Bercovitz A, Rokem JS, Goldberg I. Optimization of L-malic acid production by Aspergillus flavus in a stirred fermentor. Biotechnol Bioeng. 1991;37(11):1108-16.
126. Vishwakarma P, Lotspeich WD. The excretion of I-malic acid in relation to the tricarboxylic acid cycle in the kidney. J Clin Invest. 1959;38(2):414-23.
127. Kawai S, Suzuki H, Yamamoto K, Inui M, Yukawa H, Kumagai H. Purification and characterization of malic enzyme from the ruminal bacterium Streptococcus bovis ATCC 15352 and cloning and seqencing of its genes. Appl Environ Microbiol. 1996;62(8):2692-700.
128. Kleijn RJ, Buescher JM, Le Chat L, Jules M, Aymerich S, Sauer U. Metabolic fluxes during strong carbon catabolite repression by malate in Bacillus subtilis. J Biol Chem. 2010;285(3):1587-96.
129. Landete JM, Garcia-Haro L, Blasco A, Manzanares P, Berbegal C, Monedero V, et al. Requirement of the Lactobacillus casei MaeKR two-component system for L-malic acid utilization via a malic enzyme pathway. Appl Environ Microbiol. 2010;76(1):84-95.
130. Ezraty B, Henry C, Herisse M, Denamur E, Barras F. Commercial Lysogeny Broth culture media and oxidative stress: a cautious tale. Free Radic Biol Med. 2014;74(245-251):245-51.
131. Ipe DS, Horton E, Ulett GC. The Basics of Bacteriuria: Strategies of Microbes for Persistence in Urine. Front Cell Infect Microbiol. 2016;6:14.
132. Jiang S, Wessels MR. BsaB, a Novel Adherence Factor of Group B Streptococcus. Infection and Immunity. 2014;82(3):1007-16.
133. Manetti AG, Zingaretti C, Falugi F, Capo S, Bombaci M, Bagnoli F, et al. Streptococcus pyogenes pili promote pharyngeal cell adhesion and biofilm formation. Mol Microbiol. 2007;64(4):968-83.
134. Rinaudo C, Rosini R, Galeotti C, Berti F, Necchi F, Reguzzi V, et al. Specific involvement of pilus type 2a in biofilm formation in group B streptococcus. PLOSone. 2010;5(2):e9216.
135. Jiang SM, Cieslewicz MJ, Kasper DL, Wessels MR. Regulation of virulence by a two-component system in group B streptococcus. J Bacteriol. 2005;187(3):1105-13.
136. Jiang SM, Ishmael N, Dunning Hotopp J, Puliti M, Tissi L, Kumar N, et al. Variation in the group B Streptococcus CsrRS regulon and effects on pathogenicity. J Bacteriol. 2008;190(6):1956-65.
137. Lamy MC, Zouine M, Fert J, Vergassola M, Couve E, Pellegrini E, et al. CovS/CovR of group B streptococcus: a two-component global regulatory system involved in virulence. Mol Microbiol. 2004;54(5):1250-68.
138. Park SE, Jiang S, Wessels MR. CsrRS and environmental pH regulate group B streptococcus adherence to human epithelial cells and extracellular matrix. Infect Immun. 2012;80(11):3975-84.
139. Willoughby K. The ABC of PCR. In Practice. 2003;25(3):140-5.
140. Rosa-Fraile M, Dramsi S, Spellerberg B. Group B streptococcal haemolysin and pigment, a tale of twins. FEMS Microbiol Rev. 2014;38(5):932-46.

141. Leclercq SY, Sullivan MJ, Ipe DS, Smith JP, Cripps AW, Ulett GC. Pathogenesis of Streptococcus urinary tract infection depends on bacterial strain and β-hemolysin/cytolysin that mediates cytotoxicity, cytokine synthesis, inflammation and virulence. Scientific reports. 2016.
142. Bebien M, Hensler ME, Davanture S, Hsu LC, Karin M, Park JM, et al. The pore-forming toxin beta hemolysin/cytolysin triggers p38 MAPK-dependent IL-10 production in macrophages and inhibits innate immunity. PLoS Pathog. 2012;8(7):e1002812.
143. Lang S, Palmer M. Characterization of Streptococcus agalactiae CAMP factor as a pore-forming toxin. J Biol Chem. 2003;278(40):38167-73.

9. Appendices

Appendix 1 – Strains used in the study.

Strain	Description	Reference
GBS 874391	Human vaginal isolate; ST17	PMID: 29051249
GBS NEM316	Human neonatal isolate; Serotype III	PMID: 12354221
GBS A909	Human neonatal isolate; Serotype Ia	PMID: 16172379
874391Δ*covR*	874391 with a mutation in the *covR* gene	PMID: 28011914
874391Δ*cylE*	874391 with a mutation in the *cylE* gene	PMID: 28011914
834Δ*maeK*	834 with a mutation in the *maeK* gene	PMID: 26553467
834Δ*maeE*	834 with a mutation in the *maeE* gene	PMID: 26553467
GU0087	Clinical GBS isolate; Recurrent infection	This study
GU0127	Clinical GBS isolate; Recurrent infection	This study
GU0155	Clinical GBS isolate; Recurrent infection	This study
GU0170	Clinical GBS isolate; Recurrent infection	This study
GU0189	Clinical GBS isolate; Recurrent infection	This study
GU0216	Clinical GBS isolate; Recurrent infection	This study
GU0289	Clinical GBS isolate; Recurrent infection	This study
GU0298	Clinical GBS isolate; Recurrent infection	This study
GU0346	Clinical GBS isolate; Recurrent infection	This study
GU0359	Clinical GBS isolate; Recurrent infection	This study
GU0392	Clinical GBS isolate; Recurrent infection	This study
GU0529	Clinical GBS isolate; Recurrent infection	This study
GU0555	Clinical GBS isolate; Recurrent infection	This study
GU0602	Clinical GBS isolate; Recurrent infection	This study
GU0647	Clinical GBS isolate; Recurrent infection	This study
GU0725	Clinical GBS isolate; Recurrent infection	This study
GU0984	Clinical GBS isolate; Recurrent infection	This study
GU0994	Clinical GBS isolate; Recurrent infection	This study
GU1028	Clinical GBS isolate; Recurrent infection	This study
GU1201	Clinical GBS isolate; Recurrent infection	This study
GU1255	Clinical GBS isolate; Recurrent infection	This study
GU1258	Clinical GBS isolate; Recurrent infection	This study
GU1309	Clinical GBS isolate; Recurrent infection	This study
GU1317	Clinical GBS isolate; Recurrent infection	This study
GU1318	Clinical GBS isolate; Recurrent infection	This study
GU1319	Clinical GBS isolate; Recurrent infection	This study

GU1326	Clinical GBS isolate; Recurrent infection	This study
GU1327	Clinical GBS isolate; Recurrent infection	This study
GU1338	Clinical GBS isolate; Recurrent infection	This study
GU1344	Clinical GBS isolate; Recurrent infection	This study
GU1347	Clinical GBS isolate; Recurrent infection	This study
GU1370	Clinical GBS isolate; Recurrent infection	This study
GU1375	Clinical GBS isolate; Recurrent infection	This study
GU1376	Clinical GBS isolate; Recurrent infection	This study
GU1381	Clinical GBS isolate; Recurrent infection	This study
GU1383	Clinical GBS isolate; Recurrent infection	This study
GU1384	Clinical GBS isolate; Recurrent infection	This study
GU1388	Clinical GBS isolate; Recurrent infection	This study
GU1400	Clinical GBS isolate; Recurrent infection	This study
GU1407	Clinical GBS isolate; Recurrent infection	This study
GU1501	Clinical GBS isolate; Recurrent infection	This study
GU1504	Clinical GBS isolate; Recurrent infection	This study
GU1511	Clinical GBS isolate; Recurrent infection	This study
GU1524	Clinical GBS isolate; Recurrent infection	This study
GU1552	Clinical GBS isolate; Recurrent infection	This study
GU1559	Clinical GBS isolate; Recurrent infection	This study
GU1569	Clinical GBS isolate; Recurrent infection	This study
GU0002	Clinical GBS isolate; Asymptomatic infection	This study
GU0013	Clinical GBS isolate; Asymptomatic infection	This study
GU0014	Clinical GBS isolate; Asymptomatic infection	This study
GU0016	Clinical GBS isolate; Asymptomatic infection	This study
GU0017	Clinical GBS isolate; Asymptomatic infection	This study
GU0020	Clinical GBS isolate; Asymptomatic infection	This study
GU0024	Clinical GBS isolate; Asymptomatic infection	This study
GU0030	Clinical GBS isolate; Asymptomatic infection	This study
GU0032	Clinical GBS isolate; Asymptomatic infection	This study
GU0035	Clinical GBS isolate; Asymptomatic infection	This study
GU0040	Clinical GBS isolate; Asymptomatic infection	This study
GU0049	Clinical GBS isolate; Asymptomatic infection	This study
GU0053	Clinical GBS isolate; Asymptomatic infection	This study
GU0055	Clinical GBS isolate; Asymptomatic infection	This study
GU0072	Clinical GBS isolate; Asymptomatic infection	This study
GU0076	Clinical GBS isolate; Asymptomatic infection	This study
GU0088	Clinical GBS isolate; Asymptomatic infection	This study
GU0097	Clinical GBS isolate; Asymptomatic infection	This study

GU0100	Clinical GBS isolate; Asymptomatic infection	This study
GU0102	Clinical GBS isolate; Asymptomatic infection	This study
GU0105	Clinical GBS isolate; Asymptomatic infection	This study
GU0113	Clinical GBS isolate; Asymptomatic infection	This study
GU0117	Clinical GBS isolate; Asymptomatic infection	This study
GU0123	Clinical GBS isolate; Asymptomatic infection	This study
GU0126	Clinical GBS isolate; Asymptomatic infection	This study
GU0129	Clinical GBS isolate; Asymptomatic infection	This study
GU0153	Clinical GBS isolate; Asymptomatic infection	This study
GU0157	Clinical GBS isolate; Asymptomatic infection	This study
GU0167	Clinical GBS isolate; Asymptomatic infection	This study
GU0179	Clinical GBS isolate; Asymptomatic infection	This study
GU0187	Clinical GBS isolate; Asymptomatic infection	This study
GU0188	Clinical GBS isolate; Asymptomatic infection	This study
GU0197	Clinical GBS isolate; Asymptomatic infection	This study
GU0209	Clinical GBS isolate; Asymptomatic infection	This study
GU0210	Clinical GBS isolate; Asymptomatic infection	This study
GU0211	Clinical GBS isolate; Asymptomatic infection	This study
GU0218	Clinical GBS isolate; Asymptomatic infection	This study
GU0228	Clinical GBS isolate; Asymptomatic infection	This study
GU0232	Clinical GBS isolate; Asymptomatic infection	This study
GU0235	Clinical GBS isolate; Asymptomatic infection	This study
GU0237	Clinical GBS isolate; Asymptomatic infection	This study
GU0243	Clinical GBS isolate; Asymptomatic infection	This study
GU0250	Clinical GBS isolate; Asymptomatic infection	This study
GU0252	Clinical GBS isolate; Asymptomatic infection	This study
GU0259	Clinical GBS isolate; Asymptomatic infection	This study
GU0333	Clinical GBS isolate; Asymptomatic infection	This study
GU0387	Clinical GBS isolate; Asymptomatic infection	This study
GU0409	Clinical GBS isolate; Asymptomatic infection	This study
GU0422	Clinical GBS isolate; Asymptomatic infection	This study
GU0423	Clinical GBS isolate; Asymptomatic infection	This study
GU0426	Clinical GBS isolate; Asymptomatic infection	This study
GU0440	Clinical GBS isolate; Asymptomatic infection	This study
GU0443	Clinical GBS isolate; Asymptomatic infection	This study
GU0074	Clinical GBS isolate; Asymptomatic infection	This study
GU0114	Clinical GBS isolate; Asymptomatic infection	This study
GU0115	Clinical GBS isolate; Asymptomatic infection	This study
GU0121	Clinical GBS isolate; Asymptomatic infection	This study

