NOTTINGHAM TRENT UNIVERSITY

Profiling potentially pathogenic bacteria from neonatal feeding tubes and sepsis cases.

KHALED A. DAHMANI

A thesis submitted in partial fulfilment of the requirement of Nottingham Trent University of the degree of Doctor of Philosophy.

December 2018

Declaration;

I hereby certify that the work presented herein is the result of my own research work, except where reference has been made to published literature. I have composed the thesis and the work has not been submitted for any other degree or professional qualification. All the work was conducted in the School of Science and Technology at the Nottingham Trent University. You may copy up to 5% of this work for the private study or personal, non-commercial research. Any information used from this thesis should be fully cited.

Khaled A. Dahmani

Acknowledgements;

I am very grateful to Allah, the Almighty, for making it possible for me to conduct and complete this study

I would like to convey my special thanks to my supervisor Dr. Jody Winter for the continuous support of my PhD study and research, for her patience, motivation, enthusiasm, and immense knowledge.

Also, I would like to thank my second supervisor Dr. Michael Loughlin for his support and advice. Furthermore, I would like express my appreciation, gratitude and great thanks for Prof. Stephen Forsythe for his unlimited support of my PhD study and immense knowledge.

I sincerely wish to give my special and deepest gratitude to my family; my mother Zohra and my wife Huda for their unlimited support, encouraging and patient during the course of my PhD. Also, Lots of love to my children, Wala, Bara, Mohamed and Raghd, who made my life full of love.

I wish to give my warmest thanks to all my friends and colleagues in the Microbiology research lab and beyond who have been helpful and encouraging during the length of my PhD study. My thanks are extended to the Libyan Ministry of Higher Education and Biotechnology Research Centre for offering me the scholarship to pursue my PhD study.

Publications;

- Pauline Ogrodzki, Chi Shing Cheung, Mohamed Saad, **Khaled Dahmani**, Rebecca Coxill, Haida Liang & Stephen J. Forsythe (2017). Rapid *in situ* imaging and whole genome sequencing of biofilm in neonatal feeding tubes: A clinical proof of concept. *Springer Nature Scientific Reports* (2017), *7*(1), 1–8.

Conference Presentations (Posters)

- **K. Dahmani**, M. Saad, P. Ogrodzki, M. Elemam, J. Winter, S. Forsythe (2018). Csu fimbriae protein plays a role in biofilm formation and adhesion to Caco-2 epithelial cells in *A. baumannii* isolated from neonatal feeding tubes in neonatal intensive care. SGM Annual Conference 2018 Birmingham, UK.
- **Khaled Dahmani**, Mohamed Saad, Pauline Ogrodzki, Tareq Osaili and Stephen Forsythe (2017). Virulence Potential of *Acinetobacter baumannii* strains isolated from Neonatal Nasogastric Feeding Tube in intensive care units. The 27th ECCMID 2017 Vienna, Austria.
- **Khaled Dahmani**, Mohamed Saad, Pauline Ogrodzki, Tareq Osaili and Stephen Forsythe (2017). Phenotypic and Genotypic Characterization of Multidrug Resistant *Acinetobacter baumannii* from Neonatal Feeding Tubes in Neonatal Intensive Care Units. FEMS 2017, Valencia Spain.
- **Khaled Dahmani**, Mohamed Saad, Pauline Ogrodzki, Jody Winter and Stephen Forsythe (2017). Molecular characterization of virulence factors in *Enterobacter hormaechei* isolated from symptomatic premature babies with sepsis. SGM Annual Conference 2017 Edinburgh, UK
- **K. Dahmani**, M. Saad, and S. Forsythe (2016). Characterisation and virulence factors of *Acinetobacter baumannii* strains isolated from neonatal feeding tubes in neonatal Intensive Care Units. SGM Annual Conference 2016 Liverpool, UK.
- Mohamed Saad, **Khaled Dahmani**, Pauline Ogrodzki, Jody Winter and Stephen Forsythe (2018). Comparative study of virulence factors of *Klebsiella pneumoniae* isolated from neonatal feeding tubes and neonatal sepsis cases in neonatal intensive care. SGM Annual Conference 2018 Birmingham, UK.

- Mohamed Saad, **Khaled Dahmani** Pauline Ogrodzki, Jody Winter, Tareq Osaili and Stephen Forsythe (2017). Phenotypic and Molecular Characterization of *Klebsiella pneumoniae* Isolated From Neonatal Nasogastric Feeding Tubes. The 27th ECCMID 2017 Vienna, Austria.
- Mohamed Saad, **Khaled Dahmani**, Pauline Ogrodzki, Jody Winter and Stephen Forsythe (2017). Molecular Characterisation of Virulence and Antibacterial Resistant among *Klebsiella pneumoniae* Isolated from Neonatal with Sepsis. SGM Annual Conference 2017 Edinburgh, UK.
- Mohamed Saad, **Khaled Dahmani**, Pauline Ogrodzki, Jody Winter and Stephen Forsythe (2017). Phenotyping and Genotyping of Antibacterial Resistant *Escherichia coli* Isolated from Neonatal with Sepsis. SGM Annual Conference 2017 Edinburgh, UK.
- Mohamed Saad, **Khaled Dahmani**, Pauline Ogrodzki, Jody Winter and Stephen Forsythe (2017). Virulence Studies of *Klebsiella Pneumoniae* Isolated From Neonatal Nasogastric Feeding Tubes. FEMS 2017, Valencia Spain.
- Mohamed Saad, **Khaled Dahmani**, Tareq Osaili and Stephen Forsythe (2016). Virulence Potential of *Klebsiella pneumoniae* Isolated From Neonatal Nasogastric Feeding Tubes. SGM Annual Conference (2016) Liverpool, UK.

Manuscripts in preparation

- *In situ* colonization of nasogastric feeding tubes of neonates in intensive care by *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Escherichia coli.* **K. Dahmani**[a], M. Saad[a], P. Ogrodzki[a], S.J. Forsythe[a] ….T. Osaili[b] (others). [a]Pathogen Research Group, Nottingham Trent University, Clifton Lane, Nottingham, NG11 8NS and [b]Department of Nutrition and Food Technology, Jordan University of Science and Technology, Irbid, 22110, Jordan.

List of abbreviations;

ACT	Artemis comparison tool
ANOVA	Analysis of Variance
BSAC	British Society for Antimicrobial chemotherapy
BLAST	Basic local alignment search tool
bp	Base pairs
BPW	Buffered peptone water
Caco-2	Human colonic carcinoma epithelial cells
CASAD	Chrome Azurol S agar diffusion
CLB	Cell lysis buffer
CSB	Cell Suspension Buffer
CV	Crystal violet
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra-acetic acid
FAO	Food and Agriculture Organization of the UN
GIT	Gastrointestinal tract
HBMEC	Human brain microvascular endothelial cells
HCL	Hydrochloric acid
HDTMA	Hexadecyltrimethylammonium bromide
ICU	Intensive care units
IF	Infant formula
LB	Luria-Bertani
LPS	Lipopolysaccharide
MEM	Minimum Essential Medium
MLST	Multi-locus sequence typing
MOI	Multiplicity of infection
NEFT	Nasogastric enteral feeding tube

NICU	Neonatal intensive care unit
NTU	Nottingham Trent University
OD	Optical density
PBS	Phosphate buffered saline
PCA	Plate count agar
PCR	Polymerase chain reaction
PFGE	Pulsed Field Gel Electrophoresis
PIF	Powdered infant formula
PMA	Phorbol 12-myristate 13-acetate
SDS	Sodium dodecyl sulphate
SMS	Skimmed milk solution
SNPs	Single Nucleotide Polymorphisms
Spp.	Species
ST	Sequence type
TAE	Tris/acetate/EDTA
TBE	Tris base/ Boric acid / EDTA
TEB	Tris EDTA buffer
TSA	Tryptone Soya Agar
TSB	Tryptone Soya Broth
U937	Macrophage Cell Line
UTI	Urinary tract infection
UV	Ultraviolet
VRBGA	Violet red bile glucose agar
WHO	World Health Organization

Abstract

Recently, there has been a rise in the incidence of neonatal infections among babies born with low birth-weights and under-developed immune systems in neonatal intensive care units (NICUs). There are several risk factors to neonatal infection, the most important of which include the use of medical devices such as nasogastric enteral feeding tubes (NEFTs) and through contamination of infant feeding formula. Therefore, bacterial analysis of feeding tubes used in the NICU is important to identify infection risk factors during neonatal enteral feeding.

The aims of this study were (a) to determine the potential risk to neonates posed by ingestion of *A. baumannii* and *Enterobacter* spp., in particular *E. hormaechei* either through feeding tubes, infant formula, or by contaminated milk, (b) to determine whether some of the isolated strains originate from common sources, such as being transferred between the babies within specific neonatal units. Additionally, a longitudinal study for premature twin babies aimed to compare potentially pathogenic *E. faecium* isolates within and between the feeding tubes and faeces of twin babies over time.

PFGE indicated that all of the *A. baumannii* strains formed two different STs (ST193 and ST113). All ST113 strains were multidrug-resistant and demonstrated an ability to form significant biofilms at 37 °C in infant formula. Tolerance of acidic conditions, desiccation, resistance to human serum and persistence inside macrophages were shown by the majority of strains tested. *E. hormaechei* strains from feeding tubes exhibited similar behaviour to those isolated from sepsis cases, since both were able to adhere to and invade Caco-2 and HBMEC cell lines. Also, these strains were able to persist and replicate inside macrophages for up to 72 hours.

In the longitudinal study, all isolates of *E. faecium* isolated from preterm infant twins during their hospitalisation in the NICU were typed as ST80, belonging to clonal complex CC17. Furthermore, they were resistant to ampicillin and were found to carry several virulence-associated genes such as *esp.* All of these strains were found to be essentially the same strain based on their sequence type and genomic analysis and were shown to have high pathogenic potential. These strains isolated from different neonatal locations were indeed the same clone, showing that the bacteria were able to persist and be transferred between the two premature infants in the NICU.

This study has provided evidence of colonisation and persistence of opportunistic ESKAPE group pathogens in neonatal feeding tubes, which are important causes of nosocomial infection and dissemination of multidrug-resistant (MDR) strains.

Contents:

List of Tables

List of Figures:

Chapter 1. Introduction

The most susceptible period of a new-born's life is during the first four weeks after birth. This period is considered to pose the highest risk and is a very critical period for neonatal survival with regard to infection. There were approximately 2.6 million cases of neonatal mortality in the first month of life in 2016 worldwide. Neonatal death can result from premature birth, problems in pregnancy during labour and delivery, as well as the result of infections such as meningitis, pneumonia and sepsis (WHO, 2017). According to the World Health Organisation (2018), 75% of all neonatal mortalities in 2017 occurred during the first week of their life. One million neonates died within the first day, while in the next six days approximately another one million died.

United Nations Sustainable Development (2017) and UNICEF (2017) reported that the annual death rate of children before reaching their fifth year had dropped in 2015 by almost half (53%) when compared to the same statistics from 1990. The neonatal mortality rate over the same period has dropped by 47%. Despite the reduction in mortality rate among new-borns and children in recent decades, there has been no significant decline in infant mortality rate during the first week of their life. The World Health Organisation (WHO) reported that 46% of all neonatal deaths under the age 5 in 2016 were caused by preterm birth complications, congenital anomalies, intrapartum-related complications, acute respiratory infections, anomalies and diarrhoea, as shown in Figure 1-1. However, premature birth, or low birthweight, is one of the major risk factors recognised for neonatal deaths. According to the National Statistical Institute of the UK (2017), the infant mortality rates increased by 2.3% in 2015 compared to 2014 in England and Wales (Patel, 2015). Furthermore, a report by the national press highlighted events in the UK, which indicated that nosocomial infections and sepsis infections are associated with significant morbidity in preterm neonates in neonatal intensive care units NICUs.

Figure 1-1 Causes of death among of neonates and children under the age of five in 2016 according to the WHO. Source: http://www.who.int/gho/child_health/mortality/causes/en/

1.1. Infectious diseases in neonates

Neonates kept in NICUs are highly susceptible individuals within the paediatric group, particularly pre-term neonates with low birth-weights and under-developed immune systems. The risk of infection to immunocompromised neonates increases with prolonged hospitalisation where a wide variety of medical devices are used. There is also increased chance of acquiring nosocomial infections including pneumonia, bloodstream infections, meningitis and urinary tract infections among hospitalised infants. Nosocomial infections remain one of the major risk factor for morbidity among neonatal NICUs (Mohammed and El Seifi, 2014; Rameshwarnath and Naidoo, 2018).

The NICU environment is recognised as a potential major source of infection to the neonates. It may play a significant role in harbouring reservoirs of clinically important pathogens. A study carried out by Tajeddin et al. (2016) isolated *Staphylococcus aureus, Acinetobacter baumannii* and vancomycin-resistant enterococci from healthcare workers and environmental surfaces in the NICUs in Tehran. Their results demonstrated that these bacteria are possibly associated with hospital acquired infections (HAIs)

Another study carried out at Nottingham Trent University (Hurrell et al., 2009a; 2009b) demonstrated that neonatal enteral feeding tubes are prone to rapid colonisation by a wide variety of opportunistic pathogens of the Enterobacteriaceae, irrespective of feeding regime. Indeed, the environmental contamination issue may be a greater challenge in the NICU. Equipment and environmental surfaces in the NICU are more likely to harbour a high diversity of bacteria associated with nosocomial infection in neonates (Hewitt et al., 2013). For example, one of the most frequent nosocomial infections is neonatal sepsis. The incidence of this infection occurring in neonatal intensive care units is increasing due extended periods of hospitalisation of infants (Sadowska-Krawczenko et al., 2012).

Infants dying within the first week of life is largely attributed to cross-infection through microbial contamination of inanimate surfaces, medical devices and the indoor environment (Sankar et al., 2016). However, babies born in developing countries are at a 3 to 20 times higher risk of bacterial infections compared to those born in developed

countries. This may be due to a lack of qualified hospital staff, unhygienic delivery care, poor availability of basic requirements such as sterilised tools, and lack of education (WHO, 2010).

Bacterial infections cause serious diseases such as meningitis, sepsis (early or late onset), pneumonia and other respiratory infections. For example, neonatal sepsis is a blood infection that represents a significant cause of neonatal mortality and morbidity globally, particularly in preterm infants with a very low-birth weight (< 1500 g) (Shah and Padbury, 2014; Cortese et al., 2016). Sepsis is known as a systemic inflammatory response to pathogenic infection, however, the presence of bacteria in the blood is termed bacteraemia (Christaki and Bourboulis, 2014). Early-onset sepsis (EOS) normally occurs during the intrapartum period due to the transmission of bacteria from mothers to infants during the intrapartum process. Whereas late onset sepsis type (LOS) is defined as infection that occurs beyond 72 hours from birth, as a result of environmental contamination and lack of hygiene by the care providers in the NICU. (Shah and Padbury, 2014; Cortese et al., 2016; Mohseny et al., 2018).

1.2. Bacterial exposure to neonates through Infant Formula

Breast milk is recognised as the best sole source of nutrition for infants (Martin et al., 2016). It is recommended by the WHO (2007) that infants are exclusively breastfed for the first six months of life, however, human milk may be associated with outbreaks of infection in NICUs through collecting, storing and handling, or contamination of medical equipment (Cossey et al., 2011; Mense et al., 2014; Keim et al., 2015). Despite the benefits of breast milk in that it contains important factors required for infant growth, infant milk formula can be used when breastfeeding is not sufficient, possible or desirable. However, powdered infant formula (PIF) product is an unsterile product. Although strict and regular control measures are in place for pathogens in the manufacture process, low levels of contamination by pathogens may still occur in PIF and cause outbreaks of disease. An example of such outbreaks was reported by Acker and colleagues (2001) in a NICU in Belgium, where preterm infants with low birth-weights were fed infant formula contaminated with *Cronobacter sp*ecies. Similarly, an outbreak that occurred in Spain (2008) was reported to be associated with the

consumption of contaminated infant formula (Rodrígue et al. 2010). Recently, another outbreak was reported by the European Centre for Disease Prevention (ECDC) and Control and European Food Safety (EFSA) in 2018. This French outbreak affected 39 neonates and was associated with the consumption of infant formula contaminated with *Salmonella* Agona.

The Gram-negative organisms, *Salmonella* and *C. sakazakii*, have received more attention due their association with contaminated PIF and tendency to cause neonatal infections (Barron and Forsythe, 2007; Holý and Forsythe, 2014). It is notable that *C. sakazakii* has been isolated from milk powdered infant formula from several countries such as China, Germany, Chile and Malaysia (Sani al., 2014; Akineden et al., 2017 Fei et al., 2017; Parra-Flores et al., 2018). Also, *C. sakazakii* strains have shown a strong ability to persist in PIF for more than 1 year (Osaili et al., 2009). Most of the *Cronobacter* species are thermo-tolerant, particularly *C. sakazakii*, which can survive the production process and can be found in PIF as a contaminant after the pasteurisation process (Osaili et al., 2009; Kalyantanda et al., 2015). However, other members of the Enterobacteriaceae have also been linked to infant infections by WHO-FAO microbiological risk assessments of PIF (FAO-WHO, 2004). Enterobacteriaceae members that have previously been found to be present in PIF include *E. hormaechei, E. cloacae, E. cowanii*, *E. coli, C. freundii*, *K. oxytoca*, and *K. pneumoniae* (Muytjens et al., 1988; Townsend et al., 2008b; Araújo et al., 2015). For instance, 32 out of 75 PIF samples collected from pharmacies in Italy were contaminated by Enterobacteriaceae, including *E. hormaechei and C. freundii* (Giammanco et al., 2011).

Acinetobacter species are also considered as a cause for concern and were later included under category B (FAO-WHO, 2006). A study by Araújo et al. (2015) indicated the presence of *Acinetobacter baumannii-calcoaceticus* complex in infant milk formula in Brazil. In another study by Cho et al. (2018), it was reported that 47 *Acinetobacter* isolates from milk powder samples in Germany were obtained directly from the end of the production line of a powdered milk producer company. Indeed, there are several studies have reported that of numerous bacteria have been isolated from PIF. For example, a study conducted by Forsythe et al. (2005) showed that the most common organisms isolated from PIF were members of the Enterobacteriaceae,

Clostridium perfringens, Bacillus cereus and *S. aureus*. However, a meeting by the FAO/WHO (2004) addressed and reported the potential risk of a variety of microorganisms in PIF, and in addition, the meeting had assigned two other categories of microorganisms in PIF. These categories were created on the basis of the evidence of a causal association between the presence of these microorganisms in PIF and illness in neonates. The reorganised categorisation is shown in Table 1-1. The persistence of pathogens in PIF is of concern due to the risk of neonatal infection by the ingestion of these organisms and the reduced immunocompetence of neonates (FAO-WHO, 2006).