GU0178	Clinical GBS isolate; Asymptomatic infection	This study
GU0204	Clinical GBS isolate; Asymptomatic infection	This study
GU0205	Clinical GBS isolate; Asymptomatic infection	This study
GU0264	Clinical GBS isolate; Asymptomatic infection	This study
GU0267	Clinical GBS isolate; Asymptomatic infection	This study
GU0286	Clinical GBS isolate; Asymptomatic infection	This study
GU0290	Clinical GBS isolate; Asymptomatic infection	This study
GU0318	Clinical GBS isolate; Asymptomatic infection	This study
GU0332	Clinical GBS isolate; Asymptomatic infection	This study
GU0337	Clinical GBS isolate; Asymptomatic infection	This study
GU0344	Clinical GBS isolate; Asymptomatic infection	This study
GU0345	Clinical GBS isolate; Asymptomatic infection	This study
GU0347	Clinical GBS isolate; Asymptomatic infection	This study
GU0348	Clinical GBS isolate; Asymptomatic infection	This study
GU0349	Clinical GBS isolate; Asymptomatic infection	This study
GU0469	Clinical GBS isolate; Asymptomatic infection	This study
GU0471	Clinical GBS isolate; Asymptomatic infection	This study
GU0473	Clinical GBS isolate; Asymptomatic infection	This study
GU0481	Clinical GBS isolate; Asymptomatic infection	This study
GU0482	Clinical GBS isolate; Asymptomatic infection	This study
GU0507	Clinical GBS isolate; Asymptomatic infection	This study
GU0509	Clinical GBS isolate; Asymptomatic infection	This study
GU0516	Clinical GBS isolate; Asymptomatic infection	This study
GU0567	Clinical GBS isolate; Asymptomatic infection	This study
GU0569	Clinical GBS isolate; Asymptomatic infection	This study
GU0575	Clinical GBS isolate; Asymptomatic infection	This study
GU0582	Clinical GBS isolate; Asymptomatic infection	This study
GU0594	Clinical GBS isolate; Asymptomatic infection	This study
GU0617	Clinical GBS isolate; Asymptomatic infection	This study
GU0627	Clinical GBS isolate; Asymptomatic infection	This study
GU0636	Clinical GBS isolate; Asymptomatic infection	This study
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GU0667	Clinical GBS isolate; Asymptomatic infection	This study
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GU0689	Clinical GBS isolate; Asymptomatic infection	This study
GU0728	Clinical GBS isolate; Asymptomatic infection	This study
GU0729	Clinical GBS isolate; Asymptomatic infection	This study
GU0731	Clinical GBS isolate; Asymptomatic infection	This study

GU0737	Clinical GBS isolate; Asymptomatic infection	This study
GU0743	Clinical GBS isolate; Asymptomatic infection	This study
GU0745	Clinical GBS isolate; Asymptomatic infection	This study
GU0780	Clinical GBS isolate; Asymptomatic infection	This study
GU0802	Clinical GBS isolate; Asymptomatic infection	This study
GU0803	Clinical GBS isolate; Asymptomatic infection	This study
GU0806	Clinical GBS isolate; Asymptomatic infection	This study
GU0810	Clinical GBS isolate; Asymptomatic infection	This study
GU0824	Clinical GBS isolate; Asymptomatic infection	This study
GU0828	Clinical GBS isolate; Asymptomatic infection	This study
GU0834	Clinical GBS isolate; Asymptomatic infection	This study
GU0838	Clinical GBS isolate; Asymptomatic infection	This study
GU0839	Clinical GBS isolate; Asymptomatic infection	This study
GU0842	Clinical GBS isolate; Asymptomatic infection	This study
GU0860	Clinical GBS isolate; Asymptomatic infection	This study
GU0880	Clinical GBS isolate; Asymptomatic infection	This study
GU0885	Clinical GBS isolate; Asymptomatic infection	This study
GU0887	Clinical GBS isolate; Asymptomatic infection	This study
GU0898	Clinical GBS isolate; Asymptomatic infection	This study
GU0901	Clinical GBS isolate; Asymptomatic infection	This study
GU0910	Clinical GBS isolate; Asymptomatic infection	This study
GU0912	Clinical GBS isolate; Asymptomatic infection	This study
GU0916	Clinical GBS isolate; Asymptomatic infection	This study
GU0919	Clinical GBS isolate; Asymptomatic infection	This study
GU0920	Clinical GBS isolate; Asymptomatic infection	This study
GU0927	Clinical GBS isolate; Asymptomatic infection	This study
GU0931	Clinical GBS isolate; Asymptomatic infection	This study
GU0935	Clinical GBS isolate; Asymptomatic infection	This study
GU0936	Clinical GBS isolate; Asymptomatic infection	This study
GU0939	Clinical GBS isolate; Asymptomatic infection	This study
GU0941	Clinical GBS isolate; Asymptomatic infection	This study
GU0942	Clinical GBS isolate; Asymptomatic infection	This study
GU0943	Clinical GBS isolate; Asymptomatic infection	This study
GU0944	Clinical GBS isolate; Asymptomatic infection	This study
GU0945	Clinical GBS isolate; Asymptomatic infection	This study
GU0946	Clinical GBS isolate; Asymptomatic infection	This study
GU0947	Clinical GBS isolate; Asymptomatic infection	This study
GU0948	Clinical GBS isolate; Asymptomatic infection	This study
GU0949	Clinical GBS isolate; Asymptomatic infection	This study

GU0951	Clinical GBS isolate; Asymptomatic infection	This study
GU0956	Clinical GBS isolate; Asymptomatic infection	This study
GU0957	Clinical GBS isolate; Asymptomatic infection	This study
GU0960	Clinical GBS isolate; Asymptomatic infection	This study
GU0961	Clinical GBS isolate; Asymptomatic infection	This study
GU0963	Clinical GBS isolate; Asymptomatic infection	This study
GU0967	Clinical GBS isolate; Asymptomatic infection	This study
GU0971	Clinical GBS isolate; Asymptomatic infection	This study
GU0973	Clinical GBS isolate; Asymptomatic infection	This study
GU0974	Clinical GBS isolate; Asymptomatic infection	This study
GU0976	Clinical GBS isolate; Asymptomatic infection	This study
GU0979	Clinical GBS isolate; Asymptomatic infection	This study
GU0982	Clinical GBS isolate; Asymptomatic infection	This study
GU0983	Clinical GBS isolate; Asymptomatic infection	This study
GU0986	Clinical GBS isolate; Asymptomatic infection	This study
GU0987	Clinical GBS isolate; Asymptomatic infection	This study
GU0988	Clinical GBS isolate; Asymptomatic infection	This study
GU0991	Clinical GBS isolate; Asymptomatic infection	This study
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GU0997	Clinical GBS isolate; Asymptomatic infection	This study
GU0999	Clinical GBS isolate; Asymptomatic infection	This study
GU1000	Clinical GBS isolate; Asymptomatic infection	This study
GU1004	Clinical GBS isolate; Asymptomatic infection	This study
GU1005	Clinical GBS isolate; Asymptomatic infection	This study
GU1008	Clinical GBS isolate; Asymptomatic infection	This study
GU1009	Clinical GBS isolate; Asymptomatic infection	This study
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GU1013	Clinical GBS isolate; Asymptomatic infection	This study
GU1014	Clinical GBS isolate; Asymptomatic infection	This study
GU1016	Clinical GBS isolate; Asymptomatic infection	This study
GU1019	Clinical GBS isolate; Asymptomatic infection	This study
GU1023	Clinical GBS isolate; Asymptomatic infection	This study
GU1026	Clinical GBS isolate; Asymptomatic infection	This study
GU1027	Clinical GBS isolate; Asymptomatic infection	This study
GU1030	Clinical GBS isolate; Asymptomatic infection	This study
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GU1035	Clinical GBS isolate; Asymptomatic infection	This study
GU1037	Clinical GBS isolate; Asymptomatic infection	This study

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GU1069	Clinical GBS isolate; Asymptomatic infection	This study
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GU0091	Clinical GBS isolate; Acute infection	This study
GU0098	Clinical GBS isolate; Acute infection	This study
GU0135	Clinical GBS isolate; Acute infection	This study
GU0140	Clinical GBS isolate; Acute infection	This study
GU0150	Clinical GBS isolate; Acute infection	This study
GU0151	Clinical GBS isolate; Acute infection	This study
GU0152	Clinical GBS isolate; Acute infection	This study
GU0181	Clinical GBS isolate; Acute infection	This study
GU0185	Clinical GBS isolate; Acute infection	This study
GU0220	Clinical GBS isolate; Acute infection	This study
GU0223	Clinical GBS isolate; Acute infection	This study
GU0226	Clinical GBS isolate; Acute infection	This study
GU0247	Clinical GBS isolate; Acute infection	This study
GU0256	Clinical GBS isolate; Acute infection	This study
GU0300	Clinical GBS isolate; Acute infection	This study
GU0352	Clinical GBS isolate; Acute infection	This study
GU0364	Clinical GBS isolate; Acute infection	This study
GU0369	Clinical GBS isolate; Acute infection	This study
GU0371	Clinical GBS isolate; Acute infection	This study

GU0373	Clinical GBS isolate; Acute infection	This study
GU0382	Clinical GBS isolate; Acute infection	This study
GU0402	Clinical GBS isolate; Acute infection	This study
GU0421	Clinical GBS isolate; Acute infection	This study
GU0428	Clinical GBS isolate; Acute infection	This study
GU0441	Clinical GBS isolate; Acute infection	This study
GU0470	Clinical GBS isolate; Acute infection	This study
GU0494	Clinical GBS isolate; Acute infection	This study
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GU0673	Clinical GBS isolate; Acute infection	This study
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GU0714	Clinical GBS isolate; Acute infection	This study
GU0748	Clinical GBS isolate; Acute infection	This study
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GU0761	Clinical GBS isolate; Acute infection	This study
GU0801	Clinical GBS isolate; Acute infection	This study
GU0807	Clinical GBS isolate; Acute infection	This study
GU0872	Clinical GBS isolate; Acute infection	This study
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GU0962	Clinical GBS isolate; Acute infection	This study
GU0977	Clinical GBS isolate; Acute infection	This study
GU1012	Clinical GBS isolate; Acute infection	This study
GU1020	Clinical GBS isolate; Acute infection	This study
GU1048	Clinical GBS isolate; Acute infection	This study
GU1054	Clinical GBS isolate; Acute infection	This study

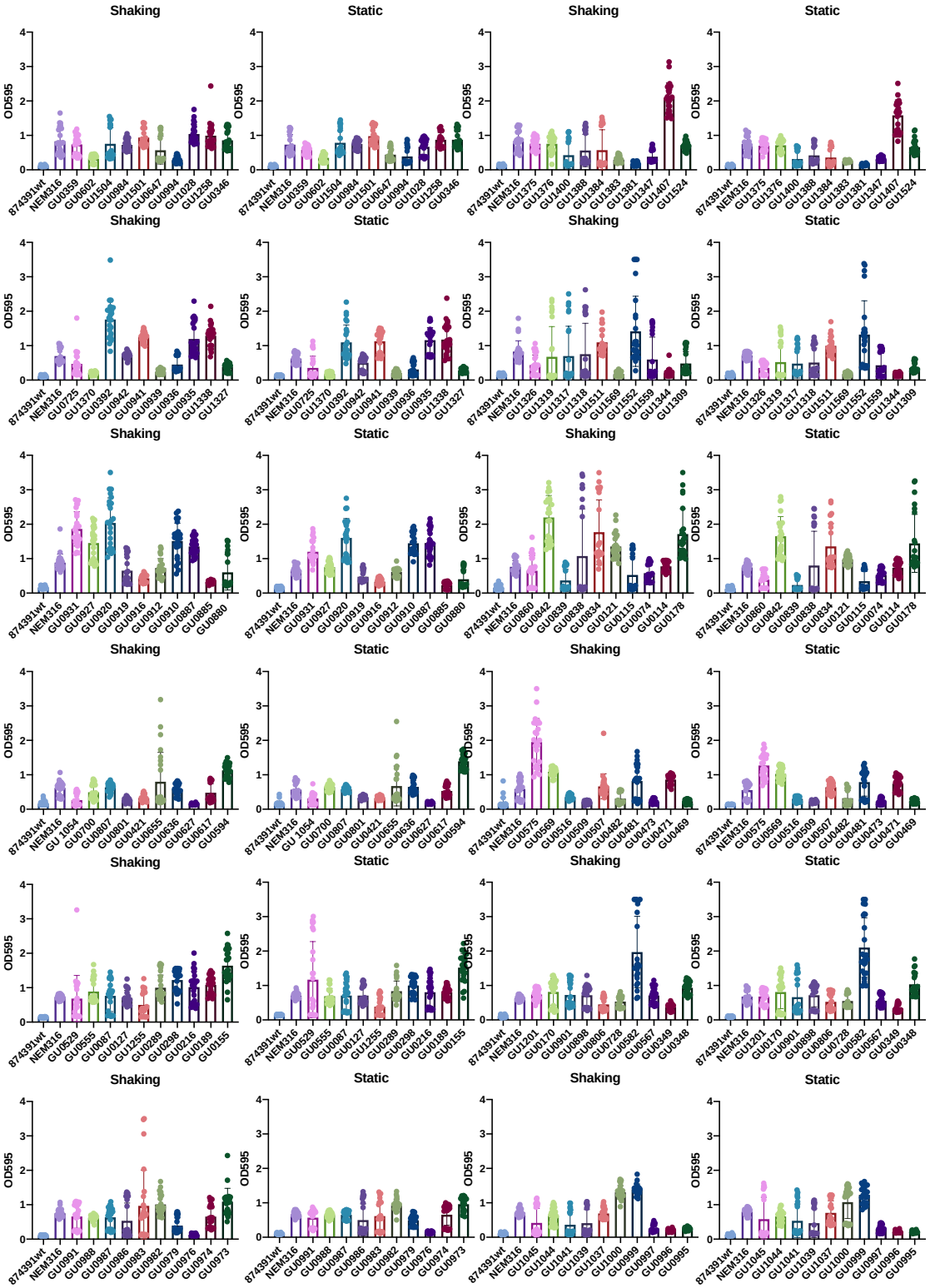
Appendix 2: SPSS output for normality of the data in Figure 2.

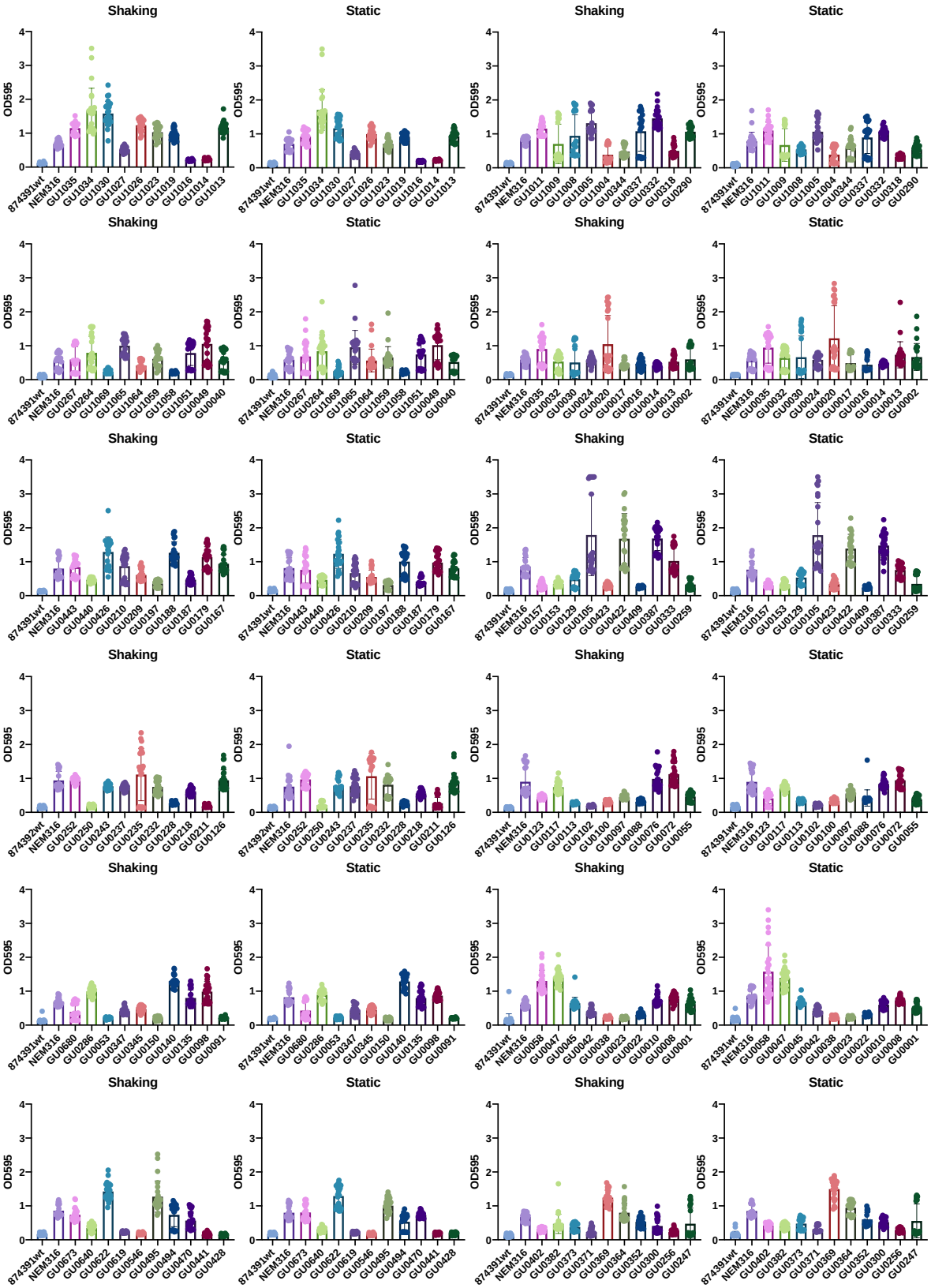
THB	Shaking	N	Valid	6
			Missing	0
		Mean		.15921
		Median		.13513
		Std. Deviation		.060464
		Skewness		.865
		Std. Error of Skewness		.845
		Kurtosis		-1.404
		Std. Error of Kurtosis		1.741
		Minimum		.105
		Maximum		.248
		Percentiles	25	.10919
			50	.13513
			75	.22806
	Static	N	Valid	6
			Missing	0
		Mean		.16258
		Median		.15987
		Std. Deviation		.029036
		Skewness		.208
		Std. Error of Skewness		.845
		Kurtosis		-1.296
		Std. Error of Kurtosis		1.741
		Minimum		.126
		Maximum		.204
		Percentiles	25	.13975
			50	.15987
			75	.18731
THB + 1% glucose	Shaking	N	Valid	6
			Missing	0
		Mean		.32775
		Median		.30232
		Std. Deviation		.170697
		Skewness		.315
		Std. Error of Skewness		.845
		Kurtosis		-1.853

		Std. Error of Kurtosis		1.741
		Minimum		.137
		Maximum		.538
		Percentiles	25	.15900
			50	.30232
			75	.52472
	Static	N	Valid	6
			Missing	0
		Mean		.29296
		Median		.25013
		Std. Deviation		.146608
		Skewness		.831
		Std. Error of Skewness		.845
		Kurtosis		-.699
		Std. Error of Kurtosis		1.741
		Minimum		.161
		Maximum		.525
		Percentiles	25	.16850
			50	.25013
			75	.43125
LB	Shaking	N	Valid	6
			Missing	0
		Mean		.13263
		Median		.12356
		Std. Deviation		.036988
		Skewness		.615
		Std. Error of Skewness		.845
		Kurtosis		-1.421
		Std. Error of Kurtosis		1.741
		Minimum		.099
		Maximum		.188
		Percentiles	25	.09956
			50	.12356
			75	.16872
	Static	N	Valid	6
			Missing	0
		Mean		.13367
		Median		.11919

		Std. Deviation		.029829
		Skewness		1.896
		Std. Error of Skewness		.845
		Kurtosis		3.437
		Std. Error of Kurtosis		1.741
		Minimum		.114
		Maximum		.191
		Percentiles	25	.11575
			50	.11919
			75	.15466
LB + 1% glucose	Shaking	N	Valid	6
			Missing	0
		Mean		.41725
		Median		.35888
		Std. Deviation		.263301
		Skewness		.477
		Std. Error of Skewness		.845
		Kurtosis		-1.786
		Std. Error of Kurtosis		1.741
		Minimum		.138
		Maximum		.776
		Percentiles	25	.17616
			50	.35888
			75	.70609
	Static	N	Valid	6
			Missing	0
		Mean		.34598
		Median		.30075
		Std. Deviation		.187894
		Skewness		.429
		Std. Error of Skewness		.845
		Kurtosis		-2.149
		Std. Error of Kurtosis		1.741
		Minimum		.175
		Maximum		.603
		Percentiles	25	.17756
			50	.30075
			75	.53934

Appendix 3: Graphs of raw data in Figure 4.