Table 1-1 Categorisation of microorganisms in PIF depends on their strength of illness in infants.

This table was adapted from FAO/WHO, (2004), * in 2008, the name *Enterobacter sakazakii* was amended to *Cronobacter* genus

Rehydration of infant feeding formula with water at 70 °C or above, was recommended by WHO and FAO to decrease the proportion of viable bacteria. Nevertheless, this instruction has not been feasible for small volume feeds as required for premature neonates, and is not adopted by all countries (Holy and Forsythe, 2014). In recent years, contamination of PIF is more likely to occur due to the preparation environment or from the staff responsible for preparing the feed than from the manufacturing process (FAO/WHO 2004). However, feeding equipment can be a source of infection through collecting, storing and handling or contamination of equipment through inadequately cleansing. It is a potential health risk to neonates, particularly among premature babies in NICUs who lack a developed immune system, and are at greater risk.

1.3. Bacterial exposure through neonatal feeding tube

Enteral feeding tubes are medical insertion devices used to feed infants, in particular premature infants who are unable to swallow milk. These tubes must be sterile and as clean as other medical inserting devices. However, a previous study by Hurrell et al. (2009b) revealed the presence of pathogenic bacteria in nasogastric enteral feeding

tubes from infants on non-formula feeding regimes. This showed that infants expose to *Cronobacter* was not exclusively through the consumption of reconstituted infant formula, but exposure to such pathogens might also be associated with general feeding practices. The same authors also reported that neonatal enteral feeding tubes are prone to rapid colonisation by a wide variety of opportunistic pathogens of the Enterobacteriaceae, irrespective of feeding regime. The most common isolates included *E. hormaechei, E. cancerogenus, K. pneumoniae, S. marcescens, Escherichia coli, Cronobacter* species and *Yersinia enterocolitica* (Hurrell et al., 2009a; 2009b). Importantly, one quarter of all *E. hormaechei* isolated from neonatal enteral feeding tubes by Hurrell (2009a) were resistant to the 3rd generation cephalosporins, cefotaxime and ceftazidime. Another related study showed that *E. coli* K1 ST95 strains isolated from neonatal nasogastric feeding tubes possessed genes encoding numerous virulence traits associated with neonatal meningitis (Alkeskas et al., 2015). Similarly, a study by Ogrodzki et al. (2017), investigated bacterial colonisation in NEFTs and faecal samples. Their study showed that *E. hormaechei* and *E. faecalis* were recovered from both feeding tubes and faecal samples. The colonisation of the enteral feeding tubes by nosocomial bacteria and their impact on earlier gut colonisation of premature new-born babies was investigated in a recent study (Moles et al., 2015). The study indicated that *E. coli, K. pneumoniae, S. aureus, S. epidermidis, Serratia marcescens, E. faecium and E. faecalis* were the predominant isolates in faeces and milk samples amongst 4,000 bacterial strains recovered (Moles et al., 2015).

The risk of infection by such bacterial strains is a concern in the NICU. An observational study was conducted by Petersen et al. (2016) revealed that even within the first day of use, nasogastric feeding tubes were colonised by high amounts of bacteria. Their study showed that out of 94 tubes, 89% yielded more than 10^3 cfu /ml bacteria and 55% yielded potentially pathogenic Enterobacteriaceae and *S. aureus*. Although several studies have identified PIF contaminated by various pathogenic bacteria (Caubilla-Barron et al., 2007a; Iversen et al., 2008; Giammanco et al., 2011; Araújo et al., 2015), microbiological safety studies should not be limited to focussing exclusively on reconstituted infant formula but also on the risk practices associated with nasogastric tube feeding. This may reduce or prevent the potential risk of exposure to

other pathogenic bacteria that can carry antibiotic resistance. Additionally, heavy colonisation by pathogenic bacteria can be prevented by avoiding placement of enteral feeding tubes for prolonged periods.

1.4. The intestinal flora of neonates

The number of bacterial species that colonise the human gastrointestinal tract is estimated to be nearly 10^{12} bacterial cells. In the last decade, clinicians and scientists recognised that the microbiota has a number of important roles, particularly bacteria that reside in the gastrointestinal tract, which include host metabolism and protective functions (Bull and Plummer, 2014; Jandhyala et al., 2015; Thursby and Juge, 2017). Neonatal intestines are considered to be sterile prior to birth but after birth the neonate is exposed to different types of microbes. The initial colonisation is linked to microbes exposed from their mother, the type of delivery and the surrounding environment (Perez-Muñoz et al., 2017; Nagpal and Yamashiro, 2017). The gut microbiome during the early life of a new-born is significant and a critical window for immunological and physiological development. This period can be altered by many factors such as antibiotic use and diet, as indicated by a number of studies (Groer et al., 2014; Milowitz et al., 2015; Tanaka and Nakayama, 2017). The first food introduced to new-borns is milk and the feeding type is believed to directly impact on the initial gastrointestinal microbiota. For example, Bezirtzoglou et al. (2011) revealed an increase in numbers of *Bifidobacterium* cells among breast-fed infants when compared to formula-fed infants. However, very low birth weight (VLBW) infants develop a very different gut microbiome when compared to full-term infants. A study by Drell et al. (2014) indicated that the gut microbiota in low birth weight infants was dominated by facultative anaerobic bacteria *Staphylococcus spp*. and Enterobacteriaceae, with relatively low prevalence of bifidobacteria, *Bacteroides*, and lactobacilli.

Furthermore, it is likely that the environments of hospitals and homes are very different in terms of inhabitant microbes and microbial resistance. The VLBW infant often requires prolonged hospitalisation in the neonatal intensive care unit. Those infants have a higher chance of developing a hospital-associated gut microbial community due to prolonged exposure to this environment. A previous study by Brooks (2014) identified

that microbes that appear in the gut of premature infants are introduced from the neonatal intensive care unit. Bacterial gut colonisation has been associated with health and disease such as inflammatory bowel disease, metabolic disorders, and asthma (Carding et al., 2015). The colonising microbiota is likely to play an important role for metabolic development and development of the immune system of neonates (Tanaka and Nakayama, 2017; Nash et al., 2017).

1.5. Bacterial outbreaks in neonatal intensive care units.

Outbreaks in NICUs have become a major concern compared with other types of intensive care units (Gastmeier et al., 2007; Kumar et al, 2018). It is evident that medical microbiological research has become concerned with NICU outbreaks due to increased risk among premature babies (Gastmeier et al., 2007; Camacho-Gonzalez et al., 2015; Hensel et al., 2017). Various factors contribute to the onset of an epidemic, such as susceptibility of the neonates in the NICU as well as virulence of the pathogen.

Outbreaks are often caused by multidrug-resistant organisms (MDROs). These organisms are predominantly bacteria and were defined as "MDR was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories" (Magiorakos et al., 2011). Over the last few years, the incidence of MDROs has increased in NICUs, which is the general problem of healthcare associated infections (Hensel et al. 2017; Yusef et al., 2018).

However, several previous studies have identified an outbreak of multidrug-resistant *E. faecium* in NICUs, especially vancomycin resistant strains. For example, a previous study by Lee et al. (2012) reported that the VRE outbreak in the NICU had occurred through 2 different *E. faecium* clones in a University Hospital in Korea. Another outbreak was reported by Carmona et al. (2012), where five cases of VRE infection were identified, with 3 deaths in a tertiary-care paediatric intensive care unit of a University Hospital in Brazil. In addition, the members of the *Enterobacter cloacae* complex can be considered as nosocomial pathogens, and *E. hormaechei* is generally the most isolated nosocomial pathogen within this group (Paauw et al., 2008), for instance, an outbreak of *E. hormaechei* was responsible for septicaemia in premature infants caused by contaminated parenteral nutrition in Brazil (Campos et al., 2007).

A. baumannii is also considered as an important nosocomial pathogen, particularly of critically-ill patients, including those in the intensive care unit (Castilho et al., 2017). It was reported by Fiji Ministry of Health and Medical Services and WHO (2017) that *A. baumannii* was responsible for the death of 12 patients in the NICU of Colonial War Memorial Hospital in Suva, Fiji. The pathogen had been isolated from blood and cerebrospinal fluid (CSF) samples and multidrug-resistant strains were identified (Lyman, 2017). Similarly, sepsis outbreaks in NICUs caused by MDR *A. baumannii* were reported during a six-month period, affecting 17 neonates, with 6 deaths in University Clinical Centre Tuzla, Bosnia and Herzegovina (Softic et al., 2013).

Improving our understanding of opportunistic bacterial pathogens with respect to neonatal health risk and ability to prevent and manage such infections in the future is of importance. There is a need to trace the dissemination of opportunistic bacterial pathogens among immunocompromised infants. Pathogenic bacteria are able to cause infection and disease, whereas non-pathogenic bacteria are considered harmless. It is presumed that pathogenic bacteria harbour additional virulence features but this requires further study of disease isolates.

1.6. The ESKAPE pathogens and diversity of antibiotic resistance

The most common microorganisms that are mainly involved in multidrug-resistant (MDR) infections have been grouped together and called ESKAPE pathogens, (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species). This group represents an important cause of nosocomial infections which significantly contributes to morbidity and mortality (Santajit and Indrawattana, 2016; El-Mahallawy et al., 2016; Karlowsky et al., 2016). The common cause of nosocomial infections is due to bacteria characterised by potential drug resistance mechanisms (Santajit and Indrawattana, 2016). Antimicrobial resistance in these pathogens is one of the most serious global public health problems and it is most likely to increase as resistance profiles change in the near future (Santajit and Indrawattana, 2016). For instance, according to the first list of priority antibiotic-resistant bacteria “priority pathogens”, which was recently published by the WHO, *A. baumannii* was being selected as priority 1 and is able to cause severe

infections (Knols et al. 2016). *E. faecium* is another important species of the ESKAPE group and the fourth most common cause of hospital-acquired infections. (Deshpande et al., 2007; Silva et al., 2017). This group is also related to significant morbidity and mortality, particularly in critically ill or immunocompromised patients (Table 1-2).

Table 1-2 Pathogenic characteristics of ESKAPE pathogens (WHO 2011).

However, the ESKAPE group pathogens have recently gained further global attention due to increasing antibiotic resistance, highlighted by the WHO (2017). Indeed, the presence of pathogens belonging to this group is a major problem in the ICU setting, thus limiting therapeutic options. A previous study conducted by Llaca-Díaz, et al. (2013) reported that 64.5% of pathogens recovered from the ICU of a University Hospital in Mexico were from the ESKAPE group. The same authors also reported that *A. baumannii* (15.8%) were most frequently isolated among the ESKAPE pathogens and strains with high resistance to carbapenems (75.3%) were detected in this study. Also *E. faecium* vancomycin-resistant isolates were detected, which were found to harbour the *vanA* gene. Moreover, multidrug-resistant *Enterobacter spp*. were commonly isolated and this organism has been implicated in several outbreaks of sepsis in NICUs (Kartali et al., 2002; Torkaman et al., 2009; Stoesser et al., 2014). Phoon et al. (2018) investigated 358 swab and fluid samples collected from a Malaysian tertiary hospital. These samples were obtained from medical devices, Health care workers' hands, the patients' immediate surroundings, and toilets. Their results showed that ESKAPE organisms were widely dispersed across the hospital wards. The majority of the isolates were MDR of *S. aureus, Acinetobacter* species*, Enterobacter* species and *K. pneumoniae*.

During the last few decades, the spread of multidrug resistance among nosocomial pathogens has been one of the growing problems for public health (Wilson et al., 2002). According to WHO (2011), these resistant bacteria are able to combat attack by antimicrobial drugs, resulting in failure to respond to treatment which leads to persistence and spread of infections (World Health Organization, 2011). In the USA,

people are infected by antibiotic-resistant bacteria each year is estimated to be nearly two million, while annual deaths of approximately 23,000 are attributable to antimicrobial resistance. The annual deaths in both the US and Europe to antimicrobial-resistant infections are estimated to be 48,000 and 700,000, respectively (Hampton, 2013; Kuehn, 2014). The European Antimicrobial Resistance Surveillance Network (EARS-Net, 2017) have pointed out that the resistance percentages to antimicrobials vary widely across Europe. For example, it was indicated that *Acinetobacter* species have high variations of antibiotic resistance across Europe, it was reported that the Baltic countries generally have high resistance percentages, as well as from Southern and South Eastern Europe (European Centre for Disease Prevention and Control, 2017).

For *E. faecium*, the percentages of vancomycin resistance were generally high as reported in Eastern and South-Eastern European countries. For example, the percentage of vancomycin resistance of *E. faecium* (VRE) has increased significantly between 2012 and 2015 in European countries. It was noted that more than 20 isolates of VRE per year was observed in 12 out of the 26 countries. However, Methicillin-resistant *Staphylococcus aureus* (MRSA) have been reported from several parts of the world. The percentages ranging from zero to 57.2% were observed among invasive of *S. aureus* isolates across Europe (European Centre for Disease Prevention and Control, 2017). Indeed, MDR has been noted for both Gram-negative and Gram-positive bacteria. According to the information from the Centers for Disease Control and Prevention (CDC), the rapid increase in infection cases is due to MRSA, VRE and fluoroquinolone-resistant *P. aeruginosa*. Also, MDR *A, baumannii, Klebsiella* species and *E. coli* which have emerged as significant pathogens worldwide (Boucher et al., 2009). However, the infections are highly associated with a rise in morbidity and mortality of critically ill patients in intensive care units (Brusselaers et al., 2011). Of particular concern are preterm infants exposed to MDR bacteria during their stay in the NICU because most of these MDR bacteria are high-risk and often belong to the ESKAPE group (Moles et al., 2015). Over the past decade, MDR bacteria have been emerged as significant pathogens that cause sepsis in the NICU.

Pathogenic bacteria utilise a number of mechanisms in order to inactivate antibacterial agents such as the beta-lactamase enzyme (β-lactamase). Such enzymes can destroy the

amide bond of the β-lactam ring, to inhibit or inactivate the antimicrobial compound. β-lactamases can be divided into four classes, based on their chemical structure: penicillins, cephalosporins, carbapenems and monobactam (Ghafourian et al., 2014; Öztürk et al., 2016). For example, Hurrell et al. (2009) reported that many *E. hormaechei* strains were found to be resistant to the 3rd generation cephalosporins. In another study, antibacterial susceptibility was investigated to determine the prevalence of *E. faecium* and *E. faecalis*. This revealed that *E. faecium* presented resistance to vancomycin (16.7%), gentamicin (77.8%), erythromycin (88.9%), ofloxacin (44.4%), teicoplanin (19.4%), and augmentin (63.9%). In contrast, *E. faecalis* showed resistance to teicoplanin (27.7%) and to vancomycin (13.8%) (Adesida et al., 2017).

The nosocomial ESKAPE pathogens are known to use a wide range of antimicrobial resistance mechanisms, including antimicrobial resistance genes, drug inactivation, and increase in expression of efflux pump or changing cell permeability through porin loss (Santajit and Indrawattana, 2016). For example, carbapenemase producing *A. baumannii* strains show resistance to both colistin and imipenem. These strains carry oxacillinase serine β-lactamases (bla$_{OXA}$) and imipenem metallo- β-lactamases (blaIMP) (Higgins et al., 2013; Cayô et al., 2015). In addition, the multidrug efflux system AcrAB has been linked with fitness of clinical *E. cloacae* isolates. Efflux pumps can expel a broad range of antibiotics and also drive the acquisition of additional resistance mechanisms (Blanco et al., 2016). Antimicrobial-resistant pathogens therefore place a significant economic burden on healthcare systems due to their association with nosocomial infection and increased treatment costs.

1.7. Potential Virulence Associated Traits

Pathogenic bacteria can use a number of mechanisms to cause disease in human hosts. For example, bacterial haemolysins are enzymes that destroy red blood cells and they are classified into different types of haemolysins, including α-haemolysin, β-haemolysin and γ-haemolysin (Kong et al., 2016). Also, pathogens may express and secrete proteases, which have a number of key roles in the ability of several bacteria to infect the host and cause disease (Culp and Wright, 2017).

Moreover, phospholipase is a lipolytic enzyme that has been identified as a virulence factor in many bacteria. For instance, phospholipase D (PLD) is a known virulence factor in *A. baumannii*, and disrupting the PLD gene results in reduced resistance to human serum as well as a decreased capacity for invading epithelial cells (Jacobs et al., 2010). Furthermore, endotoxin or lipopolysaccharide (LPS) is one of most important bacterial virulence factors associated with Gram-negative bacteria (Arenas, 2012; Tejada et al., 2015). This toxin is essential in many Gram-negative pathogens such as *E. coli* and *Salmonella* (Zhang et al., 2013a). According to a study carried out by Townsend et al. (2007b), the LPS is a heat stable endotoxin, giving bacteria their ability to persist in PIF during the manufacturing process. Furthermore, this virulence factor can enhance the translocation of enteric pathogens from the intestinal tract to the blood brain barrier, which may lead to an increased risk of infection among infants especially those with undeveloped innate immune systems (Townsend et al., 2007b). However, the important features of success for the microorganism during pathogenesis depends on its ability to detect and respond to different environments through the natural course of infection of the host. The rapid adaptation of the pathogen to the new environment allows it to colonise and survive in hostile environments (Wilson et al., 2002). Also, pathogenic bacteria are able to express a wide range of molecules that assist attachment and invasion of the host cells. The characterisation of these strategies is important for the prevention and control of bacterial diseases. The current study is focused on understanding the mechanisms used by bacterial pathogens to cause infectious disease.

Formation of biofilms is also one of the important virulence factors, which is a critical feature for the development of clinical infection (Chen and Wen, 2011; Wu et al., 2015). It plays a significant role in colonisation by microorganisms, providing an opportunity for bacteria to become resistant to environmental stresses. Additionally, biofilm formation is believed to be involved in protecting the bacteria from antimicrobial treatment and host defences (Aparna et al., 2008; Chen and Wen, 2011; Jacqueline and Caillon, 2014). The formation of biofilm is of high concern in the food industry, as it may lead to spoilage and contamination of the food products (Hartmann et al., 2010). Similarly, biofilm has emerged as a significant clinical problem. Indeed, the ability of bacteria to form biofilms on medical devices such as nasogastric enteral feeding tubes (NEFTs) and intravascular

catheters is a major concern (Francolini and Donelli, 2010; Revdiwala et al., 2012; Percival et al., 2015). It is believed that bacterial biofilms would be capable of forming on medical device surfaces and are an important risk factor for microbial spreading within the host and increases the chance of disease (Kostakioti et al., 2013).

Iron is one of the most important nutrients for the host and pathogenic bacteria. In the human body, iron is rarely found as a free and unbound molecule, and is therefore one of the major limiting factors for pathogenic microorganisms. (Hasan et al., 2015). However, Iron chelation is a vital part of establishing infections and many pathogenic bacteria release siderophores to chelate iron from iron binding proteins. Siderophores are low molecular weight chelators and considered as a virulence mechanism in bacterial pathogenicity (Eijkelkamp et al., 2011; Penwell et al., 2012; Holden and Bachman, 2015; Petrik et al., 2017). Acquisition of iron is essential in host environments to help bacteria survive and persist in the host. To counter this, pathogenic bacteria use several iron acquisition mechanisms to obtain iron from the host (Penwell et al., 2012). For example, the most iron-chelating molecule among *A. baumannii* is acinetobactin. There are various siderophores among different microbial species, some of which are presented in Table 1-3, (Balagurunathan and Radhakrishnan, 2007).

Table 1-3 Most important bacterial siderophores

Strain	Siderophore
Escherichia coli	Enterobactin
Enterobacter spp.	Enterobactin and Aerobactin
Salmonella spp.	Aerobactin
Aeromonas hydrophila	Amonabsactin
Acinetobacter calcoacaticus	Acinetobactin
Aerobacter aerogenes	Aerobacin
Klebsiella spp.	Aerobactin
Mycobacterium tuberculosis	Mycobactin
Pseudomonas aeruginosa	pyochelin and Pyoverdin
Vibrio cholerae	Vibriobactin
Yersinia pestis	Yersniabactin
Staphylococcus aureus	Aureochelin
Klebsiella spp.	Aerobactin

1.8. The interaction of bacteria with mammalian cells

Diversity and abundance of microbes living in the gastrointestinal tract leads to the microbial interaction with the host cells. The nature of these interactions can have either beneficial or detrimental effects. A variety of factors of microbes can have effects on the host epithelium, including environmental exposure to microorganisms, nutrition and antibiotic therapy, as well as the colonisation of the neonatal gut with microbes during the early period of life (Abraham and Medzhitov, 2011).

The innate immune system constitutes the first line of defence against invasion of microorganisms and their spread inside the host (Mogensen, 2009; Hato and Dagher, 2015). Nevertheless, some opportunistic pathogens can invade and proliferate inside epithelial cells and cause infections (Kim et al., 2010; Ribet and Cossart, 2015). In addition, some bacteria are able to form biofilms which protect them from various environmental stresses (Limoli et al., 2015).

New-borns and infants are considered highly susceptible to diseases due to the vulnerability of their epidermal layer, which is considered to be part of the innate immune response. For example, for neonates at 34 weeks of age the skin barrier development increases and the epidermal maturation is complete, whereas skin of the premature infant is characterised by reduced functionality in the first 2 weeks of life. The outer epidermal layer in the premature infant differs from that of the full-term baby, only consisting of three layers in premature infants by the first few weeks, whereas the epidermal layer is composed of 16 layers in a full-term infant, which may increase the chance of invasion by harmful microorganisms in premature infants (Evans and Rutter, 1986; Oranges et al., 2015).

Although there is a presence of numerous host defence mechanisms used by the host immune system comprises numerous defences, some bacterial pathogens have the ability to avoid these defence systems (Webb and Kahler, 2008). In addition, pathogenic bacteria have the ability to adhere to, and in some cases, invade host cells and tissues. Many bacterial pathogens have developed methods of avoiding the defences of immune systems by secreting various components and toxins (Kim et al., 2010). For example, pathogens can express surface capsules to prevent antibody and complement deposition on its surface (Finlay and McFadden, 2006). Some bacteria have the ability to

produce capsules which provide protection from the host immune response and antibiotics (Cress et al., 2014). Capsules may provide resistance to desiccation, serum activity and have a significant role in avoiding phagocytic killing (Ogrodzki and Forsythe, 2015).

Bacterial attachment to the host cell is an important initial step in the interaction between a pathogenic microorganism and its host and is a vital stage towards bacterial pathogenesis (Krachler and Orth, 2013; Ribet and Cossart, 2015). Several virulence factors exist which contribute to the ability of bacteria to adhere to the host cell surface, which helps them colonise and persist in the host and cause disease. Adhesion is commonly mediated by fimbriae, which are one of the most distinguished virulence factors of the surface adhesions proteins. Bacterial fimbriae or pili are filamentous surface proteins that play a crucial role in the attachment to biotic and abiotic surfaces in both Gram-positive and Gram-negative bacteria (Berne et al., 2015). For example, curli fimbriae have been described to play a vital role in the adhesion of *E. coli* and *Salmonella* to host cells. Their presence is linked with severe human infections (Cordeiro et al., 2016).

Other studies have reported that curli fimbriae play an important role in biofilm formation in many species of bacteria, and they help mediate host cell adhesion and invasion (Lasaro et al., Kim et al., 2012; Sharma et al., 2016). In addition, flagellar biosynthesis-related proteins are generally recognised to play a role in colonisation and adhesion in pathogenic bacteria and are considered to be an important virulence factor (De Maayer and Cowan, 2016). In addition to fimbriae and pili, a wide range of bacterial surface molecules are required for mediating adhesion of microorganisms to the intestinal epithelium. Adhesive matrix molecules, termed MSCRAMMs (microbial surface components recognizing adhesive matrix molecules), are recognised by several studies to play an important role in the cellular adhesion of *E. faecium*. They also mediate the attachment of *S. aureus* and *S. epidermidis*. For instance, the Acm collagen-binding adhesin of *E. faecium* contributes to the pathogenesis of experimental endocarditis. EcbA, the *E. faecium* collagen binding protein A, and fibrinogen are able to recognise adhesive matrix molecules. Moreover, the *efaA* gene product was

hypothesised to be involved in cell wall adherence (Hendrickx et al., 2009; Sava et al., 2010; Soheili et al., 2014, Yang et al., 2016).

The first step for invading bacteria usually depends on the ability of the adhesion to host cells. Pathogens can cross host barriers and get access to deeper within the host tissues where they proliferate and can result in disease (Doran et al., 2014 Ribet and Cossart, 2015). Several pathogens have been described to have the ability to enter host cells. For instance, Krzymińska et al. (2009) reported the ability of *E. hormaechei* and *E. cloacae* strains isolated from human specimens to adhere and invade to human epithelial type 2 (HEp-2) cells. Additionally, some bacterial pathogens have developed the ability to interact with macrophages, whereas others can cross the blood brain barrier and invade brain cells. Extracellular invasion occurs once the microbes are able to cause damage to host cells and tissues by invading and spreading in the host while remaining outside of host cells. Whereas intracellular invasion occurs while a microbe invades and survives within the cells of a host tissue (Wilson et al., 2002).

Macrophages are an important class of phagocytes that are commonly involved in the host response to pathogenic bacterial invasion (Tracey, 2014; Sotolongo et al., 2012). Macrophages phagocytose and destroy invading microbes in order to protect the host body. Many bacterial pathogens have showed an ability to survive and replicate inside macrophages after invasion (Ribet and Cossart 2015). For example, previous studies revealed the ability of *C. sakazakii* isolates to survive and replicate within human macrophages represented by the U937 cell line model (Townsend et al., 2008a; Almajed and Forsythe, 2016).

Bacterial cytotoxicity is another significant virulence factor for the pathogenicity of many microorganisms. Their effect on the viability of the epithelial Caco-2 cells was also assessed through mitochondrial activity, using the MTT assay. This assay is commonly used to assess and analyse cell proliferation and viability, which is a well-known biological tool to measure cell mitochondrial activity. The assay measures the reduction of tetrazolium into formazan product by the mitochondria of viable cells. (Jo et al., 2015; Riss et al., 2013). The MTT test was applied by Almajed and Forsythe (2016) to clarify the cytotoxic effect of clinical *C. sakazakii* strains on the Caco-2 and HBMEC cell lines. It

was shown that majority of isolates were able to induce cell death of both cell lines. Similarly, the MTT assay was performed by Husain and co-authors (2016), who investigated the cytotoxic potential of glutaminase-free asparaginase produced by *E. cloacae* against Human Myeloid Leukemia (HL-60) cells.

However, the presence of such virulence traits, antimicrobial resistance, biofilm formation, haemolysin activity, siderophores (iron uptake) and ability to invade and adhere to mammalian cells, is likely to increase the potential risk factors to neonatal health. Therefore, the present study has focused on determining virulence traits of a bacterial clinical collection which may form a potentially higher risk to neonatal health in NICUs.

1.9. Identification and typing methods of pathogenic bacteria

Full identification of the microbes involved in human infection is one of the important tasks of a microbiology laboratory. This provides insight into their clinical evaluation and allows differentiation between causative organisms, as well as application of an effective treatment regime. One of the main concerns is that some conventional identification techniques are not very accurate. Nevertheless, agar plate detection remains the gold standard method, due to its potential for discriminating isolates in a mixed bacterial flora on an agar surface. Culture method techniques are well understood and routinely used for discrimination, but there are also some limitations in terms of sensitivity and specificity (Méndez-Vilas and Pereira, 2013). In recent years, several genotyping methods have received more attention and have been shown to be suitable for discrimination between pathogenic bacteria. These techniques improve the sensitivity and can be used to classify bacteria into groups of closely-related isolates based on genomic diversity.

Numerous methods can discriminate a large number of isolates, such as pulsed-field gel electrophoresis (PFGE). This technique is a DNA fingerprinting method which is considered as the gold standard technique for many years to discriminate between several pathogenic organisms. This method is one of the most discriminatory for typing of bacterial strains based on their genomic DNA pattern by digestion with a restriction enzyme. Different restriction enzymes can generally be used, such as *XbaI, ApaI* and

Ascal to cut sites within the genome into several fragments. Then these fragments can be separated using agarose gel electrophoresis (Bong, 2006; Caubilla-Barron et al., 2007). Therefor PFGE was the preferred methods and considered as the method of choice when it comes to outbreaks, and allows tracking of infection sources. For example, Zarrilli et al. (2007) used PFGE to investigate multidrug-resistant *A. baumannii* strains isolated from 74 patients, between June 2003 and June 2004 in a hospital in Naples, Italy. The result of this investigation showed that the isolates revealed two different pulsotypes. PFGE pattern 1 was found across 44 patients and then the same pulsotype was found identical to that of an epidemic clone isolated during 2002 in another hospital in Naples. However, this technique has some limitations due to the expense of the equipment, requirement of highly trained technicians, it is time consuming, and non-identical strains can often give the same profile (Klaassen et al., 2002; Unemo and Dillon, 2011).

Multilocus sequence typing (MLST) is a molecular typing technique which is considered to be one of the most reliable and informative methods. This is often seen as a complementary typing method to PFGE (Tomaschek et al., 2016). MLST has been applied in identification and clustering of many pathogenic microorganisms. This technique uses partial sequence analysis of multiple housekeeping genes that scattered throughout the genome of the organism. MLST approaches have been defined and adopted for many prokaryotic and eukaryotic microorganisms. Databases of thousands of MLST profiles and sequences are now available via online sequence database provides data for over one hundred microorganisms sequences and allelic profiles

(Chen et al., 2015; Jolley et al., 2017). Traditionally, MLST was achieved by PCR amplification of seven housekeeping genes based on the MLST scheme database followed by Sanger sequencing. However, the MLST method has been further developed in recent years using a high-throughput next-generation sequencing approach (NGS), which is rapidly replacing classical Sanger sequencing (Jolley et al., 2017).

Recently, whole-genome sequencing (WGS) of bacterial pathogens has become a very attractive and provide more data than traditional subtyping methods. WGS has widespread applications in research and routine analyses in the field of epidemiology

and outbreak surveillance (Kwong et al., 2015; Scott et al., 2017). Next-Generation Sequencing (NGS) has revolutionised genetics and genomics studies and has had a huge impact on research through the rapid development in the analysis programs in the field of genomics. The development of the NGS technology has made significant progress in recent years and provides a wealth of new information for our understanding of the basic biological knowledge of microorganisms. (Seifi et al., 2017). For instance, a number of studies revealed many advantages of WGS when compared to 16S rRNA sequencing. One of the major advantages of WGS is that it provides a more accurate species level identification. The limitation of the 16S amplicon method is that it sequences only a single region while WGS can sequence all regions of the entire bacterial genome (Kwong et al., 2015; Deurenberg et al., 2017; Singh et al., 2017). Indeed, it is difficult to distinguish among closely related species by using 16S rRNA gene due to the limited resolution of this method. A recent study by Ogrodzki et al. (2017) indicated that the 8 strains isolated from 2 different feeding tubes were identified as *E. faecalis* by using the 16S rDNA gene. However, genomic profiling following NGS confirmed that these strains formed two distinct clusters according to their sequence type and SNP analysis showing as closely related strains in tube and faeces.

Genotyping techniques including PFGE, 16S rRNA and MLST were applied in the present study for accurate identification and typing of pathogens. Moreover, whole-genome sequencing and single nucleotide polymorphism (SNP) analysis was also used, in order to determine the relatedness and track the source of these isolates. Furthermore, these techniques were used to determine the presence or absence of virulence-associated genes and antibiotic resistance genes to help provide a complete picture. However, different studies have been performed which have focused on bacterial potential pathogenicity on the NEFTs rather than to study the persistence of these pathogens and their multiplication within the feeding tubes and neonatal gut. These strains only provided a snapshot of single samples and lack descriptive metadata, such as patient information. Furthermore, no follow up samples for these isolates were available, and no information regarding the persistence of these isolates (whether in the NEFTs or intestinal tract of the babies), was available. The lack of this information made it

extremely difficult to determine whether the strains that infected the babies originated from NEFTs.

1.10. Study objectives:

In this study, the overall aim of the project was to profile a clinical collection of *A. baumannii* isolated from neonatal feeding tubes from NICUs in two separate hospitals in Jordan (chapter 3), and a collection of *Enterobacter* species, in particular *E. hormaechei* isolated from feeding tubes, CSF and blood samples (sepsis cases) of premature babies in NICUs (chapter 4). The present study focussed on phenotyping and genotyping analysis for these hospital strains to evaluate potential virulence traits and to determine important risk factors with respect to neonatal infections in NICUs. Additionally, a longitudinal study of twin babies with different feeding regimes in the NICU at QMC hospital, Nottingham (chapter 5), was performed to compare potentially pathogenic *E. faecium* isolates within and between twin pairs over time. Single nucleotide polymorphism (SNP) analysis was used, in order to determine the relatedness of strains isolated from feeding tubes and faeces from both babies over time. Furthermore, this part of the study aimed to determine whether some of the strains might have been transferred between the two babies or come from common source.

Chapter 2. Materials and Methods;

2.1. Safety consideration

Health and safety code of practice for microbiology level two containment laboratories at Nottingham Trent University was considered before carrying out any experiments and protocols in this project. The procedural COSHH forms and activity risk assessment were also completed and considered. Category 2 organisms and materials were disposed of according to the recommended instructions. For tissue culture laboratories health and safety regulations, hepatitis B antibodies and vaccination were assessed before starting any tissue culture experiments.

2.2. Ethics.

Ethical approval was given by NRES Committee East Midlands. This permitted the collection of feeding tubes, faeces and metadata, but did not permit the storage of human tissue, which by definition included faeces.

2.3. Sterilisation and aseptic techniques

All buffers, solutions, media, and equipment were decontaminated by autoclave sterilisation at 121°C under 15 psi pressure for 15 minutes, 121°C for 5 minutes, filtered using 0.2 µm pore size filters (Thermo Fisher Scientific, UK) or sprayed with 70% ethanol, as appropriate.

2.4. Bacterial storage

All the studied isolates and any additional positive and negative control strains were stored at -80°C and -20°C in TSB/ glycerol (80%) (Thermo Fisher Scientific, UK) for long term storage. When required, bacteria were recovered from frozen stock and subcultured on Tryptone Soya Agar (TSA) and incubated aerobically at 37°C for 18 h. For short term storage, bacteria were streaked on TSA and stored at 4°C.

2.5. General stock reagents and buffers

2.5.1. Phosphate buffered saline (PBS)

The PBS solution was prepared by dissolving one tablet of PBS (Sigma Aldrich, UK) into 100 ml of distilled water and autoclaved at 121°C for 15 minutes.

2.5.2. Saline Solution (0.85 %)

For preparing the 0.85% saline solution, one tablet of saline (Thermo Fisher Scientific, UK) was dissolved into 500 ml of distilled water and autoclaved at 121°C for 15 minutes.

2.5.3. Hydrochloric acid (HCl)

One molar hydrochloric acid (HCl) was prepared by adding 86ml of 37% HCl (Thermo Fisher Scientific, UK) to 914 ml of distilled water. The mixture was gently mixed and used to adjust the pH of solutions used in this project.

2.5.4. TRITON X-100 (1%)

In order to prepare 1% Triton X-100, 1 ml of TRITON X-100 (Thermo Fisher Scientific, UK) was added to 99 ml of distilled water, then autoclaved and then stored at room temperature until use.

2.5.5. 1 M Tris-HCl (pH8)

For preparing 1 M Tris-HCL, 60.55 g of Tris base (Thermo Fisher Scientific, UK) was dissolved into 400 ml of distilled water and then the mixture was adjusted to pH 8 by adding HCl and measured by pH meter (HANNA, USA). The volume was completed with distilled water to be 500 ml and then autoclaved at 121°C for 15 minutes.

2.5.6. EDTA (Ethylenediamine tetra-acetic acid, sodium hydroxide) (pH8)

The preparation of 0.5 EDTA was obtained by dissolving 93 g of EDTA (Sigma Aldrich, UK) into 400 ml of distilled water and the mixture then was adjusted to pH 8 by adding sodium hydroxide pellets (NaOH) (Sigma Aldrich, UK) and the pH was measured by a pH meter. The total volume of the mixture was made up to 500 ml and then it was autoclaved at 121°C for 15 minutes.