Shaking
Static
OD595
874391wt
NEM316
GU1035
GU1034
GU1030
GU1027
GU1026
GU1023
GU1019
GU1016
GU1014
GU1013
GU1011
GU1009
GU1008
GU1005
GU1004
GU0344
GU0337
GU0332
GU0318
GU0290
GU0267
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GU0002
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GU0440
GU0426
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GU0209
GU0197
GU0188
GU0187
GU0179
GU0167
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GU0387
GU0333
GU0259
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GU0250
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GU0211
GU0126
GU0123
GU0117
GU0113
GU0102
GU0100
GU0097
GU0088
GU0076
GU0072
GU0055
GU0680
GU0286
GU0053
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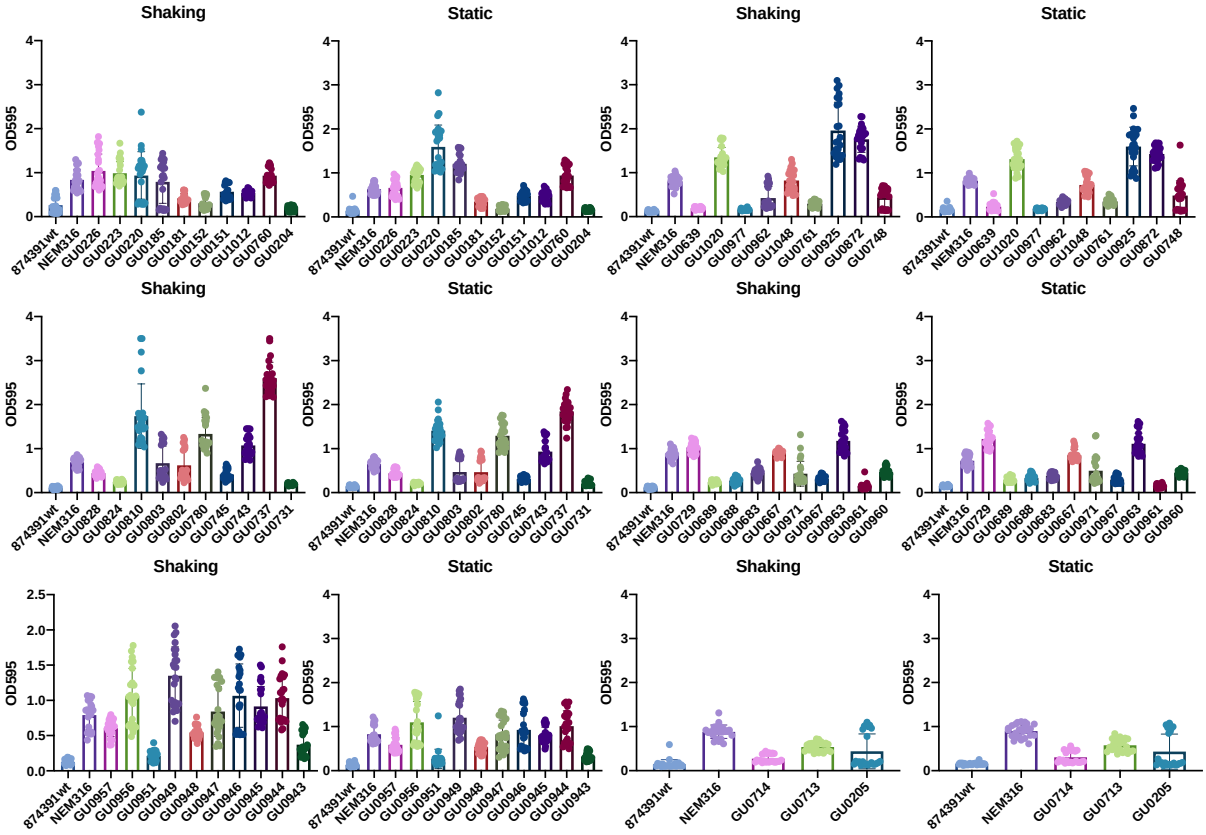
GU0345
GU0150
GU0140
GU0135
GU0098
GU0091
GU0058
GU0047
GU0045
GU0042
GU0038
GU0023
GU0022
GU0010
GU0008
GU0001
GU0673
GU0640
GU0622
GU0619
GU0546
GU0495
GU0494
GU0470
GU0441
GU0428
GU0402
GU0382
GU0373
GU0371
GU0369
GU0364
GU0352
GU0300
GU0256
GU0247


Shaking
Static
Shaking
Static
Shaking
Static
Shaking
Static
Shaking
Static
Shaking
Static
OD595

Appendix 4: SPSS output for normality of the data in Figure 4 and 5.

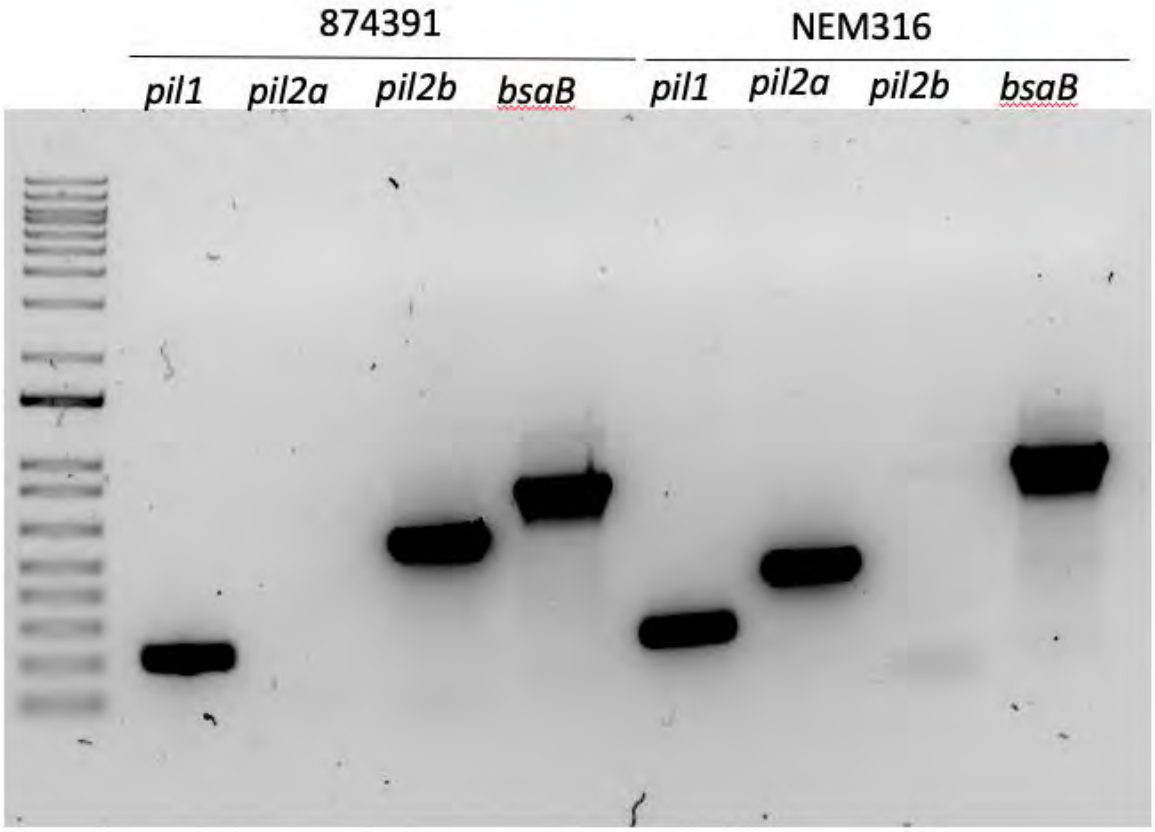
Growth Condition	Clinical Presentation			Strain	OD600
Shaking	Recurrent	N	Valid	141	141
			Missing	0	0
		Mean			.75979
		Std. Error of Mean			.046495
		Median			.70125
		Std. Deviation			.552098
		Skewness			.942
		Std. Error of Skewness			.204
		Kurtosis			.600
		Std. Error of Kurtosis			.406
		Percentiles	25		.25581
			50		.70125
			75		1.09694
	Asymptomatic	N	Valid	552	552
			Missing	0	0
		Mean			.75938
		Std. Error of Mean			.024218
		Median			.62806
		Std. Deviation			.568997
		Skewness			1.420
		Std. Error of Skewness			.104
		Kurtosis			2.521
		Std. Error of Kurtosis			.208
		Percentiles	25		.30409
			50		.62806
			75		1.08256
	Acute	N	Valid	183	183
			Missing	0	0
		Mean			.62655
		Std. Error of Mean			.034603
		Median			.48925
		Std. Deviation			.468100
		Skewness			1.264
		Std. Error of Skewness			.180
		Kurtosis			1.832
		Std. Error of Kurtosis			.357
		Percentiles	25		.23463
			50		.48925
			75		.89988
Static	Recurrent	N	Valid	141	141
			Missing	0	0
		Mean			.67493
		Std. Error of Mean			.040549
		Median			.60575
		Std. Deviation			.481498
		Skewness			1.045
		Std. Error of Skewness			.204
		Kurtosis			1.036
		Std. Error of Kurtosis			.406
		Percentiles	25		.24338
			50		.60575
			75		.90419
	Asymptomatic	N	Valid	552	552

		Missing	0	0
	Mean			.69018
	Std. Error of Mean			.020234
	Median			.57869
	Std. Deviation			.475387
	Skewness			1.237
	Std. Error of Skewness			.104
	Kurtosis			2.037
	Std. Error of Kurtosis			.208
	Percentiles	25		.29784
		50		.57869
		75		.97306
Acute	N	Valid	183	183
		Missing	0	0
	Mean			.63252
	Std. Error of Mean			.033008
	Median			.49988
	Std. Deviation			.446519
	Skewness			1.149
	Std. Error of Skewness			.180
	Kurtosis			.704
	Std. Error of Kurtosis			.357
	Percentiles	25		.26687
		50		.49988
		75		.86913

Appendix 5: SPSS output for normality of data for Figure 8.

Media	Growth Condition			Strain	OD595
LB + 1% glucose	Shaking	N	Valid	15	15
			Missing	0	0
		Mean			1.00873
		Median			1.01200
		Std. Deviation			.563698
		Skewness			-.243
		Std. Error of Skewness			.580
		Kurtosis			.332
		Std. Error of Kurtosis			1.121
		Minimum			.098
		Maximum			2.142
		Percentiles	25		.87200
			50		1.01200
			75		1.36100
	Static	N	Valid	15	15
			Missing	0	0
		Mean			1.09893
		Median			1.05700
		Std. Deviation			.687627
		Skewness			.357
		Std. Error of Skewness			.580
		Kurtosis			.491
		Std. Error of Kurtosis			1.121
		Minimum			.112
		Maximum			2.635
		Percentiles	25		.69700
			50		1.05700
			75		1.58900
LB +1% glucose + Malic acid	Shaking	N	Valid	15	15
			Missing	0	0
		Mean			.49573
		Median			.50700
		Std. Deviation			.283022
		Skewness			.356
		Std. Error of Skewness			.580
		Kurtosis			-.604
		Std. Error of Kurtosis			1.121
		Minimum			.119
		Maximum			1.004
		Percentiles	25		.30100
			50		.50700
			75		.72300
	Static	N	Valid	15	15
			Missing	0	0
		Mean			.45813
		Median			.39300
		Std. Deviation			.296957
		Skewness			.925
		Std. Error of Skewness			.580
		Kurtosis			.056
		Std. Error of Kurtosis			1.121
		Minimum			.138
		Maximum			1.100

Appendix 6: PCR screen for the four genes in the controls.