2.5.7. 10 X TBE Buffer (Tris base, boric acid and EDTA buffer)

The preparation of 10X TBE buffer was obtained by dissolving 108 g Tris base (Thermo Fisher Scientific, UK), 55 g boric acid (Thermo Fisher Scientific, UK) into 800 ml of distilled water. Once the chemicals were completely dissolved, 40 ml of 0.5 EDTA pH 8 was added and the mixture volume was adjusted to 1000 ml by adding distilled water. The prepared mixture was then autoclaved at 121°C for 15 minutes.

2.5.8. 1 X TAE buffer (1 X Tris-acetate-EDTA buffer)

In order to prepare 1X TAE buffer, 20 ml of 50X TAE buffer (National Diagnostics, UK) was diluted with 980 ml of distilled water. The 1X TAE diluted buffer was used for preparing agarose gel and filling the gel electrophoresis tanks.

2.5.9. Glycerol (80%)

The preparation of 80% of glycerol was completed by adding 80 ml of glycerol (Thermo Fisher Scientific, UK) to 20 ml of distilled water. The mixture was autoclaved and stored at room temperature until use.

2.5.10. Iron III Solution

This solution was prepared by dissolving 0.0027 g of FeCl3-6H2O (Sigma Aldrich, UK) into 10 ml of 10 mM HCl.

2.5.11. Chrome Azurol Sulphate (CAS) SOLUTION

CAS solution was prepared by mixing 0.06 g CAS (Sigma Aldrich, UK) with 50ml of sterile distilled water.

2.5.12. Hexadecyltrimethylammonium bromide (HDTMA)

Exactly 0.1458 g of HDTMA (H6268, Sigma Aldrich, UK) was dissolved in 80 ml of distilled water.

2.5.13. Sodium hydroxide solution.

This solution was prepared by dissolving 10 g of NaOH into 20 ml distilled water.

2.5.14. Lysozyme Solution (for Gram-positive bacteria only)

The instructions for stock solution of lysozyme were followed as explained by the manufacturer. To prepare 1 ml of Lysozyme Solution (L4919), were dissolved in 1 mL of Gram-Positive Lysis Solution (L7539). For each DNA preparation to be performed, 200 µL of Lysozyme Solution was required.

2.6. General culture media

2.6.1. Tryptone Soya Agar (TSA)

According to manufacturer's instructions for TSA (Thermo Fisher Scientific, UK) the media was prepared by dissolving 40 g TSA agar in 1 L of distilled water. The mixture was boiled to completely melt the agar and then autoclaved at 121°C for 15 minutes. The media was cooled to 50°C and then dispensed into petri dishes about 20 ml each. These plates were dried and stored at 4°C until use.

2.6.2. Trypticase soy broth (TSB)

Exactly 15 g of TSB (Thermo Fisher Scientific, UK) were dissolved into 500 ml of distilled water. This mixture was dispensed to 100 ml in separate bottles. These 100 ml bottles were autoclaved at 121°C for 15 minutes and then stored at room temperature.

2.6.3. MacConkey agar

To prepare 1 litre, 51.5 g of MacConkey agar (Thermo Fisher Scientific, UK) were added to 1 litre of distilled water. The mixture was boiled to completely dissolve the powder and then autoclaved at 121°C for 15 minutes after which it was cooled to 50°C and dispensed into petri dishes and stored at 4°C until needed.

2.6.4. Violet Red Bile Glucose Agar (VRBGA)

The medium was prepared by dissolving 38.5 g of VRBGA (Thermo Fisher Scientific, UK) into 1 litre of distilled water. The medium was boiled then cooled to 50°C to be dispensed into petri dishes.

2.6.5. Luria-Bertani agar (LBA).

The manufacturer's directions were followed to prepare Luria-Bertani Agar (Merck KgaA, Darmstad, Germany; 1102830). Thirty-seven gram were dissolved in 1 litre of water, mixed and then sterilized for 15 min at 121°C.

2.6.6. Xylose lysine deoxycholate agar (XLD)

Fifty-three grams of XLD (Thermo Fisher Scientific, UK) agar was added to 1 litre of distilled water and heated with frequent agitation until the mixture boiled. The medium was transferred immediately to a 50°C water bath and then poured into petri dish plates.

2.6.7. De Man, Rogosa, Sharpe Agar (MRS agar)

Sixty-two grams of MRS (Oxoid Thermo Fisher Scientific, UK) agar was added to 1 litre of distilled water. The mixture was boiled to completely dissolve the powder and then autoclaved at 121°C for 15 minutes after which it was cooled to 50°C, and dispensed into petri dishes.

2.6.8. Bifidus Selective Medium Agar (BSM-Agar)

55.5 g BSM-Agar (Sigma-Aldrich, 88517) was added to 1 litre of distilled water. The final pH was adjusted to 6.8 and then autoclaved at 121°C for 15 minutes. After cooling to 55°C, 116 mg BSM supplement (Cat. No. 83055; suspended in 5 ml sterile water) was added. Then the agar was mixed gently and dispensed into sterile Petri dishes.

2.6.9. Brain Heart Infusion Agar (BHIA) Brain Heart Infusion Agar (CM131B, Oxoid

Thermo Fisher Scientific) was prepared according to the manufacturer's instructions. Briefly, 47 g/ L of BHIA was suspended in 1 litre of distilled water, and sterilised at 121°C for 15 minutes. After cooling to 45-50°C it was mixed gently and dispensed into sterile Petri dishes. These were stored in the fridge at 4°C until required.

2.6.10. Calcofluor media.

Cellulose production was investigated by streaking the bacterial strains on supplemented LBA medium with Calcofluor White Stain (18909 Sigma, UK). Calcofluor media was prepared by dissolving 5 g of tryptone, 2.5 grams yeast extract and 7.5 g of technical agar (LP0013 Agar No 3; Oxoid Thermo Fisher; UK) in 500 ml of distilled water.

It was mixed until completely dissolved and autoclaved at 121°C for 15 minutes. Then it was cooled to 50°C. The agar was supplemented with 25 ml of Calcofluor White Stain (18909 Sigma, UK), and about 15 - 20 ml of media was poured into each sterile Petri dish. The plates were then stored in a dark and cool place for up to 2 weeks at 4°C, followed by incubation 18 h at 37°C and 48h at 30°C.

2.6.11. Tributyrin agar, base (activity).

The lipase activity assay was performed by using Tributyrin agar (pre-prepared Oxoid, UK). The pre-prepared agar was rehydrated and then poured into petri dishes. The petri dish plates were left at room temperature to dry for two to three days and after that strains were streaked on tributyrin agar and incubated for 72 h at 37°C. Each strain was evaluated for lipase activity by visual observation. A positive result was defined as a clear zone around the bacterial colonies.

2.6.12. Iso-Sensitest agar (ISA)

Exactly 31.4 g were suspended in 1 litre of distilled water and then the mixture was boiled to dissolve the agar and then autoclaved at 121°C for 15 minutes. The medium was cooled to 50°C and dispensed into petri dishes.

2.7. Identification methods and molecular typing

2.7.1. Bacterial strains and their sources.

A total of 100 strains were isolated from different clinical sources used in this study. Forty-three *A. baumannii* strains were used in this project, which were isolated from nasogastric enteral feeding tubes from neonates in NICUs, from Princesses Rahma Hospital (PRH) and King Abdulla Hospital (KAH) in Jordan. The strains had been collected from two hospitals between 15/7/2011 and 21/12/2011 as described in chapter 3. Twenty-six *Enterobacter* spp. were selected from the Nottingham Trent University culture collection, which had not previously been studied. These strains were isolated from Blood culture and CSF specimens. The collection were from sporadic cases at either Nottingham City Hospital (NCH) or Queen Medical Centre (QMC) collected during 7 years between 2009 and 2015. In addition, 7 strains from blood, nasogastric enteral feeding tubes and faeces samples from QMC in Nottingham were also included in the collection

as described in chapter 4. Further, 24 *E. faecium* isolates from two premature babies (twins) from the neonatal intensive care unit of Queens Medical Centre were studied, as described in chapter 5. The samples were collected from feeding tubes and faecal samples from both neonates at different times. The first sample was collected at 4 weeks of age and the last sample was collected at 18 weeks. The neonates were fed breast milk, fortified and pre-med formula during this period of time (chapter 5). Isolates information including source, site and date of isolation are presented in Table 2-1, Table 2-2 and Table 2-3

Table 2-1 *A. baumannii* strains used in this study isolated from enteral feeding tubes of babies in NICU at Jordan Hospitals (chapter 3).

NTU strain	Strain	Hospital	D.O.I	Country	Age (d)	Source	**Feeding information	*A	*B	*C	*D
1591	*A. baumannii*	PRH	15/07/2011	Jordan	12	FIF	bebelac/12 meal	X	X	X	X
1594	*A. baumannii*	KAH	01/12/2011	Jordan	8	Tube	neosure/6 meal	X			
1595	*A. baumannii*	KAH	01/12/2011	Jordan	8	Tube	neosure/6 meal	X	X	X	X
1596	*A. baumannii*	KAH	07/12/2011	Jordan	12	Tub	neosure/6 meal	X			
1597	*A. baumannii*	KAH	07/12/2011	Jordan	5	FIF	neosure/6 meal	X	X		
1598	*A. baumannii*	KAH	07/12/2011	Jordan	5	FIF	neosure/6 meal	X			
1599	*A. baumannii*	KAH	07/12/2011	Jordan	24	Tube	neosure/6 meal	X			
1600	*A. baumannii*	KAH	07/12/2011	Jordan	24	Tube	neosure/6 meal	X			
1601	*A. baumannii*	PRH	10/12/2011	Jordan	12	Tube	S26L/12 meal	X	X	X	X
1602	*A. baumannii*	PRH	10/12/2011	Jordan	12	Tube	S26L/12 meal	X			
1603	*A. baumannii*	PRH	10/12/2011	Jordan	12	FIF	S26L/12 meal	X			
1604	*A. baumannii*	PRH	10/12/2011	Jordan	12	FIF	S26L/12 meal	X	X	X	X
1605	*A. baumannii*	PRH	10/12/2011	Jordan	15	Tube	S26L/12 meal	X			
1606	*A. baumannii*	PRH	10/12/2011	Jordan	15	Tube	S26L/12 meal	X			
1607	*A. baumannii*	PRH	10/12/2011	Jordan	15	FIF	S26L/12 meal	X	X		
1608	*A. baumannii*	PRH	10/12/2011	Jordan	8	FIF	S26L/12 meal	X			
1609	*A. baumannii*	PRH	10/12/2011	Jordan	8	FIF	S26L/12 meal	X	X		
1610	*A. baumannii*	PRH	12/12/2011	Jordan	14	Tube	S26L/12 meal	X	X		
1611	*A. baumannii*	PRH	12/12/2011	Jordan	14	Tube	S26L/12 meal	X			
1612	*A. baumannii*	PRH	12/12/2011	Jordan	13	FIF	S26L/12 meal	X			
1613	*A. baumannii*	PRH	12/12/2011	Jordan	11	FIF	S26L/12 meal	X	X	X	X
1614	*A. baumannii*	PRH	12/12/2011	Jordan	13	Tube	S26L/12 meal	X			
1615	*A. baumannii*	PRH	12/12/2011	Jordan	13	Tube	S26L/12 meal	X	X	X	X
1616	*A. baumannii*	PRH	15/12/2011	Jordan	13	FIF	S26L/12 meal	X			
1617	*A. baumannii*	PRH	15/12/2011	Jordan	13	Tube	S26L/12 meal	X	X		
1618	*A. baumannii*	PRH	15/12/2011	Jordan	14	Tube	S26L/12 meal	X	X	X	X
1619	*A. baumannii*	NA	NA	Jordan	NA	NA	-	X			
1620	*A. baumannii*	PRH	15/12/2011	Jordan	10	Tube	S26/12 meal	X	X		
1621	*A. baumannii*	PRH	15/12/2011	Jordan	10	Tube	S26/12 meal	X			
1622	*A. baumannii*	PRH	15/12/2011	Jordan	10	FIF	S26/12 meal	X			
1623	*A. baumannii*	PRH	15/12/2011	Jordan	10	FIF	S26/12 meal	X	X	X	X
1624	*A. baumannii*	PRH	15/12/2011	Jordan	7	FIF	S26/12 meal	X	X	X	X
1625	*A. baumannii*	PRH	15/12/2011	Jordan	14	FIF	S26/12 meal	X			
1626	*A. baumannii*	NA	NA	Jordan	NA	NA	NA	X			
1627	*A. baumannii*	KAH	21/12/2011	Jordan	29	FIF	Similac (a)/6 meal	X			
1628	*A. baumannii*	KAH	21/12/2011	Jordan	47	FIF	neasure/6 meal	X	X		
1630	*A. baumannii*	KAH	21/12/2011	Jordan	16	Tube	Similac (a)/6 meal	X	X	X	X
1631	*A. baumannii*	KAH	21/12/2011	Jordan	16	Tube	Similac (a)/6 meal	X			
1632	*A. baumannii*	PRH	20/12/2011	Jordan	5	FIF	S26/12 meal	X			
1633	*A. baumannii*	PRH	20/12/2011	Jordan	12	Tube	S26/12 meal	X			
1634	*A. baumannii*	PRH	12/12/2011	Jordan	14	FIF	S26/12 meal	X			
1635	*A. baumannii*	PRH	20/12/2011	Jordan	7	FIF	S26/12 meal	X			
1636	*A. baumannii*	PRH	20/12/2011	Jordan	12	FIF	S26/12 meal	X	X		

NTU: Nottingham Trent University strain identity number. *A: Pulsed Field Gel Electrophoresis was performed. *B: Physiological characterization was performed. *C: whole-genome sequencing was performed. *D: tissue culture was performed. PRH = Princes Rahma Hospital. KHA = King Abdulla Hospital. NA = Data not available, D.O.I = Date of isolation. FIF = Flushed infant formula. ** = Various feed regime were in practices at the hospitals. These were various powdered infant formula from different manufacturers

Table 2-2 Enterobacter strains used in this study isolated from sepsis, enteral feeding tubes and faecal samples on NICU at Nottingham Hospitals UK (chapter 4).

NTU culture	Organism	Source	Year	Hospital	Country	*A	*B	*C	*D
1448	*E. hormaechei*	Neonatal sepsis	2010	QMC	UK	X	X	X	
1977	*E. hormaechei*	CSF	2013	QMC	UK	X	X	X	X
1988	*E. hormaechei*	Neonatal sepsis	2012	QMC	UK	X	X	X	X
1439	*E. hormaechei*	Neonatal sepsis	2010	QMC	UK	X	X	X	X
450	*E. hormaechei*	Neonatal sepsis	NA	QMC/NCH	UK	X	X	X	X
667	*E. hormaechei*	Neonatal sepsis	NA	QMC/NCH	UK	X	X	X	
668	*E. hormaechei*	Neonatal sepsis	NA	QMC/NCH	UK	X	X	X	X
460	*E. hormaechei*	Neonatal sepsis	NA	QMC/NCH	UK	X	X	X	X
665	*E. hormaechei*	Neonatal sepsis	NA	QMC/NCH	UK	X	X	X	
462	*E. hormaechei*	Neonatal sepsis	NA	QMC/NCH	UK	X	X	X	X
495	*E. hormaechei*	Neonatal sepsis	NA	QMC/NCH	UK	X	X	X	X
664	*E. hormaechei*	Neonatal sepsis	NA	QMC/NCH	UK	X	X	X	
663	*E. hormaechei*	Neonatal sepsis	NA	QMC/NCH	UK	X	X	X	
660	*E. hormaechei*	Neonatal sepsis	NA	QMC/NCH	UK	X	X	X	
666	*E. aerogenes*	Neonatal sepsis	NA	QMC/NCH	UK	X	X		
1987	*E. aerogenes*	Neonatal sepsis	2012	QMC	UK	X	X		
451	*E. aerogenes*	Neonatal sepsis	NA	QMC/NCH	UK	X	X		
457	*E. aerogenes*	Neonatal sepsis	NA	QMC/NCH	UK	X	X		
2254	*E. cloacae*	Neonatal sepsis	2013	QMC	UK	X	X	X	
604	*E. cloacae*	Neonatal sepsis	NA	QMC/NCH	UK	X	X		
662	*E. cloacae*	Neonatal sepsis	NA	QMC/NCH	UK	X	X		
602	*S. marcescens*	Neonatal sepsis	NA	QMC/NCH	UK	X		X	
461	*E. cloacae*	Neonatal sepsis	NA	QMC/NCH	UK	X	X		
661	*E. cloacae*	Neonatal sepsis	NA	QMC/NCH	UK	X	X		
2215	*E. cloacae*	Neonatal sepsis	2013	QMC	UK	X	X		
669	*C. sakazakii*	Neonatal sepsis	NA	QMC/NCH	UK				
3215	*E. hormaechei*	Feeding Tube	2015	QMC	UK		X	X	X
2316	*E. hormaechei*	Faecal	2015	QMC	UK		X	X	
2317	*E. hormaechei*	Faecal	2015	QMC	UK		X	X	
2318	*E. hormaechei*	Feeding Tube	2015	QMC	UK		X	X	X
2319	*E. hormaechei*	Faecal	2015	QMC	UK		X	X	
2320	*E. hormaechei*	Feeding Tube	2015	QMC	UK		X	X	X
2307	*E. hormaechei*	Neonatal sepsis	2015	QMC	UK		X	X	X

NTU: Nottingham Trent University strain identity number. *A: Pulsed Field Gel Electrophoresis was performed. *B: Physiological characterization was performed. *C: whole-genome sequencing was performed. *D: tissue culture was performed. QMC: Queen Medical Centre. NCH: Nottingham City Hospital. NA: Data not available.

Table 2-3 *E. faecium* strains used in this study isolated from enteral feeding tubes and faecal samples on NICU at Queen Medical Centre Hospitals UK (chapter 5).

NTU Strain	Organism	Neonate	Collection date	Sample source	Hospital	Country	Feeding regime	*A	*C	*B	*D
2367	*E. faecium*	1	23/07/15	Faecal	QMC	UK	pre-made formula + breast milk + fortifier		X	X	X
2368	*E. faecium*	1	10/08/15	Faecal	QMC	UK			X	X	X
2372	*E. faecium*	1	24/08/15	Faecal	QMC	UK			X	X	X
2375	*E. faecium*	1	15/09/15	Faecal	QMC	UK			X	X	X
2376	*E. faecium*	1	24/09/15	Faecal	QMC	UK			X	X	X
2379	*E. faecium*	1	20/10/15	Faecal	QMC	UK			X	X	X
2382	*E. faecium*	1	22/10/15	Faecal	QMC	UK			X	X	X
2393	*E. faecium*	1	10/08/15	NG tube	QMC	UK			X	X	X
2395	*E. faecium*	1	19/08/15	NG tube	QMC	UK			X	X	X
2397	*E. faecium*	1	08/09/15	NG tube	QMC	UK			X	X	X
2399	*E. faecium*	1	27/09/15	NG tube	QMC	UK			X	X	X
2401	*E. faecium*	1	01/10/15	NG tube	QMC	UK			X	X	X
2407	*E. faecium*	2	31/07/15	Faecal	QMC	UK			X	X	X
2409	*E. faecium*	2	16/08/15	Faecal	QMC	UK			X	X	X
2410	*E. faecium*	2	20/08/15	Faecal	QMC	UK			X	X	X
2413	*E. faecium*	2	10/09/15	Faecal	QMC	UK			X	X	X
2417	*E. faecium*	2	30/09/15	Faecal	QMC	UK			X	X	X
2418	*E. faecium*	2	16/10/15	Faecal	QMC	UK			X	X	X
2425	*E. faecium*	2	20/10/15	Faecal	QMC	UK			X	X	X
2430	*E. faecium*	2	16/07/15	NG tube	QMC	UK			X	X	X
2433	*E. faecium*	2	17/08/15	NG tube	QMC	UK			X	X	X
2436	*E. faecium*	2	08/09/15	NG tube	QMC	UK			X	X	X
2439	*E. faecium*	2	11/10/15	NG tube	QMC	UK			X	X	X
2462	*E. faecium*	2	12/10/15	NG tube	QMC	UK			X	X	X

NTU: Nottingham Trent University strain identity number. *A: Pulsed Field Gel Electrophoresis was performed. *B: Physiological characterization was performed. *C: whole-genome sequencing was performed. *D: tissue culture was performed.

2.7.2. Biochemical confirmation;

Suspected colonies were picked for confirmation by biochemical testing. The colonies selected based on colony morphology on each media, were first subjected to Gram staining, catalase test and oxidase test. After that, the presumptive isolates were subjected to various phenotypic and genotypic analysis.

2.7.2.1. Gram stain

Gram staining is a very crucial first step for identifying, differentiation and classifying bacteria either Gram positive or Gram negative. There are four components required for Gram staining. In the Gram stain procedure single pure colonies were heat fixed onto a clean and labelled microscope slide by passing it over a flame. Then the smear was flooded with primary crystal violet dye for about 45 seconds. Before applying Gram's iodine, the slide was washed off with sterile water or tap water to remove primary stain; the Gram's iodine was then added for about 25 seconds. The Gram's iodine was washed off with sterile water. Then ethanol 95% was used to wash the dye off. Finally safranin (red dye) was used for 45 seconds, the slide was gently washed off and then dried before it was viewed under a light microscope on the oil immersion 100 X objective lens. The Gram-staining components that were used in this study were from Remel™ Gram Stain Kit (Thermo Scientific, UK).

2.7.2.2. Oxidase test

Oxidase reaction was determined with filter paper saturated with N,N,N,N'-tetramethyl-p-phenylene diamine dihydrochloride (Alfa Products, Damers, MA). Isolates were streaked onto the filters with wooden applicator sticks. Purple to black discoloration within 10 seconds constituted a positive reaction

2.7.2.3. Catalase test

A well-grown 18-24 hours old culture on TSA was picked up and spread on clean glass slide, a drop of 3 per cent hydrogen peroxide (H_2O_2) (Thermo Scientific, UK) added on the smear and observed for the immediate development of bubbles which was considered as positive for catalase.

2.7.3. Phenotyping

Four different media were used to assess the diversity of the colony morphology of examined strains according to recovery on TSA, MRS, VRBGA and BSM-Agar.

2.7.4.Genotyping assays

2.7.4.1. Pulsed field gel electrophoresis (PFGE)

PFGE was used in order to determine whether there indistinguishable strains were collected from four different hospitals and the sites or departments. The experiment used has been standardised according to the CDC and PulseNet (2004) protocol. PFGE analysis for these collections was performed by using the three restriction enzymes *Xbal*, *ApaI* and *AscaI* (Promega, UK). CHEF-DR II system (BIO-RAD, Belgium) was used to separate the bands at 6V, 14°C for 20 h with initial and final switch of 5 and 50 sec respectively. BioNumerics software version 7.1 (Applied Maths, Belgium) was used to analysed DNA bands profiles. Less than 95 % of band similarity value was used to consider the isolates to be non-clonal (Tenover *et al*., 1995).

2.7.4.2. Tris EDTA Buffer (TEB)

TEB was prepared aseptically by diluting 10 ml of 1M Tris pH 8 and 2 ml of 0.5 M EDTA pH 8 with 988 ml distilled water. This buffer was used for making and washing the PFGE plugs.

2.7.4.3. Cell Suspension Buffer (CSB)

The preparation of CSB was obtained aseptically by diluting 10 ml of 1 M Tris pH 8 and 20 ml of 0.5 M EDTA pH 8 with 70 ml of distilled water.

2.7.4.4. Cell Lysis Buffer (CLB)

CLB was prepared aseptically by diluting 2.5 ml of 1 M Tris pH 8 and 5 ml of 0.5 M EDTA pH 8 with 42.5 ml distilled water. 1% Sarkosyl NL (N-Dodecanoyl-N-methylglycine sodium salt) (Sigma Aldrich, UK) was dissolved into the mixture before adding 20 mg/mL of proteinase K (Sigma Aldrich, UK).

2.7.4.5. DNA preparation in agarose plugs

The strains of interest and a marker strain *Salmonella* enterica serovar Typhimurium reference standard H9812 (NTU 732) were screened in this project. About two full loops of overnight culture on TSA of the isolates and reference strain were individually suspended into cell suspension buffer and centrifuged at 7000 rpm for 4 minutes. The

previous step was repeated two times and then, using the same buffer, the optical density (OD) of each bacterial suspension was adjusted to be between 1.35-1.5 at 610 nm. Exactly 400 µl of bacterial suspension was pipetted into a 1.5 ml Eppendorf tube and while they were incubated in water bath at 37°C for 10 minutes, 1% agarose gel in TEB was microwaved for melting and 0.5% sodium dodecyl sulfate (SDS) was added and the gel was left in 55°C water bath. Each Eppendorf tube was mixed with 25 µl proteinase K and then mixed with 400 µl of TEB agarose and immediately about 100 µl of this mixture was dispensed into five well plug molds. The plugs were transferred into 15 ml falcon tubes contains 5 ml of CLB and incubated in a shaking water bath at 50°C for 2 hours. After the period of incubation, the plugs were washed twice with 15 ml of warmed sterile distilled water and incubated in shaking water bath at 50°C for 15 minutes. Finally, the DNA plugs were washed three times into 15 ml warmed TEB in a shaking water bath at 55°C for 15 minutes and stored in 5 ml of TEB at 4°C until needed.

2.7.4.6. Digestion and Electrophoresis

Three restriction enzymes *Xbal*, *ApaI* and *Ascal* (Promega, UK) were used for digestion. About 2 millimetres of each plug was transferred into 2 ml Eppendorf tube contain 2 µl BSA, 20 µl 10X buffer D and 178 µl sterile distilled water and incubated in a water bath 37°C for 15 minutes. After the incubation, the content of tubes was removed and digestion was performed on each plug by adding 2 µl BSA, 20 µl 10X buffer D, 5 µl *Xbal* and 173 µl sterile distilled water and incubated in water bath at 37°C for 4 hours. While, two restriction enzymes *ApaI* and *Ascal* transferred into 2 ml Eppendorf tube contain 2 µl BSA, 20 µl 10X buffer B, 3 µl *ApaI* or *Ascal* enzyme and 176 µl sterile distilled and incubated in a water bath at 37°C for 16 hours (overnight). 1% agarose gel was prepared in 100 ml of 0.5XTBE buffer and kept in 55°C water bath. The restricted plugs were then loaded on a 15-tooth comb (BIO-RAD Laboratory; Belgium). The comb was inserted in the gel tray and the 1% agarose gel was carefully poured into the gel tray. After the gel solidified, the comb was removed, it was placed in an electrophoresis cell and covered with 2400 ml of 0.5X TBE buffer. The system used for PFGE was CHEF-DR II system (BIO-RAD, Belgium), it was performed for *Xbal* at 14°C, 6V for 20 hours, and initial and final switch was 5 and 50 seconds respectively. While for *ApaI* and *Ascal,* at 14°C, 6V for 20 hours, and initial and final switch was 5 and 50 seconds respectively.

2.7.4.7. Staining and Analysis

The gel was stained with ethidium bromide (Sigma Aldrich, UK) 0.1 µg/ml for 45 minutes, visualised under UV light and photographed using In Genius® gel documentation system (Syngene, UK). The DNA band profiles were analysed using BioNumerics software version 7.1 (Applied Maths, Belgium).

2.7.4.8. DNA extraction

GeneEluteTM kit (NA2110-1KT, Sigma, UK) was used for DNA extraction from target strains. The instructions were followed as explained by the manufacturer. The DNA concentration was confirmed by using a NanoDrop® ND-2000 UV-Vis spectrometer (Thermo Scientific, UK), and the DNA was stored at -20°C for up to 6 months.

2.7.4.9. PCR 16S rRNA Sequence Analysis

The partial sequencing of the 16S rRNA loci (528 bp) Table 2-4 was performed using primers described by (Ogrodzki and Forsythe, 2017). Cycling conditions were an initial denaturation at 95°C for 10 minutes; 30 cycles of denaturation at 95°C for 30 sec, primer annealing at 62.6°C for 30 sec, extension at 72°C for 45 sec; followed by a final extension step of 72°C for 10 minutes. Regarding to *E. faecium*, the partial sequencing of the 16S rRNA loci (520 bp) Table 2-4 was performed using primers described by (Jiménez et al., 2008). Cycling conditions were an initial denaturation of 94°C for 5 minutes; 25 cycles of 94°C for 30 seconds, primer annealing at 48°C for 30 seconds, extension at 72°C for 45 seconds and followed by a final extension step of 72°C for 4 minutes.

2.7.4.10. Multilocus sequence typing (MLST)

The MLST scheme is based on sequences of the conserved regions of the seven housekeeping genes, for *A. baumannii* using the MLST scheme database of the Institute of Pasteur (http:/pubmlst.org/abaumannii). Seven housekeeping genes of *A. baumannii* were amplified using the primers; *fusA* (elongation factor EF-G), *gltA* (citrate synthase), *pyrG* (CTP synthase), *recA* (homologous recombination factor), *rplB* (50S ribosomal protein L2), *rpoB* (RNA polymerase subunit B) and cpn60 (60-kDa chaperonin), as showed in Table 2-4. Polymerase chain reaction (PCR) amplification product was performed using PCR machine (Techne, UK). A total volume of PCR reaction was of 20 µl

composed of GoTaq® Green Master Mix (Promega, UK) which diluted with 50% SDW, 2% of primers and 1 µl of bacterial DNA. Reaction condition for all the MLST primers was as follows: initial denaturation at 94°C for 2 min; 35 cycles of denaturation at 94°C for 30 s; primer annealing at 50°C for 30 s; extension at 72°C for 30 s; followed by a final extension step of 72°C for 5 min. With regards to the MLST analysis *E.* hormaechei and *E. faecium* isolates, the MLST interface on Centre for Genomic Epidemiology (CGEMLST) database was used to analyse MLST sequence types using whole-genome data (https://cge.cbs.dtu.dk/services/MLST-2.0/). Sequence files were uploaded onto the server in order to determine allele identity and sequence types.

2.7.4.11. Screening for *rpoB* gene for *Acinetobacter* isolates

For screening of RNA polymerase β-subunit (*rpoB*) gene was performed as described by Gundi et al. (2009). DNA. GoTaq® Green Master Mix (Promega, UK) was diluted to 50% with sterile distilled water and 2% of primers showed in Table 2-4, was added. One microliter of DNA of each isolate was added into 19 µl of master mix with 2% primer and the PCR condition was as following: initial denaturation at 94°C for 2 min; 35 cycles of denaturation at 94°C for 30 second; primer annealing at 52°C for 30 second; extension at 72°C for 1 min; followed by a final extension step of 72°C for 7 min.

2.7.4.12. Screening for *fusA* gene for *Enterobacter* isolates

Sequence typing of the *fusA* allele was performed according to the information in MLST database http://www.pubmlst.org. DNA. GoTaq® Green Master Mix (Promega, UK) was diluted to 50% with sterile distilled water and 2% of primers showed in Table 2-4, was added. One microliter of DNA of each isolate was added into 19 µl of master mix with 2% primer and the PCR condition was as following: initial denaturation at 94°C for 90 second; 40 cycles of denaturation at 94°C for 10 second; primer annealing at 55°C for 20 second; extension at 72°C for 50 second; followed by a final extension step of 72°C for 5 min.

2.7.4.13. Screening of OXA-51-like β-lactamases

The screening of *bla*OXA-51-like gene was performed as described by Feizabadi et al. (2008). One microliter of DNA of each isolate was added into 19 µl of master mix with

2% primer and the PCR condition was as following: initial denaturation at 94°C for 4 min; 34 cycles of denaturation at 94°C for 45 second; primer annealing at 52°C for 45 second; extension at 72°C for 30 second; followed by a final extension step of 72°C for 10 min.

2.7.4.14. Screening of Phospholipase Genes

The PCR method was used to screen for phospholipase A, C and D. Primers were synthesized and designed by Eurofins Genomics. The polymerase chain reactions (PCR) were applied: Initial denaturation 95°C for 5 min followed by 95°C for 1 min, annealing ranged from 52.5 to 64.5°C for 1 min (according to the probes as shown in Table 2-4.), and extension at 72°C for 1 min, final extension at 72°C for 5 min. The numbers of cycles of these PCRs were 35.

Table 2-4 Primers used to amplify 16S rRNA, seven housekeeping genes and virulence factor genes for *A. baumannii*

Primer name		Primer Sequence	Product size(bp)	Annealing Temp. (°C)
rplB	F	GTAGAGCGTATTGAATACGATCCTAACC	330	55
	R	CACCACCACCRTGYGGGTGATC		
pyrG	F	GGTGTTGTTTCATCACTAGGWAAAGG	297	55
	R	ATAAATGGTAAAGAYTCGATRTCACCMA		
gltA	F	AATTTACAGTGGCACATTAGGTCCC	722	55
	R	GCAGAGATACCAGCAGAGATACACG		
fusA	F	ATCGGTATTTCTGCKCACATYGAT	633	55
	R	CCAACATACKYTGWACACCTTTGTT		
cpn60	F	ACTGTACTTGCTCAAGC	479	55
	R	TTCAGCGATGATAAGAAGTGG		
rpoB	F	GGCGAAATGGCAGTGAAGAACCA	456	55
	R	GAAGTCCTTCGAAGTTGTAACC		
recA	F	CCTGAATCTTCYGGTAAAAC	372	55
	R	GTTTCTGGGCTGCCAAACATTAC		
rpoB	F	TAYCGYAAAGAYTTGAAAGAAG	350	55.5
	R	CMACACCYTTGTTMCCRTGA		
fusA	F	GCTGGATGCGGTAATTGA	438	58.0
	R	CCCATACCAGCGATGATG		
OXA-51	F	TAATGCTTTGATCGGCCTTG	353	55.3
	R	TGGATTGCACTTCATCTTGG		
plA	F	GCCGACACCTTAGCCCCTGT	870	52.5
	R	TCCGCGCAGCTTGCCACTAA		
plC	F	AACCCAGCAACGGCAGACCG	390	64.5
	R	GACCCAACCGCCACGACTCC		
plD	F	GGTTGCGCTTATTGGTGGGCG	410	62.7
	R	ACGCCGAACGTGGGTCAAAGT		
16S rRNA	F	AGAGTTTGATCCTGGCTCAG	520	48
	R	GGCTGCTGGCACGTAGTTAG		

2.7.4.15. Confirmation of PCR products by agarose gel electrophoresis

Agarose gel was used to visualize the amplified and size of PCR products. The gel was prepared as follows: 1% (w/v) agarose (Fisher Scientific, UK) was made up in 1X Tris-acetate-EDTA (TAE) buffer (Geneflow, UK). The solution was heated in a microwave oven to dissolve the agarose. 0.1 µ/ml (v/v) of SYBR® Safe DNA gel stain (Life Technologies –

Invitrogen, UK) was added and dissolved well in the agarose solution. The gel was then dispensed into the tray of gel and left for 20 to 30 minute to cool. After making wells, 10 µl of 1kb ladder (Promega, UK) was loaded as marker into two wells and 10 µl PCR products were loaded into each of the well. Gel electrophoresis was performed for 40 minutes at 100 V in 1X TAE buffer. The gel was then visualized under ultraviolet (UV) light. DNA bands were observed using the InGenius® gel documentation system (Syngene, UK).

2.7.4.16. Purification of PCR product

The amplified products were purified using the MinElute PCR Purification Kits (Qiagen, UK) according to the manufacturer's protocol. The concentration and purity of the samples was checked by using the Nano drop 2000 (Thermo Scientific, UK). The purity of DNA samples with minimum 260/280 nm values of 1.8 and concentration with minimum 260/230 nm values of 15 ng were used. The products were finally eluted in 50 µl of molecular biology grade water (Fisher Scientific, UK).

2.7.4.17. PCR product sequencing

PCR products were Sanger sequenced by Eurofins MWG Operon (London, UK) and source Bioscience (Nottingham, UK). The nucleotide sequences were determined on each DNA strand using nested sequencing primers.

2.7.4.18. Whole Genome Sequencing

Bacterial DNA was extracted from 1-day old cultures using GenElute bacterial genome kit (Sigma Aldrich, UK) using the manufacturer's protocol. Genomes were sequenced on an Illumina MiSeq by Pauline Ogrodzki. Genome comparisons were applied to find the key physiological and virulence genes of the organisms such as environmental stress, attachment, invasion, capsule, cellulose, curli fimbriae and antimicrobial resistance genes. This comparative analysis was performed using WebACT comparative tool, Artimes comparative tool (ACT) for genome alignment, which has been developed by Carver *et al*. (2005). In addition, BLAST searches were performed using NCBI BLAST research facility at; https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_ TYPE=BlastSearch&LINK _LOC=blasthome. A collaborative approach was taken in this

study, and credit must be given to my colleague Pauline Ogrodzki (NTU), who carried out the whole-genome sequencing, as part of a parallel PhD study.