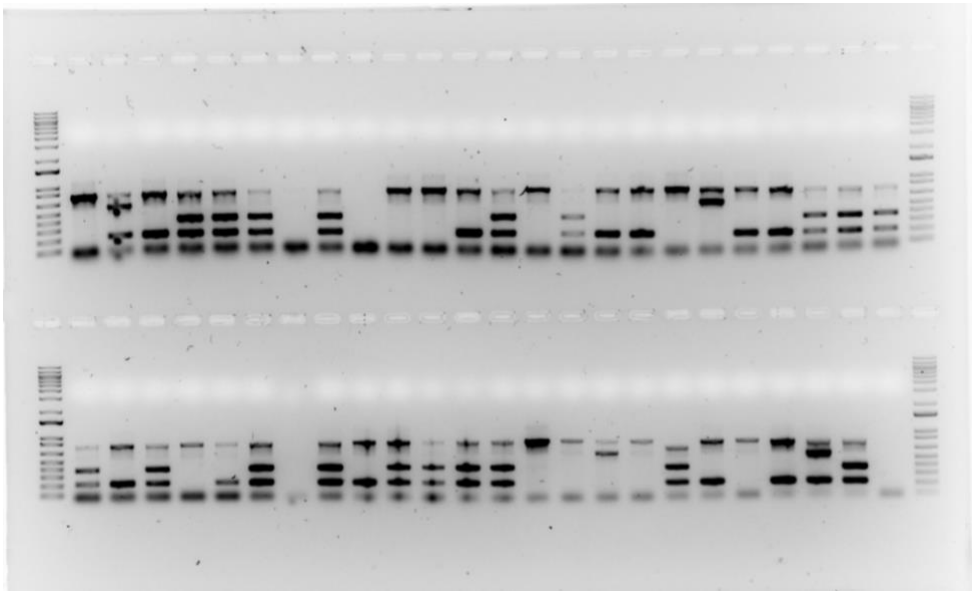


Appendix 7: PCR screens for the four genetic loci in the 292 isolates.

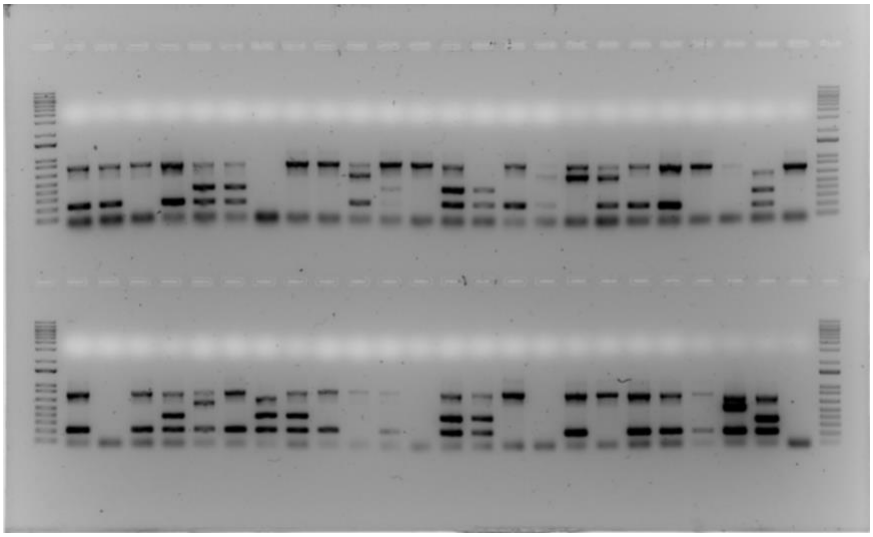
Highlighted cells were repeated

	1	2	3	4	5	6	7	8	9	10	11	12
A	GU0002	GU0013	GU0014	GU0016	GU0017	GU0024	GU0030	GU0032	GU0035	GU0040	GU0049	GU0053
B	GU0055	GU0072	GU0076	GU0088	GU0097	GU0100	GU0102	GU0105	GU0113	GU0117	GU0123	GU0126
C	GU0129	GU0153	GU0157	GU0167	GU0179	GU0187	GU0188	GU0197	GU0209	GU0210	GU0211	GU0218
D	GU0228	GU0235	GU0237	GU0250	GU0252	GU0333	GU0423	GU0426	GU0440	874391	NEM316	No DNA
E	GU0469	GU0471	GU0481	GU0482	GU0507	GU0516	GU0575	GU0594	GU0617	GU0627	GU0636	GU0655
F	GU0667	GU0683	GU0688	GU0689	GU0729	GU0731	GU0737	GU0743	GU0780	GU0802	GU0803	GU0810
G	GU0824	GU0828	GU0834	GU0838	GU0839	GU0842	GU0860	GU0880	GU0885	GU0887	GU0910	GU0912
H	GU0916	GU0919	GU0920	GU0927	GU0913	GU0935	GU0936	GU0939	GU0941	874391	NEM316	No DNA

Row A-D

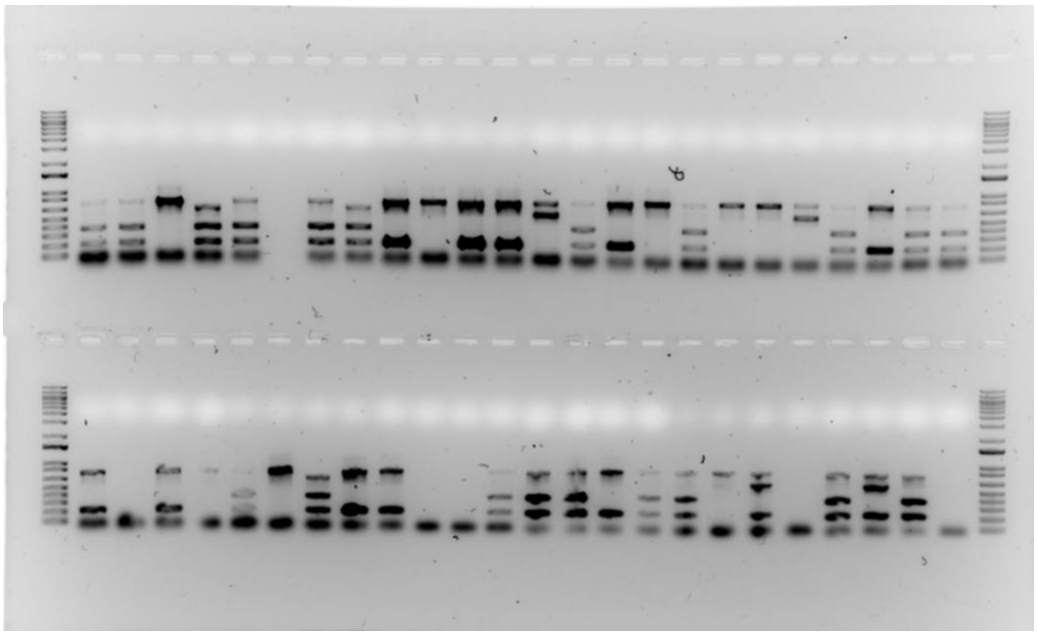


Row E-H

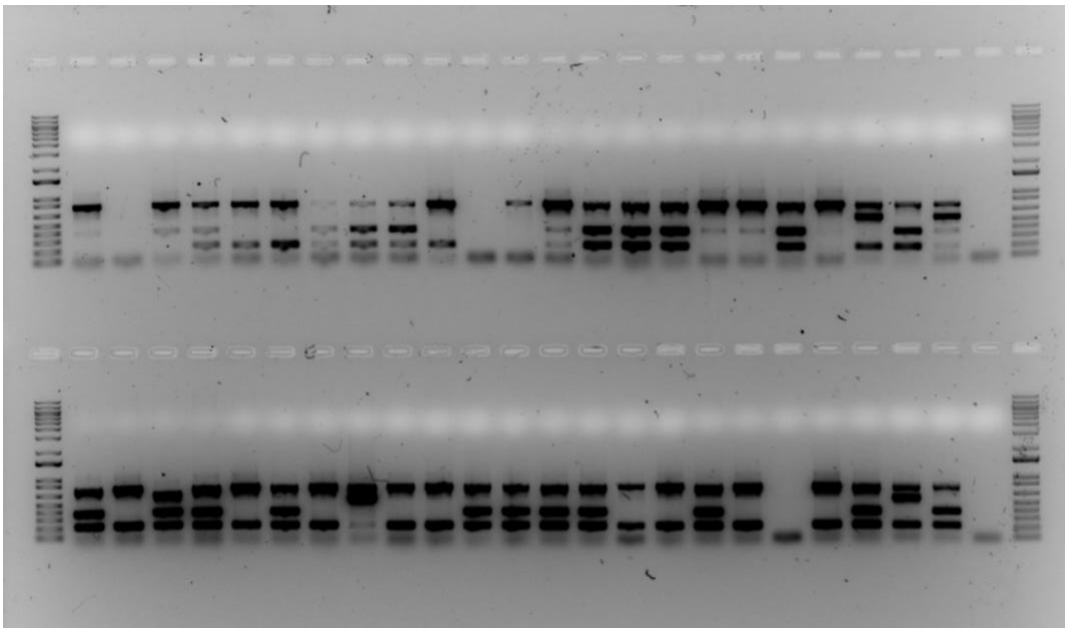


	1	2	3	4	5	6	7	8	9	10	11	12
A	GU0942	GU0943	GU0944	GU0945	GU0946	GU0947	GU0948	GU0949	GU0951	GU0956	GU0957	GU0960
B	GU0961	GU0967	GU0973	GU0976	GU0979	GU0982	GU0983	GU0986	GU0987	GU0988	GU0991	GU0995
C	GU0996	GU0997	GU1004	GU1005	GU1009	GU1011	GU1013	GU0974	GU1016	GU1027	GU1035	GU1037
D	GU1039	GU1039	GU1058	GU1059	GU1069	GU0074	GU0114	GU0115	GU0121	874391	NEM316	No DNA
E	GU0178	GU0205	GU0267	GU0286	GU0290	GU0318	GU0337	GU0344	GU0345	GU0347		GU0567
F	GU0728	GU0806	GU0008	GU0045	GU0047	GU0058	GU0135	GU0140	GU0150	GU0151	GU0152	
G	GU0226	GU0364	GU0369	GU0382	GU0402	GU0428	GU0470	GU0546	GU0619	GU0673	GU0700	GU0713
H	GU0872	GU0232	GU0243	GU0422	GU0443	GU0473	GU0509	GU0569	GU0745	874391	NEM316	No DNA