2.7.5. Physiological assays

Based on PFGE analysis and associated neonatal metadata, representative isolates were selected for further characterisation of various virulence traits.

2.7.5.1. Haemolysis reaction

Isolates were plated on 5% horse-blood (Oxoid Fischer Scientific, UK) agar and incubated at 37 °C for 24 h. The resultant colony morphology was recorded after 24 h to determine the formation of either α-haemolysis, γ-haemolysis or β-haemolysis. *Staphylococcus aureus* NCTC 10788 was used as a positive control for β haemolysis, *Streptococcus pneumoniae* was used as a positive control for α haemolysis and *Staphylococcus epidermidis* was used as a negative control for λ haemolysis.

2.7.5.2. Iron siderophore detection

Siderophore detection assay was performed as described by (Shin *et al*., 2001). Two solutions were used to prepare the Chrome azurolsulphate (CAS) agar. The first solution which is a dark blue liquid was prepared by using 10 ml of iron III solution (Section 2.5.10), 50 ml of CAS solution (Section 2.5.11), and 40 ml of HDTMA (Section 2.5.12) and then autoclaved at 121°C for 15 minutes, (the total volume of dark solution is 100 ml). The second solution was prepared by mixing 900 ml of DW, 15 g agar, 30.24 g PIPES (Sigma Aldrich, UK) and 12 g NaOH and then autoclaved at 121°C for 15 minutes. After autoclaving, the first solution was mixed with the second solution and then the media was dispensed into the petri dishes. Immediately before use, 5mm diameter holes were punched into the agar using 5mm diameter gel plug cutter. The bacterial suspension was prepared by inoculating five colonies from TSA culture into 10 ml TSB broth containing 200 µM of 2,2´-dipyridyl (Sigma Aldrich, UK) and incubated at 37°C in shaking incubator at 200 rpm for 20 h. Then the samples were centrifuged at 5000 rpm for 10 minutes and 70 µl of the supernatant was placed into a specific hole. The agar was incubated at 37^{o}C for up to 8 hours and observed for the presence of an orange zone around the hole which indicated that the strain is positive for siderophore production. Ye*rsinia*

enterocolitica strain 1880 and *C. sakazaki* 520 were used as positive and negative controls respectively.

2.7.5.3. Protease activity

Ten percent skimmed milk powder (Thermo Fisher Scientific, UK) was used to prepare the skimmed milk solution (SMS) and autoclaved for 5 minutes at 121°C. PCA plate count agar was prepared by adding 22.5 g to 1 litre of distilled water and autoclaved at 121°C under 15 psi pressures for 15 minutes. 20 ml of 10 % SMS was aseptically mixed with 980 ml of PCA and then dispensed into Petri dishes to be stored at room temperature for 2 days. The tested strains were streaked on the dried plates and incubated at 37°C for 72 h and monitored every 24 h. *Bacillus cereus* was used as a positive control while *E. coli* DH5α NTUCC407 was used as a negative control.

2.7.5.4. Cellulose production

LB agar without salt was prepared and supplemented with calcofluor white stain (Fluka, UK) as mentioned in 2.6.10. The medium was poured in petri dishes and left to dry for 2 days. Representative strains were streaked and incubated either at 37°C for 24 h and then the cellulose production was visualised by using ultraviolet light at 366 nm.

2.7.5.5. Congo red morphotype

To prepare LB agar without salt, 5 g of tryptone (Thermo Fisher Scientific, UK), 2.5 g of yeast extract (Melford laboratories, UK) and 7.5 g agar (Thermo Fisher Scientific, UK) were mixed in 500 ml distilled water and then autoclaved. Congo red solution dye was prepared by dissolving 32 mg of Congo red powder (Sigma Aldrich, Uk) in 8 ml sterile distilled water (SDW) and the solution was filtered with 0.20 µm pore filter. After this, LB agar without salt was cooled to 55°C, 5 ml of filtered Congo red solution was added, gently mixed and dispensed into petri dishes.

2.7.5.6. Biofilm Formation

The biofilm formation was performed according to the crystal violet (CV) method as described by Zhang et al., (2004) with some modification. Eighteen hour TSB cultures were used to inoculate 5 ml of TSB and adjusted to O.D = 0.3 reading at 600 nm. In three 96 plates, 600 µl of each inoculated TSB was dispensed into 3 wells (200 µl into each

well) of each 96 well plate. Also liquid infant formula (Cow and Gate Premium 1) was used instead of TSB to detect and compare the ability of examined isolates forming biofilm in both media. After measuring the O.D of TSB culture the milk was adjusted to a cell density of an O.D = 0.3. Loading plates were incubated for 24 h at 25°C and 37°C. After 24 hours of incubation the plates were emptied from the TSB or the milk and washed twice with sterile distilled water (SDW) the plates then left for 10 minutes at room temperature to dry. Two hundred microliter of 1% crystal violet (CV) was added to each well and left for 30 minutes after which all the wells were washed three times with SDW. Finally, 200 µl of absolute ethanol was added to each well and after 15 minutes the content of the plates was transferred into new plates and read using ELx800 absorbance microplate reader (BioTek, UK) at absorbance 600 nm.

2.7.5.7. Capsule production assay

For detecting the ability of the strains to produce capsule, two types of media were used; formula whey (Cow and Gate Premium 1) and soy-based (Cow and Gate infasoy). For preparing milk agar 800 ml of Plate Count Agar (PCA) was made according to the manufacture of instructions. Gently 200 ml of milk (20% final concentrated) was added to PCA and mixed and the media then dispensed into petri dishes. All plates were left at room temperature for drying. The test strains were plated on the PCA with milk agar and incubated at 37°C for 24h.

2.7.5.8. Serum resistance

A single isolated colony was inoculated into TSB and incubated at 37 °C for 24 h with shaking. The bacterial suspension was then diluted 1:10 in new TSB and incubated under the same conditions for 2 h. Forty microliters of the bacterial culture were added to 360 µl of 50% human serum (Sigma, UK). Strains were compared with negative and positive controls which were *Escherichia coli* K-12 and *Salmonella* Eenteritidis respectively. The remaining viable bacterial cells after 24 h were counted using the Miles and Misra technique. All strains were tested in triplicate and in three independent experiments.

2.7.5.9. Acid tolerance

Acid tolerance was studied following the method of Edelson-Mammel *et al*. (2006) with some modification. One colony from a fresh TSA plate was used to inoculate 5 ml of TSB and incubated in shaking incubator for overnight at 37°C. To mimic the stomach acid, the pH of a liquid infant formula (Cow and Gate Premium 1) was adjusted to pH value of 3.5 units with 1 M of hydrochloric acid. One millilitre of the bacterial cell suspension was inoculated into acidified infant formula at pH 3.5 in a water bath at 37 °C. Viable cells were enumerated after 0, 15, 30, 60, 90 and 120 min using the Miles and Misra technique. All experiments were performed in triplicate from separate overnight cultures.

2.7.5.10. Heat tolerance

Thermal resistance of representative strains were investigated at 55°C. The heat tolerance of tested strains were determined as described by (Breeuwer et al., 2003) with some modification. Briefly, 1 ml overnight culture was suspended in 9 ml of temperature equilibrated sterile liquid infant formula (Cow & Gate Premium 1) in a water bath at 55°C. At zero time 20 µl of suspension were transferred to one well of the 96 well plates. This was repeated every 10 minutes for 30 minutes. Serial dilutions of the bacterial suspension (20 µl) were diluted in saline to 10^{-7}. The Miles and Misra enumeration technique on TSA plates was used to determine the number of cells surviving exposure to 55°C. The number of survivors at this temperature was plotted against time. The best fit-line was extrapolated and the D values were determined (-1/slope of the regression line). Each single number presented in this thesis is an average of three replicate experiments. The standard deviations of the D value were calculated.

2.7.5.11. Desiccation stress

All strains were grown on TSA plates overnight at 37°C. A single colony from a purity TSA plate was used to inoculate 5 ml of TSB and incubated in shaking incubator overnight at 37°C. The overnight bacterial growth was diluted to 1:10 using liquid infant formula (Cow and Gate Premium 1) and the bacterial cell density was approximately 10^9 CFU/ml. Aliquots 0.2 ml of the suspension were transferred into six-well plates and air-dried overnight in a class II cabinet at room temperature (20 to 25°C) as described by Caubilla-Barron and Forsythe (2007). After desiccation, the reconstitution time points were 24 h

and 14 days. The test strains were re-suspended in 0.2 ml of sterile water and viable counts were determined in triplicate using the Miles and Misra method on TSA.

2.7.5.12. Antimicrobial susceptibility assay

Susceptibility to antimicrobial agents were tested by the Kirby-Bauer method, as described by British Society for Antimicrobial Chemotherapy guidelines (BSAC, 2015). The antibiotics tested were augmentin, imipenem, meropenem, ceftazidime, ampicillin, chloramphenicol, gentamicin, tobramycin, cefotaxime, vancomycin, doxycycline and ciprofloxacin. ESBL production was determined by the combination of cefotaxime + clavulanate, cefpodoxime + clavulanate discs according to the manufacturer's instructions (Mast Diagnostics, Bootle, United Kingdom). Four colonies of fresh TSA culture were suspended in 3 ml of sterile normal saline and the OD adjusted to be equivalent to a 0.5 McFarland standard. Suspensions were swabbed onto ISA (OXOID, UK) and then the antibiotic disks were applied onto the surface of the ISA plates. The plates were incubated at 37 °C for 20 h. The diameters of zones of inhibition were measured and interpreted according to the BSAC Protocol (2015). *Escherichia coli* strain 10418 and *Escherichia coli* 13353 were used as negative and positive controls respectively.

2.7.6. Determination of bacterial pathogenicity using tissue culture

2.7.6.1. Bacterial strains

Salmonella Eenteritidis strain number NCTC 3046 358 and *Citrobacter koseri* strain number SMT319 48, were used as positive controls for HBMEC and Caco-2 cell lines. *E. coli* K12 MG1655 was the negative control for all cell lines. A single colony of each test and control strain was inoculated into 5 ml of TSB and incubated in a shaking incubator at 200 rpm at 37°C for 18 h. 120 µl of overnight culture was added to 5 ml of appropriate infection culture media as described below in Section 2.7.6.2 and incubated for a further 2 h to reach the OD of 0.3-0.5 at 600 nm using the spectrophotometer (JENWAH, UK), to obtain $4x10^6$ cfu/ml, which equated to a multiplicity of infection (MOI) 1:100 on the cells.

2.7.6.2. Culture Media for Caco-2 and HBMEC cell lines

All media and reagents for cell culture were obtained from Sigma Aldrich, UK unless otherwise stated. Growth medium for Caco-2 cells was Minimum Essential Medium (MEM) with 10% fetal bovine serum (FBS), 1% non-essential amino acids solution and 1% Penicillin-Streptomycin. HBMEC cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 1% non-essential amino acid solution and 1% Penicillin-Streptomycin. The infection medium for the two cell lines was same as the growth medium but lacking the 1% Penicillin-Streptomycin.

2.7.6.3. Human cell lines

Human eukaryotic cell lines were used to determine the bacterial adhesion and invasion ability as shown in Table 2-5. All cell lines were stored in liquid nitrogen. When a cell line was required, a vial was taken from the liquid nitrogen and thawed quickly. The content of the cell line tube was mixed into 6 ml of suitable pre-warmed growth medium, as explained above in Section 2.7.6.2, in a 15 ml falcon tube. The tube was then centrifuged at 1200 rpm for 5 minutes to harvest the cells. After discarding the supernatant, the cell pellet was re-suspended into 6 ml pre-warmed growth medium and transferred into a 25 cm^3 tissue culture flask. Finally, the flask was incubated for 48 hours at 37°C in the presence of 5% CO_2. After the cell line achieved a confluent monolayer, the medium was decanted out and the cells were detached using 5 ml of TrypLe™ express (Life Technologies, UK). The cell suspension was mixed with 5 ml pre-warmed growth medium and centrifuged at 1200 rpm for 5 minutes. The supernatant was discarded and the cell pellet was re-suspended in pre-warmed growth medium and then transferred into a new 75 cm^3 tissue culture flask containing 20 ml of pre-warmed growth medium. The cell lines were maintained and split routinely twice a week to keep the continuity of growth.

Table 2-5 Cell lines used in this study.

Cell line	Reference code	Source
Human colonic carcinoma epithelial cells (Caco-2)	ECACC #86010202	European Collection of Cell Cultures
Human brain microvascular endothelial cells (HBMEC)	#P10354	American Type Culture Collection
Macrophage cell line (U937)	ATCC#CRL-1593.2	American Type Culture Collection

2.7.6.4. Determination of the bactericidal concentration of gentamicin

Before tissue culture assays could be carried out, preliminary experiments were needed in order to determine the susceptibility of test strains to gentamicin as this is used to discriminate between attached and internalized bacterial cells. Strains were tested at the different gentamicin concentrations. Due to the resistance of these strains, this concentration was increased to 125 µg/ml and strains were re-tested. The antibiotic susceptibility testing was conducted in accordance with the (BSAC guidelines, 2015). The inhibitory effects of the gentamicin were determined by the micro-dilution method (BSAC, 2015). Bacterial cultures were grown in TSA for 18-20h at 37°C. Their turbidity was adjusted to McFarland Standard 0.5 (~$1.5x10^8$ cfu /ml) before being diluted to 1:10 and 1:100. Equal volumes of these dilutions and the gentamicin solution were added at a final concentration of 31.2, 62.5, 125 and 250 µg/ml to 96-well plates. Blank bacterial suspensions (without gentamicin) were also included as controls for each strain. Plates were incubated for 20h at 37 °C. Aliquots (~10 µl) of these overnight suspensions were inoculated into TSA plates. The presence or absence of growth recovery was checked 24 h later. Duplicate results were recorded for each strain.

2.7.6.5. Attachment to human cells

For Caco-2 and HBMEC the cells were grown as described above in Section 2.7.6.3. The adhesion assay was conducted as described by Townsend et al., (2008b). The cells were seeded into 24-well plates (Sarstedt, Germany) at a concentration of $4x10^4$ cells/well in growth medium and incubated at 37°C under 5% CO_2 for 48 h to achieve a confluent monolayer. After achieving the confluent monolayer, the bacterial strains of interest and control strains were grown as described in Section 2.7.6.1. Bacterial suspension was added to the wells at a concentration of $4x10^6$ cfu /well, which equated to an MOI of

1:100 on the cells, and 24-well plates were incubated at 37°C with 5% CO_2 for 2 h. After the incubation period, the plates were washed 3 times with PBS (Sigma Aldrich, UK) to remove unbound bacteria, and attached bacterial cells were released by lysing the human cells with 1% Triton X-100 (Thermo Fisher Scientific, UK). Serial dilution and Miles Misra method on TSA were performed to determine the overall number of viable attached bacterial cells. All bacterial strains were added to cells in similar numbers and the cfu/well of the original bacterial suspensions were determined.

2.7.6.6. Invasion of human cells

The preparation of cell lines and bacterial infection dose were used the same method as that of the adhesion assay as described in Section 2.7.6.1 and 2.7.6.3. However, after 3 times washing with PBS the wells were filled with 500 µl infection media supplemented with gentamicin at a concentration of 125 µg/ml and the plates were incubated in same condition for 1 h to kill any extracellular bacteria. The plates were washed a further 3 times with PBS (Sigma Aldrich, UK) and invasive bacterial cells were released by lysing the human cells with 1% Triton X-100 (Thermo Fisher Scientific, UK). Serial dilution and Miles Misra method on TSA were performed to determine the overall number of viable invaded bacterial cells.

2.7.6.7. Uptake and persistence into macrophage cell line U937

2.7.6.7.1. Culture media

The macrophage cell line U937 was grown in RPMI medium containing 2 mM L-glutamine, 10 mM HEPES, and 1 mM sodium pyruvate. The growth medium was supplemented with 10% FBS, 1% non-essential amino acids solution and 1% Penicillin-Streptomycin while infection medium was supplemented just with 10% FBS and 1% non-essential amino acid solution. The condition of incubation was 37°C under 5% CO_2 for 24 h.

2.7.6.7.2. Macrophage Assays

The experiment was performed as described by (Townsend et al., 2008b). The macrophage cells were seeded into four 24-well plates (Sarstedt, Germany) at a concentration of $4x10^4$ cells/well in growth medium and incubated at 37°C under 5%

CO_2 for 72 h to achieve a confluent monolayer. However, before seeding the cells into 24-well plates, the macrophage cell suspension was supplemented with phorbol 12-myristate 13-acetate (PMA; Sigma Aldrich, UK) at a concentration of 0.1 µg/ml to promote cell adhesion. The seeded plates were washed 3 times with PBS before cells were infected with the determined infection dose. All tested and control strains were prepared as described previously in Section 2.7.6.1; however, the infection dose was at a concentration of $4x10^5$ cfu/ml which is equivalent to an MOI of 1:10. The plates were incubated at 37°C under 5% CO_2 for 1 h and the bacterial infection medium was replaced with infection medium supplemented with gentamicin at a concentration of 125 µg/ml and incubated in the same condition for further one hour. Three plates were washed 3 times with PBS and infection medium with gentamicin at a concentration of 50 µg/ml was added into the wells and incubated for intervals period 24 h, 48 h and 72 h. The infection medium with gentamicin was changed every day. At different time points 1 h, 24 h, 48 h and 72 h, each plate was washed 3 times with PBS and treated with 1% Triton X-100 to lyse the macrophage cells. Serial dilution and Miles Misra method on TSA were performed to determine the overall number of viable intracellular bacteria.

2.7.6.8. Bacterial cytotoxic activity (MTT)

This assay is based on the reduction of MTT (Sigma Aldrich, UK) by viable cells to its insoluble form formazan, which has a purple colour. This experiment was applied as described previously (Krzymińska *et al.* 2009) with some modifications. Human cells, Caco-2, were grown in 24-well plates at $4x10^4$ cell/well. The plates were then incubated for 48 hours at 37°C in 5% CO_2. Bacterial suspensions were prepared by growing bacteria for 18 hours prior to infection. These were then added to the wells at $4x10^6$ cfu/well to achieve MOI of 100. The plates were then incubated in 5% CO_2 at 37°C for 1 hour. The wells were then washed three times using PBS before adding 0.5 ml fresh tissue culture infection medium. A volume of 50 µl of MTT at a concentration of 5 mg/ml (w/v) was added to each well. The plates were then incubated in 5% CO_2 for 1 hours at 37°C. Next, the medium containing MTT was removed and formazan was solubilised in dimethyl sulfoxide (DMSO; Fisher Scientific, UK). The plates were then shaken for 10 minutes before measuring the absorbance at 600nm using a plate reader (BioTek, UK). The negative control for the assay consisted of uninfected cells.