Row A-D

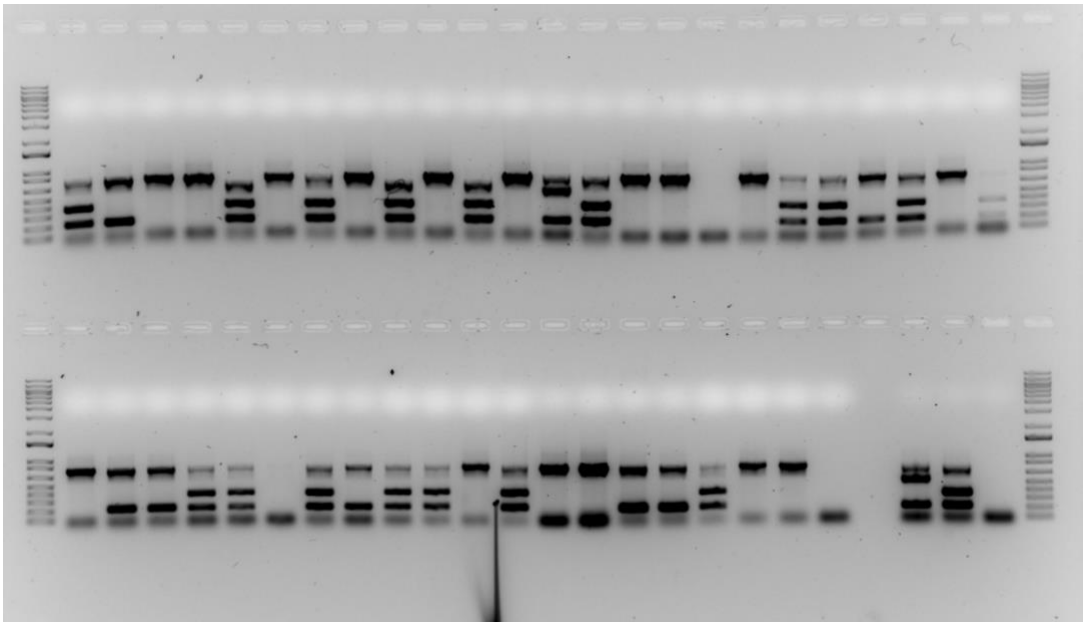


Row E-H

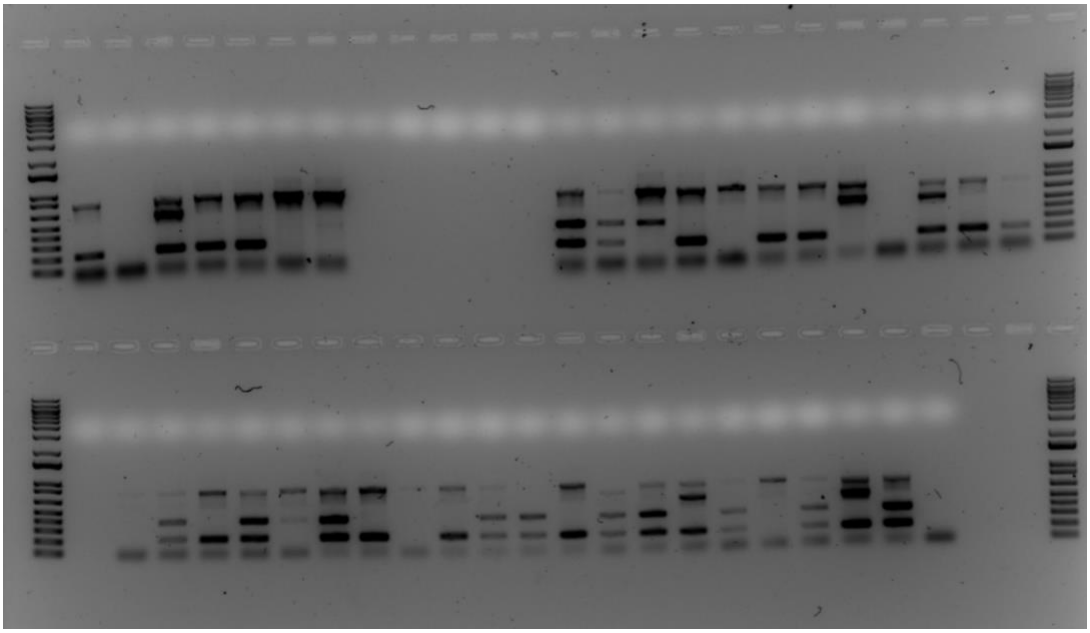


	1	2	3	4	5	6	7	8	9	10	11	12
A	GU0127	GU0087	GU0555	GU0529	GU0216	GU0170	GU0189	GU0155	GU0298	GU0289	GU1258	GU1255
B	GU1201	GU1309	GU1318	GU1319		GU1317	GU1326	GU1327	GU1552	GU1559	GU1511	
C	GU1407	GU1347	GU1383	GU1384	GU1400	GU1381	GU1376	GU1375	GU1370	GU0725	GU0346	GU0359
D	GU1501	GU1504	GU0602	GU0647	GU0994	GU1028	GU0392			874391	NEM316	No DNA
E	GU0001	GU0912	GU0247	GU0010	GU0022	GU0038	GU0098					
F	GU0256	GU300	GU0421	GU0494	GU0680	GU0760	GU0925	GU0962	GU1054	GU0807	GU0409	GU0091
G	GU0023	GU0042	GU0220	GU0223	GU0352	GU0371	GU0373	GU0441	GU0495	GU0622	GU0639	GU0640
H	GU0748	GU0761	GU0776	GU0801	GU0997	GU1012	GU1020	GU1048	874391	NEM316	No DNA	

Row A-D

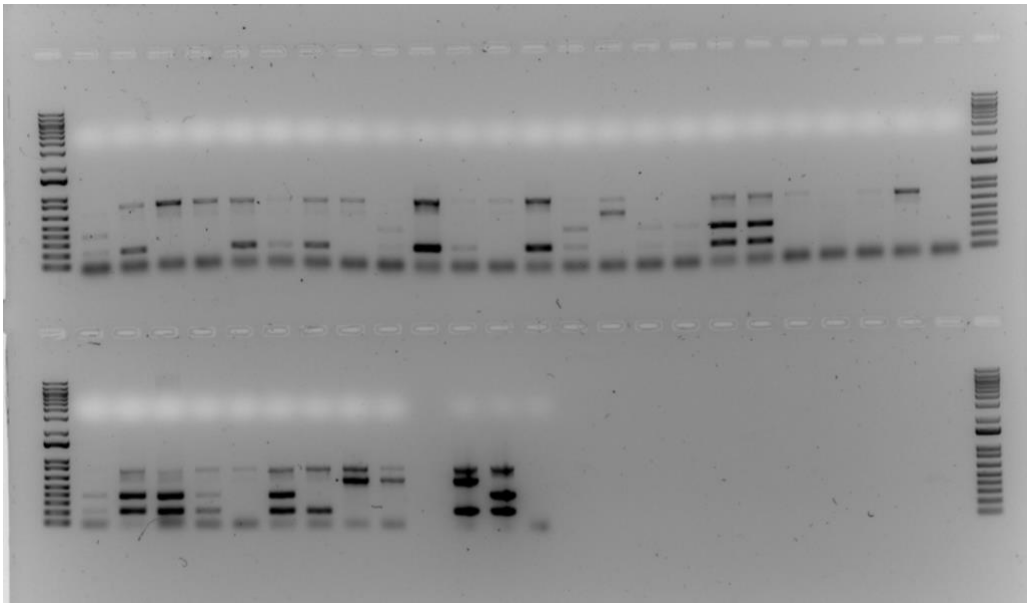


Row E-H

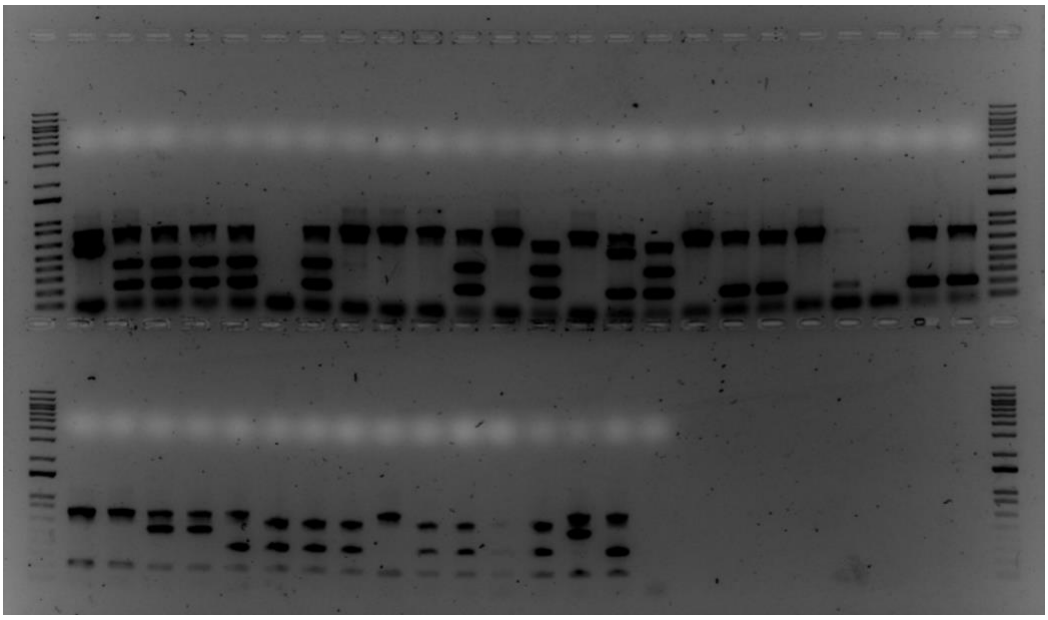


	1	2	3	4	5	6	7	8	9	10	11	12
A	GU0963	GU0971	GU0999	GU1000	GU1008	GU1014	GU1019	GU1023	GU1026	GU1027	GU1030	GU1034
B	GU1041	GU1044	GU1045	GU1064	GU1065	GU0204	GU0264	GU0332	GU0349	GU0582	GU0984	
C	GU0901	GU0020	GU0008	GU0181	GU0185	GUO259	GU0387	GU1344	GU1569	874391	NEM316	No DNA

Row A-C



	1	2	3	4	5	6	7	8	9	10	11	12
A	GU1054	GU1381	GU1388	GU1524	GU0188	GU0205	GU0032	GU0030	GU0035	GU1034	GU1035	GU1051
B	GU0342	GU0528	GU0349	GU0898	GU1338	GU0575	GU0509	GU0802	GU0828	GU0927	GU0947	GU0945
C	GU0023	GU0042	GU0714	GU0977	GU0115	GU0216	GU0298	GU1258	GU0912	GU0803	GU0860	GU0945
D	GU0369	874391	NEM316	No DNA								



Appendix 8: SPSS output for chi-square analysis of PCR results.

Table 8.1: Comparing genetic loci across the three clinical presentations.

Genetic Loci		Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Point Probability
bsaB	Pearson Chi-Square	.418[a]	2	.812	.826	
	Likelihood Ratio	.425	2	.809	.790	
	Fisher's Exact Test	.425			.865	
	Linear-by-Linear Association	.054[b]	1	.816	.890	.110
	N of Valid Cases	290				
pil1	Pearson Chi-Square	4.688[c]	2	.096	.097	
	Likelihood Ratio	4.469	2	.107	.116	
	Fisher's Exact Test	4.576			.104	
	Linear-by-Linear Association	4.164[d]	1	.041	.042	.009
	N of Valid Cases	290				
pil2a	Pearson Chi-Square	.253[e]	2	.881	.884	
	Likelihood Ratio	.252	2	.882	.884	
	Fisher's Exact Test	.286			.870	
	Linear-by-Linear Association	.100[f]	1	.752	.813	.060
	N of Valid Cases	290				
pil2b	Pearson Chi-Square	2.429[g]	2	.297	.324	
	Likelihood Ratio	2.274	2	.321	.326	
	Fisher's Exact Test	2.502			.272	
	Linear-by-Linear Association	.779[h]	1	.377	.412	.061
	N of Valid Cases	290				

a. 1 cells (16.7%) have expected count less than 5. The minimum expected count is 3.89.
b. The standardized statistic is -.233.
c. 0 cells (.0%) have expected count less than 5. The minimum expected count is 13.94.
d. The standardized statistic is 2.041.
e. 0 cells (.0%) have expected count less than 5. The minimum expected count is 18.80.
f. The standardized statistic is -.316.
g. 0 cells (.0%) have expected count less than 5. The minimum expected count is 5.83.
h. The standardized statistic is -.883.

Table 8.2: Comparison of proportion of strains with *pil1* between asymptomatic and acute strains.

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Point Probability
Pearson Chi-Square	.227[a]	1	.634	.740	
Continuity Correction[b]	.096	1	.757		
Likelihood Ratio	.224	1	.636	.740	
Fisher's Exact Test				.622	
Linear-by-Linear Association	.226[c]	1	.635	.740	.116
N of Valid Cases	243				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 16.57.
b. Computed only for a 2x2 table
c. The standardized statistic is .475.

Table 8.3: Comparison of proportion of strains with *pil1* between asymptomatic and recurrent strains.

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Point Probability
Pearson Chi-Square	4.684[a]	1	.030	.034	
Continuity Correction[b]	3.941	1	.047		
Likelihood Ratio	4.468	1	.035	.048	
Fisher's Exact Test				.048	
Linear-by-Linear Association	4.663[c]	1	.031	.034	.015
N of Valid Cases	229				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 13.96.
b. Computed only for a 2x2 table
c. The standardized statistic is 2.159.

Table 8.4: Comparison of proportion of strains with *pil1* between acute and recurrent strains

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Point Probability
Pearson Chi-Square	1.981[a]	1	.159	.223	
Continuity Correction[b]	1.450	1	.229		
Likelihood Ratio	1.975	1	.160	.223	
Fisher's Exact Test				.223	
Linear-by-Linear Association	1.963[c]	1	.161	.223	.061
N of Valid Cases	108				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 16.54.
b. Computed only for a 2x2 table
c. The standardized statistic is 1.401.

Table 8.5: Comparison of the presence of four genes between low, medium and high biofilm forming strains under shaking conditions.

Gene Loci		Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Point Probability
bsaB	Pearson Chi-Square	1.681[a]	2	.432	.440	
	Likelihood Ratio	1.645	2	.439	.450	
	Fisher's Exact Test	1.726			.423	
	Linear-by-Linear Association	.333[b]	1	.564	.625	.066
	N of Valid Cases	580				
pil1	Pearson Chi-Square	27.954[c]	2	.000	.000	
	Likelihood Ratio	27.700	2	.000	.000	
	Fisher's Exact Test	27.613			.000	
	Linear-by-Linear Association	27.661[d]	1	.000	.000	.000
	N of Valid Cases	580				
pil2a	Pearson Chi-Square	9.138[e]	2	.010	.010	
	Likelihood Ratio	9.383	2	.009	.009	
	Fisher's Exact Test	9.281			.010	
	Linear-by-Linear Association	3.766[f]	1	.052	.054	.007
	N of Valid Cases	580				
pil2b	Pearson Chi-Square	12.718[g]	2	.002	.002	
	Likelihood Ratio	12.384	2	.002	.002	
	Fisher's Exact Test	12.062			.002	
	Linear-by-Linear Association	9.773[h]	1	.002	.002	.000
	N of Valid Cases	580				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 10.84.
b. The standardized statistic is .577.
c. 0 cells (.0%) have expected count less than 5. The minimum expected count is 38.85.
d. The standardized statistic is 5.259.
e. 0 cells (.0%) have expected count less than 5. The minimum expected count is 52.40.
f. The standardized statistic is 1.941.
g. 0 cells (.0%) have expected count less than 5. The minimum expected count is 16.26.
h. The standardized statistic is 3.126.

Table 8.6: Comparison of the presence of four genes between low and medium biofilm forming strains under shaking conditions.

Gene Loci		Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Point Probability
bsaB	Pearson Chi-Square	.417[a]	1	.518	.594	
	Continuity Correction[b]	.219	1	.640		
	Likelihood Ratio	.418	1	.518	.594	
	Fisher's Exact Test				.594	
	Linear-by-Linear Association	.416[c]	1	.519	.594	.115
	N of Valid Cases	449				
pil1	Pearson Chi-Square	7.584[d]	1	.006	.006	
	Continuity Correction[b]	6.995	1	.008		
	Likelihood Ratio	7.626	1	.006	.006	
	Fisher's Exact Test				.006	
	Linear-by-Linear Association	7.567[e]	1	.006	.006	.002
	N of Valid Cases	449				
pil2a	Pearson Chi-Square	.604[f]	1	.437	.447	
	Continuity Correction[b]	.465	1	.495		
	Likelihood Ratio	.605	1	.437	.447	
	Fisher's Exact Test				.447	
	Linear-by-Linear Association	.603[g]	1	.437	.447	.056
	N of Valid Cases	449				
pil2b	Pearson Chi-Square	9.453[h]	1	.002	.002	
	Continuity Correction[b]	8.625	1	.003		
	Likelihood Ratio	9.670	1	.002	.002	
	Fisher's Exact Test				.002	
	Linear-by-Linear Association	9.432[i]	1	.002	.002	.001
	N of Valid Cases	449				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 16.81.
b. Computed only for a 2x2 table
c. The standardized statistic is -.645.
d. 0 cells (.0%) have expected count less than 5. The minimum expected count is 55.38.
e. The standardized statistic is 2.751.
f. 0 cells (.0%) have expected count less than 5. The minimum expected count is 95.92.
g. The standardized statistic is -.777.
h. 0 cells (.0%) have expected count less than 5. The minimum expected count is 30.16.
i. The standardized statistic is 3.071.

Table 8.7: Comparison of the presence of four genes between low and high biofilm forming strains under shaking conditions.

Gene Loci		Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Point Probability
bsaB	Pearson Chi-Square	.533[a]	1	.465	.570	
	Continuity Correction[b]	.292	1	.589		
	Likelihood Ratio	.524	1	.469	.570	
	Fisher's Exact Test				.456	
	Linear-by-Linear Association	.531[c]	1	.466	.570	.113
	N of Valid Cases	358				
pil1	Pearson Chi-Square	28.127[d]	1	.000	.000	
	Continuity Correction[b]	26.860	1	.000		
	Likelihood Ratio	27.536	1	.000	.000	
	Fisher's Exact Test				.000	
	Linear-by-Linear Association	28.048[e]	1	.000	.000	.000
	N of Valid Cases	358				
pil2a	Pearson Chi-Square	5.489[f]	1	.019	.023	
	Continuity Correction[b]	4.969	1	.026		
	Likelihood Ratio	5.583	1	.018	.023	
	Fisher's Exact Test				.023	
	Linear-by-Linear Association	5.474[g]	1	.019	.023	.006
	N of Valid Cases	358				
pil2b	Pearson Chi-Square	6.725[h]	1	.010	.013	
	Continuity Correction[b]	5.948	1	.015		
	Likelihood Ratio	7.239	1	.007	.009	
	Fisher's Exact Test				.009	
	Linear-by-Linear Association	6.706[i]	1	.010	.013	.004
	N of Valid Cases	358				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 12.08.
b. Computed only for a 2x2 table
c. The standardized statistic is .729.
d. 0 cells (.0%) have expected count less than 5. The minimum expected count is 38.06.
e. The standardized statistic is 5.296.
f. 0 cells (.0%) have expected count less than 5. The minimum expected count is 48.30.
g. The standardized statistic is 2.340.
h. 0 cells (.0%) have expected count less than 5. The minimum expected count is 19.39.
i. The standardized statistic is 2.590.

Table 8.8: Comparison of the presence of four genes between medium and high biofilm forming strains under shaking conditions.

Gene Loci		Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Point Probability
bsaB	Pearson Chi-Square	1.688[a]	1	.194	.229	
	Continuity Correction[b]	1.207	1	.272		
	Likelihood Ratio	1.640	1	.200	.229	
	Fisher's Exact Test				.229	
	Linear-by-Linear Association	1.683[c]	1	.195	.229	.068
	N of Valid Cases	353				
pil1	Pearson Chi-Square	8.204[d]	1	.004	.006	
	Continuity Correction[b]	7.561	1	.006		
	Likelihood Ratio	8.127	1	.004	.006	
	Fisher's Exact Test				.006	
	Linear-by-Linear Association	8.181[e]	1	.004	.006	.002
	N of Valid Cases	353				
pil2a	Pearson Chi-Square	8.899[f]	1	.003	.003	
	Continuity Correction[b]	8.238	1	.004		
	Likelihood Ratio	9.075	1	.003	.003	
	Fisher's Exact Test				.003	
	Linear-by-Linear Association	8.874[g]	1	.003	.003	.001
	N of Valid Cases	353				
pil2b	Pearson Chi-Square	.003[h]	1	.958	1.000	
	Continuity Correction[b]	.000	1	1.000		
	Likelihood Ratio	.003	1	.958	1.000	
	Fisher's Exact Test				1.000	
	Linear-by-Linear Association	.003[i]	1	.958	1.000	.156
	N of Valid Cases	353				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 10.76.
b. Computed only for a 2x2 table
c. The standardized statistic is 1.297.
d. 0 cells (.0%) have expected count less than 5. The minimum expected count is 47.50.
e. The standardized statistic is 2.860.
f. 0 cells (.0%) have expected count less than 5. The minimum expected count is 51.21.
g. The standardized statistic is 2.979.
h. 0 cells (.0%) have expected count less than 5. The minimum expected count is 11.13.
i. The standardized statistic is .053.

Table 8.9: Comparison of the presence of four genes between low, medium and high biofilm forming strains under static conditions.

Genetic Loci		Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Point Probability
bsaB	Pearson Chi-Square	.673[a]	2	.714	.701	
	Likelihood Ratio	.669	2	.716	.701	
	Fisher's Exact Test	.797			.701	
	Linear-by-Linear Association	.042[b]	1	.838	.887	.110
	N of Valid Cases	290				
pil1	Pearson Chi-Square	14.783[c]	2	.001	.001	
	Likelihood Ratio	14.389	2	.001	.001	
	Fisher's Exact Test	14.328			.001	
	Linear-by-Linear Association	14.165[d]	1	.000	.000	.000
	N of Valid Cases	290				
pil2a	Pearson Chi-Square	4.832[e]	2	.089	.091	
	Likelihood Ratio	5.003	2	.082	.084	
	Fisher's Exact Test	4.880			.089	
	Linear-by-Linear Association	2.172[f]	1	.141	.150	.022
	N of Valid Cases	290				
pil2b	Pearson Chi-Square	6.216[g]	2	.045	.043	
	Likelihood Ratio	6.170	2	.046	.048	
	Fisher's Exact Test	5.777			.058	
	Linear-by-Linear Association	5.579[h]	1	.018	.023	.006
	N of Valid Cases	290				

a. 1 cells (16.7%) have expected count less than 5. The minimum expected count is 4.80.
b. The standardized statistic is .204.
c. 0 cells (.0%) have expected count less than 5. The minimum expected count is 17.20.
d. The standardized statistic is 3.764.
e. 0 cells (.0%) have expected count less than 5. The minimum expected count is 23.20.
f. The standardized statistic is 1.474.
g. 0 cells (.0%) have expected count less than 5. The minimum expected count is 7.20.
h. The standardized statistic is 2.362.