2.8. Statistical analysis

All the conducted experiments in this study were repeated at least twice. The statistical analysis was carried out using unpaired t-test and One-way and Two-way ANOVA (Graph Pad Prism Software Version 7.0) to determine statistical significance. A p value of < 0.05, < 0.01 and < 0.001 was considered statistically significant, highly significant and very highly significant respectively.

Chapter 3. Profiling a clinical collection of *Acinetobacter baumannii* strains isolated from nasogastric enteral feeding tubes

3.1. Introduction

Premature babies are highly susceptible to infection and one of the major routes of infection is through contamination of infant feeding formula. There are several risk factors to neonatal infection; the most important of which would include the use of medical devices such as nasogastric enteral feeding tubes (NEFTs) and intravascular catheters. Easily penetrable epithelial and mucosal barriers and underdeveloped immune systems are also factors which predispose neonatal infection (Hurrell et al., 2009b; Ramasethu, 2017). The gastrointestinal tract of neonates may be sterile at birth; however, it is rapidly colonised by bacteria afterwards, making up part of the normal microflora (Rodríguez et al., 2015; Chong et al., 2018).

Premature infants are often fed through a NEFT, with nutrition provided through different food types ranging from fortified breast milk, to reconstituted powdered infant formula (PIF) (Hunter et al., 2008). None of these nutritional sources can be considered as a sterile food type (Drudy et al., 2006). The lumen of feeding tubes acts as a locus where bacterial cells can adhere, colonise, and produce multispecies biofilm, before sloughing off as clumps of bacteria and entering the neonatal gastrointestinal tract each time fresh feed passes through the tube (Hurrell et al., 2009a; Hurrell et al., 2009b; Ogrodzki et al., 2017). These clumps of bacteria are likely to be resistant to stress conditions such as neonatal low gastric pH, and are therefore able to survive in higher numbers (Ogrodzki et al., 2017).

A potential major source of neonatal infection would be the hospital environment itself. It has been reported in previous studies that healthcare practice in neonatal intensive care units (NICUs), such as antibiotic exposure play vital role in the nosocomial infections (Khan, 2015). Previous investigations carried out at Nottingham Trent University (Hurrell et al., 2009a and 2009b) have demonstrated that neonatal enteral feeding tubes are prone to rapid colonisation by a wide variety of opportunistic pathogens of the Enterobacteriaceae, irrespective of feeding regime. It is estimated that microbial

infections are responsible for up to 50% of infant mortality cases, of which, bacteria are considered to be the major cause accounting for 26% of neonatal deaths worldwide.

In recent years, there has been a rise in the incidence of neonatal infections among babies born with low birth weight and undeveloped immune systems, especially among those fed via nasogastric tubes, which presents an important risk factor in respect to neonatal infections in NICUs. Several different organisms are responsible for causing infections in the NICU. Among them, the Gram-negative bacterium *Acinetobacter baumannii*, has received more attention and it is reported widely as an important and problematic opportunistic pathogen in the clinical setting (Gonzalez and Garduno, 2016). For example, A study by Softic et al., (2013) reported that the outbreaks of sepsis in neonatal intensive care units caused by multidrug-resistant *Acinetobacter baumannii* in the University Clinical Centre in Bosnia and Herzegovina (2012).

The *Acinetobacter* genus is a member of the Moraxellaceae family of Gammaproteobacteria. It is described as a Gram-negative, non-lactose fermenting, strictly aerobic, oxidase-negative, indole negative, non-motile, catalase-positive, and rod-shaped coccobacillus. Having been discovered since 1911, it was not until the late 1970's that it gained recognition as a significant hospital-acquired pathogen (Howard et al., 2011). In 1986, 12 phylogenetic groups of *Acinetobacter* were defined using DNA-DNA hybridization and 4 new species of this genus were proposed, which included *A. baumannii* (Chan et al., 2012).

3.1.1. Clinical importance of *A. baumannii*

Acinetobacter species which are commonly isolated from hospitals typically belong to the *Acinetobacter calcoaceticus-Acinetobacter baumannii* (ACB) complex. However, among these species, *A. baumannii* has attracted significant attention over the preceding decades as one of the most globally important pathogens in healthcare institutions (Joly-Guillou, 2005). The incidence rate of *A. baumannii* infection is very high in most hospitals and its emergence is becoming a major concern because of its ability to make use of a wide range of multidrug resistance mechanisms. For instance, Uwingabiye et al., (2017) reported that among 964 patients hospitalized in the ICUs in a Moroccan teaching hospital, 81 (8.4%) developed *A. baumannii* infections. In other

study by Cai et al. (2017) reported that the Prevalence of Carbapenem-Resistant infections in different hospitals between 2009 and 2013 in the United States Predominated by *Acinetobacter* baumannii and *Pseudomonas aeruginosa*.

Additionally, of concern is its ability to persist in the hospital environment for prolonged periods of time (Lăzureanu et al., 2016). Study by Juma et al. (2016) to investigate the persistence of desiccated *Acinetobacter* in infant formula; their study showed that the ability of *A. baumanni*, *A. calcoaceticus* can survive in desiccated infant formula and maintain its viability during long-term desiccation for 6 and 9 months. Antimicrobial resistance by *A. baumannii* has recently been identified by the World Health Organization (WHO) 2017 as a critical priority pathogen. Additionally, it is reported as a one of the most serious ESKAPE pathogens leading cause of nosocomial infections throughout the worldwide (Santajit and Indrawattana 2016) . The ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) are important of nosocomial infection and multidrug resistant strains (Santajit and Indrawattana 2016; El-Mahallawy et al., 2016; Karlowsky et al., 2017; Silva et al., 2017).

3.1.2. Infections caused by *Acinetobacter baumannii*

A. baumannii is of particular concern because it has emerged as a clinically significant antimicrobial-resistant pathogen that is ubiquitous in the clinical environment (Peleg et al., 2008). It is an important nosocomial pathogen, particularly of critically-ill patients, including those in the intensive care unit ICU (Alsan and Klompas, 2010; Almasaudi, 2018). *A. baumannii* is considered as a typical opportunistic pathogen. It causes a variety of different infections, including bacteraemia, pneumonia, wound infection, urinary tract infection (UTI), meningitis, and nosocomial post-neurosurgical infections. *Acinetobacter* meningitis is becoming increasingly more common, with many other Gram-negative species also proving to be problematic in post-operative care (Howard et al., 2012; Santajit and Indrawattana 2016).

In recent times, *Acinetobacter* infections, including those of human skin and soft tissue, have become highly problematic among inpatients. It was reported that *Acinetobacter* was responsible for around 3% of ICU-acquired skin and soft tissue infection (Howard et

al., 2012). *A. baumannii* was also reported to cause ICU-acquired bloodstream infections, which are responsible for the third highest mortality rate among patients in the ICU. For example, it was found that *A. baumannii* was responsible for around 1.5% of all nosocomial bloodstream infections among 24 hospital in the United States (Wisplinghoff et al., 2000). While in the Brazil *Acinetobacter* spp caused 9.2% prospective surveillance nosocomial bloodstream infections at 16 hospitals among paediatric patients (Pereira et al., 2013). A previous study has reported that the mortality rates for *A. baumannii* at Hamad General Hospital, Qatar, was 31% among patients had underlying medical conditions (Al Samawi et al., 2016). However, infections are more common in patients who have undergone major surgical procedures or suffering from an underlying disease. With regards to acquiring the pathogen within the hospital environment, *A. baumannii* can enter the body easily through open wounds, ingestion, mechanical ventilators and intravascular catheters.

3.1.3. Potential virulence-associated traits

A. baumannii strains demonstrate several potential virulence-associated genes due to their involvement in both epithelial cell attachment and invasion and human serum resistance. Genes encoding phospholipases *plACD* have been found to play an important role in the virulence of *A. baumannii* and are also responsible for its survival in human serum (Camarena *et al*., 2010; Jacobs *et al*., 2010; Stahl *et al*., 2015). Also, outer membrane proteins including (OmpA) have been confirmed to be involved in epithelial cell invasion and apoptosis (Kim et al., 2010; Kim et al., 2016). Additionally, lipopolysaccharides (LPS) may have a role in causing septic shock as result of *A. baumannii* passing into the bloodstream (Ketter et al., 2014). Other potential virulence-associated traits include carbapenem-resistance genes which are believed to be a major problem in nosocomial infections, especially oxacillinases (OXA). Multidrug resistance of *A. baumannii* infections is the major concern in critical care patients in the ICU (Sileem et al., 2017; Blanco et al., 2018). Over the last few years, the rapid increase in the number of antibiotic resistance of *A. baumannii* has become a global issue, due to use of extensive of broad- spectrum antibiotics (Gonzalez and Garduno, 2016; Gao et al., 2017). In 2016, European Centre for Disease Prevention and Control (ECDC) has reported the increase of carbapenem-resistant *A. baumannii* in Europe. Furthermore, it is a one

of the most serious ESKAPE pathogens, which they are known for important health concerns due to their antibiotic resistant nature (Xie et al., 2018). For instance, in 17 European countries about 96 carbapenem-resistant *Acinetobacter baumannii* strains were isolated from 25 hospitals (Towner et al., 2011). Another study by Novovic (2015) showed that Twenty-eight clinical isolates of carbapenem-resistant *A. baumannii* strains were isolated from pediatric hospital in Serbian, their study showed that the all isolates harboured intrinsic OXA-51, AmpC cephalosporinase and OXA-23 was detected in 16 isolates.

3.1.4. Biofilm formation

In addition to the virulence factors mentioned above, biofilm formation is also reported as a virulence-associated trait by helping to maintain cell survival on biotic and abiotic surfaces (Tomaras et al., 2003; Gaddy and Actis; 2009). In the last three decades, bacterial biofilms have been studied extensively, and biofilm formation is recognised as a well-known pathogenic mechanism which is involved in a multitude of serious chronic infections (Joo and Otto, 2013). Among Gram-negative pathogenic bacteria responsible for biofilm-associated infection, *A. baumannii* has received the most attention concerning this due to its ability to form strong biofilms (He et al., 2015; Longo et al., 2014). This organism has been found to be frequently associated with the surface of medical devices such as catheters, intubation tubes, cleaning instruments and water lines (Tomaras et al., 2003; Abdi-Ali et al., 2014; Pakharukova et al., 2018). Biofilms are complex biological matrices that contain nucleic acids, ions, proteins and polysaccharide polymers. One of the major components of biofilms, which has been well described in *Staphylococcus aureus* and *Staphylococcus epidermidis*, is poly-β-(1-6)-N-acetylglucosamine (PNAG) (Itoh et al., 2005; Ferreirinha et al., 2016). Choi (2009) reported that synthesis of PNAG in *A. baumannii* is controlled by an operon (*pgaABCD*) which is also present in the genomes of several other Gram-negative bacteria, such as *Pseudomonas fluorescens*, *Escherichia coli, Y. enterocolitica* and *Yersinia pestis*. It has also been reported that deletion of the *pgaABC* genes in *A. baumannii* resulted in mutants that are defective for PNAG production and led to strong decrease biofilm phenotype (Choi et al., 2009). Another study (Tomaras et al., 2003), reported that the presence of pili-like structures on the surface of *A. baumannii* leads to biofilm formation.

The disruption of the *csuE* and *csuC* resulted in non-piliated cells and eliminated biofilm formation and cell attachment (Tomaras et al., 2003). Bacteria in biofilms are also associated with the expression of several virulence factors, including the quorum sensing system and outer membrane protein *OmpA* (Kim et al., 2016; Subhadra et al., 2016). Moreover, it has been hypothesised that biofilm formation in *Acinetobacter* mediates prolonged survival of the organism in healthcare settings (Espina et al., 2012). The biofilm-forming ability of this microorganism can be considered one of the main virulence factors shared among the vast number of clinical *A. baumannii* isolates (Rumbo et al., 2013; Longo et al., 2014; Reena et al., 2017).

3.1.5. Aim and objectives

Acinetobacter baumannii is recognised as a pathogen of considerable clinical significance. The organism has been associated with several epidemics and has become one of the major concerns in healthcare settings worldwide (Almasaudi, 2018). The spread of *A. baumannii* in the clinical environment has been reported to be responsible for nosocomial infections associated with blood stream infection, pneumonia and mortality rates may reach 35% (Antunes et al., 2014). It has been proposed that the organism could cause bacteraemia and spread throughout the body of the neonate. For example, those born with low birth weight and undeveloped immune systems. This could be of particular concern for neonates fed via a nasogastric tube, which is an important risk factor with respect to infections in the NICU. Additionally, nasogastric feeding tubes may act as a location for bacterial colonisation due to the formation of biofilm in the lumen of the tube (Hurrell et al., 2009b).

Therefore, the overall aim of the project was to profile a clinical collection of *A. baumannii* isolated from neonatal feeding tubes from NICUs in two separate hospitals, Princesses Rahma Hospital (PRH) and King Abdulla Hospital (KAH) in Jordan. This was achieved according to the following objectives:

- Identify their species, source and relatedness of these strains, using PFGE method, and MLST techniques to determine whether some of the strains might originate from common sources, such as being transferred to multiple patients within specific neonatal units.

- A range of physiological traits, such as capsule and biofilm formation, acid, serum resistance and desiccation tolerance will be characterised to evaluate the possible risk to neonates acquiring these organisms through feeding tubes.
- Determine the correlation between biofilm formation and ability to adhere. Indeed, the quantitative differences in biofilm formation and ability to adhere among clinical isolates have been poorly investigated thus far.
- Genomic analysis of representative isolates to identify virulence and antibiotic resistance genes.
- *In vitro* virulence characterization of representative isolates by using epithelial cell line Caco-2, to determine bacterial attachment, invasion, cytotoxicity and their persistence within the human macrophage cell line U937.

3.2. Materials and Methods

The methodology of this chapter is described in detail in Chapter 2 above (Section 2). Forty-three *A. baumannii* strains were used in this project, which were isolated from nasogastric enteral feeding tubes from neonates in NICUs, from Princesses Rahma Hospital (PRH) and King Abdulla Hospital (KAH) in Jordan. The strains had been collected from two hospitals between 15/7/2011 and 21/12/2011. However, neonatal information regarding sample source, birth of neonate, and date of isolation are presented in Table 3-1. Eighteen representative strains of *A. baumannii* were selected for further investigation based on pulsed field gel electrophoresis (PFGE), hospital, neonatal sample source information, and birth of neonate. Of the 18 strains tested, 10 representative strains 1591, 1595, 1601, 1604, 1613, 1615, 1618, 1623, 1624 and 1630 were selected based on PFGE groups, sample source and virulence factors for whole genome sequencing (WGS) and tissue culture. Tissue culture-based virulence studies, using the human colonic epithelial cell line Caco-2, were performed in order to determine bacterial attachment, invasion, and cytotoxicity. The attachment and the gentamicin protection assay was used to assess the invasion ability of *A. baumannii* to invade and to survive within the cell line. In addition, the macrophage cell line U937 was used with representative strains in order to investigate their ability to internalise and persist within human macrophages.

Table 3-1 *A. baumannii* strains used in this study.

NTU strain	Hospital	D.O.I	Neonate age (d)	Source
1591	PRH	15/07/2011	12	FIF
1594	KAH	01/12/2011	8	Tube
1595	KAH	01/12/2011	8	Tube
1596	KAH	07/12/2011	5	Tube
1594	KAH	01/12/2011	8	Tube
1597	KAH	07/12/2011	5	FIF
1598	KAH	07/12/2011	5	FIF
1599	KAH	07/12/2011	24	Tube
1600	KAH	07/12/2011	24	Tube
1601	PRH	10/12/2011	12	Tube
1602	PRH	10/12/2011	12	Tube
1603	PRH	10/12/2011	12	FIF
1604	PRH	10/12/2011	12	FIF
1605	PRH	10/12/2011	15	Tube
1606	PRH	10/12/2011	15	Tube
1607	PRH	10/12/2011	15	FIF
1608	PRH	10/12/2011	8	FIF
1609	PRH	10/12/2011	8	FIF
1610	PRH	12/12/2011	14	Tube
1611	PRH	12/12/2011	14	Tube
1612	PRH	12/12/2011	13	FIF
1613	PRH	12/12/2011	11	FIF
1614	PRH	12/12/2011	13	Tube
1615	PRH	12/12/2011	13	Tube
1616	PRH	15/12/2011	13	FIF
1617	PRH	15/12/2011	13	Tube
1618	PRH	15/12/2011	13	Tube
1619	-	-	-	-
1620	PRH	15/12/2011	10	Tube
1621	PRH	15/12/2011	10	Tube
1622	PRH	12/12/2011	10	FIF
1623	PRH	15/12/2011	10	FIF
1624	PRH	15/12/2011	7	FIF
1625	PRH	15/12/2011	14	FIF
1626	-	-	-	-
1627	KAH	21/12/2011	29	FIF
1628	KAH	21/12/2011	47	FIF
1630	KAH	21/12/2011	16	Tube
1631	KAH	21/12/2011	16	Tube
1632	PRH	20/12/2011	5	FIF
1633	PRH	20/12/2011	12	Tube
1634	PRH	12/12/2011	14	FIF
1635	PRH	20/12/2011	7	FIF
1636	PRH	20/12/2011	12	FIF

PRH = Rahma Hospital. KHA = Abdulla Hospital (Jordan).
NA = Data not available, D.O.I = Date of isolation. FIF = Flushed infant formula.

3.3. Results

3.3.1. Genotyping Profiles

3.3.1.1. Pulsed Field Gel Electrophoresis (PFGE)

Molecular typing by PFGE was performed using the BioNumerics software to study the potential relatedness of 43 *A. baumannii* isolates. These isolates were digested using two restriction enzymes *ApaI* and *AscaI*. They would be considered to be non-clonal when they have less than 95% of band similarity value (Tenover et al., 1995).