Table 8.10: Comparison of the presence of four genes between low and medium biofilm forming strains under static conditions.

Genetic Loci		Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Point Probability
bsaB	Pearson Chi-Square	.280[a]	1	.597	.632	
	Continuity Correction[b]	.080	1	.777		
	Likelihood Ratio	.280	1	.597	.632	
	Fisher's Exact Test				.632	
	Linear-by-Linear Association	.279[c]	1	.598	.632	.169
	N of Valid Cases	232				
pil1	Pearson Chi-Square	3.040[d]	1	.081	.096	
	Continuity Correction[b]	2.535	1	.111		
	Likelihood Ratio	3.059	1	.080	.096	
	Fisher's Exact Test				.096	
	Linear-by-Linear Association	3.027[e]	1	.082	.096	.027
	N of Valid Cases	232				
pil2a	Pearson Chi-Square	.173[f]	1	.677	.693	
	Continuity Correction[b]	.080	1	.777		
	Likelihood Ratio	.173	1	.677	.693	
	Fisher's Exact Test				.693	
	Linear-by-Linear Association	.172[g]	1	.678	.693	.097
	N of Valid Cases	232				
pil2b	Pearson Chi-Square	3.828[h]	1	.050	.058	
	Continuity Correction[b]	3.119	1	.077		
	Likelihood Ratio	3.881	1	.049	.058	
	Fisher's Exact Test				.058	
	Linear-by-Linear Association	3.812[i]	1	.051	.058	.023
	N of Valid Cases	232				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 8.92.
b. Computed only for a 2x2 table
c. The standardized statistic is -.528.
d. 0 cells (.0%) have expected count less than 5. The minimum expected count is 28.75.
e. The standardized statistic is 1.740.
f. 0 cells (.0%) have expected count less than 5. The minimum expected count is 49.57.
g. The standardized statistic is -.415.
h. 0 cells (.0%) have expected count less than 5. The minimum expected count is 15.86.
i. The standardized statistic is 1.952.

Table 8.11: Comparison of the presence of four genes between low and high biofilm forming strains under static conditions.

Genetic Loci		Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Point Probability
bsaB	Pearson Chi-Square	.125[a]	1	.724	.783	
	Continuity Correction[b]	.006	1	.940		
	Likelihood Ratio	.123	1	.726	.783	
	Fisher's Exact Test				.783	
	Linear-by-Linear Association	.124[c]	1	.725	.783	.201
	N of Valid Cases	173				
pil1	Pearson Chi-Square	14.828[d]	1	.000	.000	
	Continuity Correction[b]	13.499	1	.000		
	Likelihood Ratio	14.383	1	.000	.000	
	Fisher's Exact Test				.000	
	Linear-by-Linear Association	14.742[e]	1	.000	.000	.000
	N of Valid Cases	173				
pil2a	Pearson Chi-Square	3.313[f]	1	.069	.095	
	Continuity Correction[b]	2.734	1	.098		
	Likelihood Ratio	3.393	1	.065	.070	
	Fisher's Exact Test				.095	
	Linear-by-Linear Association	3.294[g]	1	.070	.095	.026
	N of Valid Cases	173				
pil2b	Pearson Chi-Square	4.028[h]	1	.045	.065	
	Continuity Correction[b]	3.161	1	.075		
	Likelihood Ratio	4.484	1	.034	.044	
	Fisher's Exact Test				.065	
	Linear-by-Linear Association	4.004[i]	1	.045	.065	.024
	N of Valid Cases	173				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 5.36.
b. Computed only for a 2x2 table
c. The standardized statistic is .352.
d. 0 cells (.0%) have expected count less than 5. The minimum expected count is 17.10.
e. The standardized statistic is 3.840.
f. 0 cells (.0%) have expected count less than 5. The minimum expected count is 21.46.
g. The standardized statistic is 1.815.
h. 0 cells (.0%) have expected count less than 5. The minimum expected count is 8.38.
i. The standardized statistic is 2.001.

Table 8.12: Comparison of the presence of four genes between medium and high biofilm forming strains under static conditions.

Genetic Loci		Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Point Probability
bsaB	Pearson Chi-Square	.648[a]	1	.421	.555	
	Continuity Correction[b]	.259	1	.611		
	Likelihood Ratio	.625	1	.429	.555	
	Fisher's Exact Test				.555	
	Linear-by-Linear Association	.644[c]	1	.422	.555	.162
	N of Valid Cases	175				
pil1	Pearson Chi-Square	5.674[d]	1	.017	.020	
	Continuity Correction[b]	4.905	1	.027		
	Likelihood Ratio	5.588	1	.018	.020	
	Fisher's Exact Test				.020	
	Linear-by-Linear Association	5.642[e]	1	.018	.020	.008
	N of Valid Cases	175				
pil2a	Pearson Chi-Square	4.639[f]	1	.031	.034	
	Continuity Correction[b]	3.956	1	.047		
	Likelihood Ratio	4.763	1	.029	.034	
	Fisher's Exact Test				.034	
	Linear-by-Linear Association	4.612[g]	1	.032	.034	.013
	N of Valid Cases	175				
pil2b	Pearson Chi-Square	.311[h]	1	.577	.776	
	Continuity Correction[b]	.073	1	.787		
	Likelihood Ratio	.321	1	.571	.776	
	Fisher's Exact Test				.776	
	Linear-by-Linear Association	.309[i]	1	.578	.776	.202
	N of Valid Cases	175				

a. 1 cells (25.0%) have expected count less than 5. The minimum expected count is 4.64.
b. Computed only for a 2x2 table
c. The standardized statistic is .803.
d. 0 cells (.0%) have expected count less than 5. The minimum expected count is 20.88.
e. The standardized statistic is 2.375.
f. 0 cells (.0%) have expected count less than 5. The minimum expected count is 22.54.
g. The standardized statistic is 2.148.
h. 1 cells (25.0%) have expected count less than 5. The minimum expected count is 4.97.
i. The standardized statistic is .556.

Appendix 9: SPSS output for chi-square of haemolytic activity of clinical isolates.

Table 9.1: Comparison of haemolytic activity of asymptomatic, acute and recurrent strains.

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Point Probability
Pearson Chi-Square	14.808[a]	6	.022	.022	
Likelihood Ratio	17.554	6	.007	.009	
Fisher's Exact Test	12.943			.032	
Linear-by-Linear Association	5.025[b]	1	.025	.025	.003
N of Valid Cases	292				

a. 3 cells (25.0%) have expected count less than 5. The minimum expected count is .97.
b. The standardized statistic is -2.242.

Table 9.2: Comparison of low and medium haemolytic activity of asymptomatic and recurrent strains.

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Point Probability
Pearson Chi-Square	2.916[a]	1	.088	.098	
Continuity Correction[b]	2.319	1	.128		
Likelihood Ratio	3.088	1	.079	.098	
Fisher's Exact Test				.098	
Linear-by-Linear Association	2.901[c]	1	.089	.098	.035
N of Valid Cases	195				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 13.62.
b. Computed only for a 2x2 table
c. The standardized statistic is -1.703.

Table 9.3: Comparison of low and high haemolytic activity of asymptomatic and recurrent strains.

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Point Probability
Pearson Chi-Square	5.461[a]	1	.019	.028	
Continuity Correction[b]	4.397	1	.036		
Likelihood Ratio	6.713	1	.010	.017	
Fisher's Exact Test				.017	
Linear-by-Linear Association	5.428[c]	1	.020	.028	.010
N of Valid Cases	166				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 6.87.
b. Computed only for a 2x2 table
c. The standardized statistic is -2.330.

Table 9.4: Comparison of medium and high haemolytic activity of asymptomatic and recurrent strains.

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Point Probability
Pearson Chi-Square	1.354[a]	1	.245	.322	
Continuity Correction[b]	.677	1	.411		
Likelihood Ratio	1.484	1	.223	.322	
Fisher's Exact Test				.322	
Linear-by-Linear Association	1.339[c]	1	.247	.322	.150
N of Valid Cases	89				

a. 1 cells (25.0%) have expected count less than 5. The minimum expected count is 3.71.
b. Computed only for a 2x2 table
c. The standardized statistic is -1.157.

Table 9.5: Comparison of low and medium haemolytic activity of asymptomatic and acute strains.

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Point Probability
Pearson Chi-Square	2.162[a]	1	.141	.176	
Continuity Correction[b]	1.693	1	.193		
Likelihood Ratio	2.240	1	.135	.176	
Fisher's Exact Test				.176	
Linear-by-Linear Association	2.151[c]	1	.142	.176	.047
N of Valid Cases	207				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 17.35.
b. Computed only for a 2x2 table
c. The standardized statistic is -1.467.

Table 9.6: SPSS output for chi-square analysis of low and high haemolytic activity of asymptomatic and acute strains.

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Point Probability
Pearson Chi-Square	4.303[a]	1	.038	.047	
Continuity Correction[b]	3.441	1	.064		
Likelihood Ratio	4.879	1	.027	.047	
Fisher's Exact Test				.047	
Linear-by-Linear Association	4.279[c]	1	.039	.047	.019
N of Valid Cases	176				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 8.73.
b. Computed only for a 2x2 table
c. The standardized statistic is -2.069.

Table 9.7: SPSS output for chi-square analysis of medium and high haemolytic activity of asymptomatic and acute strains.

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Point Probability
Pearson Chi-Square	.956[a]	1	.328	.405	
Continuity Correction[b]	.482	1	.487		
Likelihood Ratio	1.003	1	.316	.405	
Fisher's Exact Test				.405	
Linear-by-Linear Association	.946[c]	1	.331	.405	.147
N of Valid Cases	95				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 5.73.
b. Computed only for a 2x2 table
c. The standardized statistic is -.973.

Table 9.8: SPSS output for chi-square analysis of low and medium haemolytic activity of recurrent and acute strains.

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Point Probability
Pearson Chi-Square	.117[a]	1	.732	.811	
Continuity Correction[b]	.010	1	.920		
Likelihood Ratio	.118	1	.732	.811	
Fisher's Exact Test				.811	
Linear-by-Linear Association	.116[c]	1	.733	.811	.182
N of Valid Cases	102				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 9.71.
b. Computed only for a 2x2 table
c. The standardized statistic is .341.

Table 9.9: SPSS output for chi-square analysis of low and high haemolytic activity of recurrent and acute strains.

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Point Probability
Pearson Chi-Square	.308[a]	1	.579	.690	
Continuity Correction[b]	.017	1	.897		
Likelihood Ratio	.316	1	.574	.690	
Fisher's Exact Test				.690	
Linear-by-Linear Association	.304[c]	1	.581	.690	.291
N of Valid Cases	86				

a. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 2.65.
b. Computed only for a 2x2 table
c. The standardized statistic is .552.

Table 9.10: SPSS output for chi-square analysis of medium and high haemolytic activity of recurrent and acute strains.

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Point Probability
Pearson Chi-Square	.113[a]	1	.736	1.000	
Continuity Correction[b]	.000	1	1.000		
Likelihood Ratio	.115	1	.734	1.000	
Fisher's Exact Test				1.000	
Linear-by-Linear Association	.109[c]	1	.741	1.000	.347
N of Valid Cases	28				

a. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 2.36.
b. Computed only for a 2x2 table
c. The standardized statistic is .331.