The *ApaI* restriction enzyme digest results shown in Figure 3-1, indicated that the 43 strains clustered into five pulsotypes. The majority of strains clustered in pulsotype Ac1, with a similarity coefficient of 100%, while only one strain, 1623, was shown to be highly related with a similarity coefficient of 97%. This was caused by a difference of one band extra after restriction with *ApaI* enzyme. Of particular interest was that all strains of pulsotype Ac1 were isolated from NICUs in 2 hospitals, over a 5-month period, from neonates ranging from 5 to 47 days old, and described as receiving different types of feeding milk. However, after the initial isolation on 15/07/2011, no further isolates were recovered until a period between 01/12/2011 and 21/12/2011. Pulsotype Ac5 comprised two strains, 1594 and 1595, which were isolated on the same day from the same patient, aged 8 days old. One strain, 1624, belonged to Ac3 which was a unique pulsotype. Also, two strains, (1603 and 1604) which belonged to pulsotypes Ac1 and Ac4 respectively, were isolated from the same patient in the same hospital. The same strain belonging to pulsotype Ac1 was isolated twice from the same neonate on the same day. For example, the two strains 1597 and 1598 belonging to pulsotype Ac1 were isolated from the same neonate at the same time and date. Pulsotype Ac1, containing the largest number of strains (n = 38), was also the most frequently detected pulsotype among strains isolated from two hospitals.

According to hospital, neonatal sample source information, birth of neonate, and pulsotype, 18 representative strains of *A. baumannii* were selected for further study. The representative strains similarly grouped into 5 pulsotypes with the second restriction enzyme *AscI*, confirming the *ApaI* results, as shown in Figure 3-2.

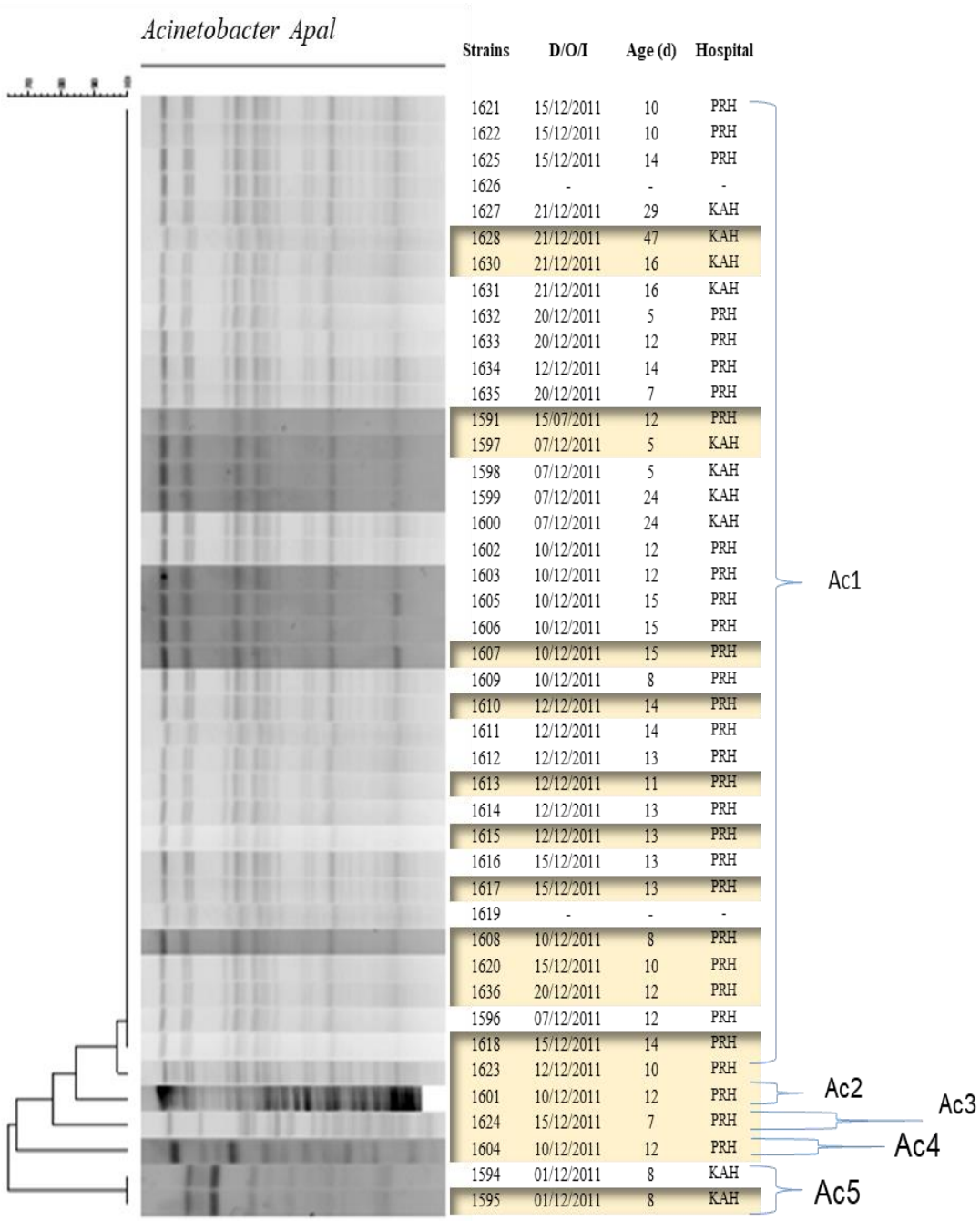


Figure 3-1: Apal PFGE profiles of 43 *Acinetobacter baumannii* strains isolated from neonatal enteral feeding tubes.

It was revealed that the strains clustered into five pulsotypes. Representative strains are highlighted in bink color were selected according to pulsotype, hospital, neonatal sample source and birth of neonate. Dendrogram construction and band assignment was achieved using BioNumerics software version 7.1. For cluster analysis, dice coefficient, un-weighted pair group method with arithmetic mean (UPGMA) was used. Band similarity <95% of value was used to consider the isolates to be non-clonal. The tolerance and optimization of the bands was 1.5%. AC: indicates PFGE cluster groups, DOI: Date of isolation, PRH: Princesses Rahma Hospital, KAH: King Abdulla Hospital.

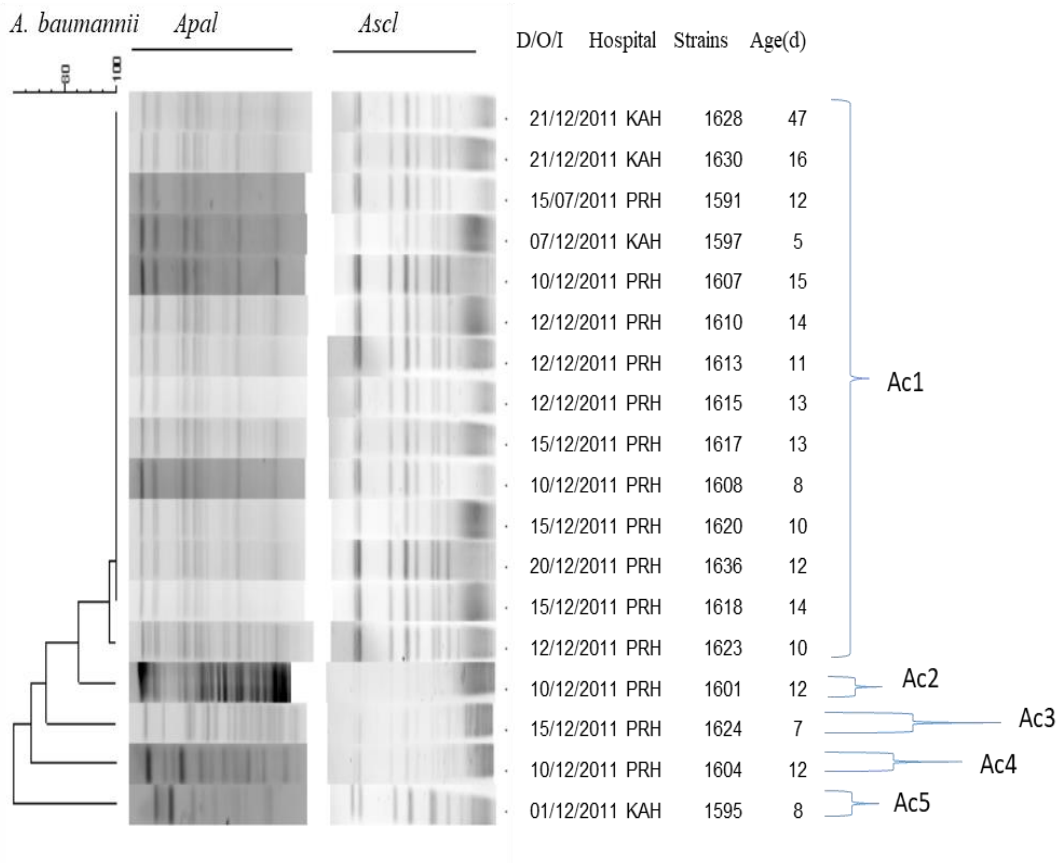


Figure 3-2: Apal and Ascl PFGE profiles of 18 representative *Acinetobacter baumannii* strains isolated from neonatal enteral feeding tube. These strains were selected according to pulsotype, hospital, neonatal sample source and birth of neonate.

Dendrogram construction and band assignment was achieved using BioNumerics software version 7.1. For cluster analysis, dice coefficient, un-weighted pair group method with arithmetic mean (UPGMA) was used. Band similarity <95% of value was used to consider the isolates to be non-clonal. The tolerance and optimization of the bands was 1.5%.AC: indicates PFGE cluster groups, DOI: Date of isolation, PRH: Princesses Rahma Hospital, KAH: King Abdulla Hospital.

Table 3-1 summarises the results of Figure 3-2. The 43 *A. baumannii* strains were isolated from neonatal feeding tubes as a biofilm, from two Jordanian hospitals in 2011. The strains were clustered into five pulsotypes; Ac1, Ac2, Ac3, Ac4 and Ac5 and are described briefly below.

Table 3-1: Summary of PFGE analysis of forty-three A. baumannii strains.

Species	Hospital	Strain Number	P.T	Number of Strains	Period of isolation	Patients
A. baumannii	KAH & PRH	1591, 1596, 1597, 1698, 1599, 1600, 1602, 1603, 1605. 1606, 1607, 1608, 1609, 1610, 1611, 1612, 1613, 1614, 1615, 1616, 1617. 1618, 1619, 1620, 1621, 1622, 1623, 1625, 1626, 1627, 1628, 1630, 1631, 1632, 1633, 1634, 1635, 1636	Ac1	38	Over five month	17
	PRH	1601	Ac2	1	Same day	1
		1624	Ac3	1	Same day	1
		1604	Ac4	1	Same day	1
	KAH	1594, 1595	Ac5	2	Same day	1

PRH: Princesses Rahma Hospital, KAH: King Abdulla Hospital, P.T: pulsotype, Ac: PFGE cluster groups,

3.3.1.2. Partial *rpoB* sequence analysis for *Acinetobacter* genus identification

The *rpoB* gene (350 bp) was amplified and sequenced for 18 representative isolates. Based on multiple alignments, sequence similarities of *rpoB* gene were compared with strains of *Acinetobacter* from GenBank database. All strains were identified as *A. baumannii* and found to be the closest to the *A. baumannii* strain type in GenBank, yet well differentiated from other *Acinetobacte* spp. reference strains. Most of the isolates were identified to the species level with high similarity to *A. baumannii*. A phylogenetic tree was constructed from partial *rpoB* sequence analysis using the neighbour-joining method. It was clear from this analysis that all the species had separated into distinct clusters. The phylogenetic tree, which is based on a sequence analysis in zone 1 of the *rpoB* gene, showed homogeneous grouping of *A. baumannii* (Figure 3-3). Two isolates were misidentified by API phenotyping as *Acinetobacter lwoffi* in the hospital. However, *rpoB* sequencing confirmed the identity of isolates of *Acinetobacter* to the species level.

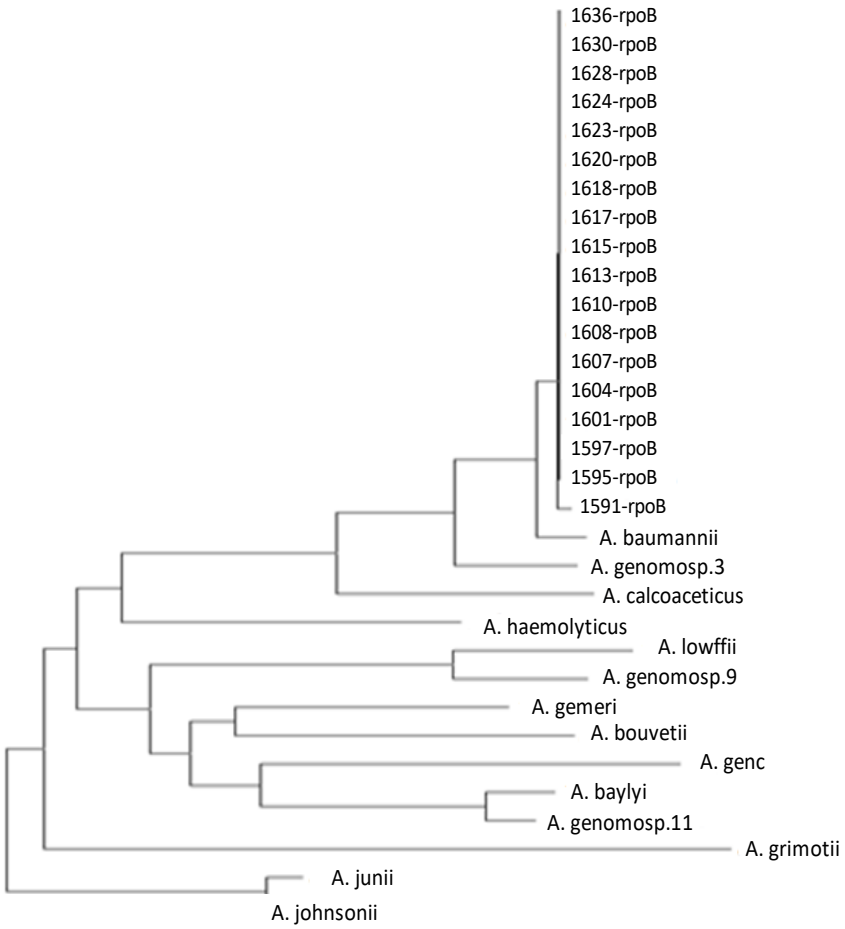


Figure 3-3: Phylogenetic relationships within different *Acinetobacter* spp.

Representative isolates, rpoB gene sequences of 18 reference strains included in this study), as obtained by rooted dendrogram construction on the basis of rpoB gene sequences. The cluster analysis was performed using the MEGA 7 software and was based on the Maximum-likelihood tree using species of the closest related genus.

3.3.1.3. Multi locus sequence typing (MLST)

The *A. baumannii* MLST scheme is based on sequences of the conserved regions of the seven housekeeping genes: *fusA, gltA, pyrG, recA, rplB, rpoB* and *cpn60*, using the MLST scheme database of the Institute of Pasteur, where publicly accessible databases are available from the pubMLST website (http:/pubmlst.org/abaumannii). In this study, seven MLST loci have been effectively amplified and sequenced for representative strains, as shown in Table 3-3. Two STs were identified by MLST analysis among the 18 representative of *A. baumannii* isolates, ST113 (94%), and ST193 (6%). ST113, representing the 17 strains belonging to pulsotypes Ac1, Ac2, Ac3 and Ac4, was identified as the predominant sequence type. This ST is largely prevalent among neonates from the two hospitals, KAH and PRH. On the contrary, the single isolate of

pulsotype Ac5 that was identified as ST193, appeared in only one hospital (KAH). It was interesting to note that strains isolated initially on 15/7/2011 when compared to strains isolated 5 months later on 21/12/2011, have identical pulsotypes and belong to the same sequence type, ST113.

Table 3-2: representative *A. baumannii* isolates, used in this study and their MLST profiles.

Strain	P.T	Hospital	Isolate source	*fusA*	*gltA*	*pyrG*	*recA*	*rplB*	*rpoB*	*cpn60*	ST
1595	Ac5	KAH	BM	1	7	1	7	2	4	3	193
1601	Ac2	PRH	BM	3	3	4	7	4	4	3	113
1604	AC4	PRH	FM	3	3	4	7	4	4	3	113
1624	Ac3	PRH	FM	3	3	4	7	4	4	3	113
1591	Ac1	PRH	FM	3	3	4	7	4	4	3	113
1597		KAH	FM	3	3	4	7	4	4	3	113
1607		PRH	FM	3	3	4	7	4	4	3	113
1608		PRH	FM	3	3	4	7	4	4	3	113
1610		PRH	BM	3	3	4	7	4	4	3	113
1613		PRH	FM	3	3	4	7	4	4	3	113
1615		PRH	BM	3	3	4	7	4	4	3	113
1617		PRH	BM	3	3	4	7	4	4	3	113
1618		PRH	BM	3	3	4	7	4	4	3	113
1620		PRH	BM	3	3	4	7	4	4	3	113
1623		PRH	FM	3	3	4	7	4	4	3	113
1628		KAH	FM	3	3	4	7	4	4	3	113
1630		KAH	BM	3	3	4	7	4	4	3	113
1636		PRH	FM	3	3	4	7	4	4	3	113

MLST scheme is based on sequences of the conserved regions of the seven housekeeping genes: elongation factor (*fusA*), citrate synthase (*gltA*), homologous recombination factor (recA), CTP synthase (*pyrG*), ribosomal protein (*rplB*), RNA polymerase subunit B (*rpoB*) and chaperonin (*cpn60*). Two STs were identified by MLST analysis among the 10 representative strains. PRH: Princesses Rahma Hospital, KAH: King Abdulla Hospital, FM: Flushed milk from neonatal nasogastric feeding tubes, BM: Biofilm materials from neonatal nasogastric feeding tubes, P.T: pulsotype, Ac: PFGE cluster groups, ST: sequence typing.

3.3.1.4. OXA-51-like β-lactamases

The *bla*$_{OXA}$-51-like gene is reported to be present in the majority of isolates of *A. baumannii* and generally has been found to be chromosomally encoded (Turton et al., 2006). Therefore, all 18 representative isolates were subjected to conventional PCR assay to detect *bla*$_{OXA}$-51-like genes. The results showed that the *bla*$_{OXA}$-51-like gene was present in all tested isolates (Table 3-6).

3.3.1.5. Detection of Phospholipase Genes

The PCR method was used in this study to investigate the phospholipase A, C, and D genes. Primers were designed and synthesised by Eurofins Genomics. The details for the selected genes are presented in Table 2-4. The results showed that the detection of phospholipase encoding three genes for phospholipases A, C, and D were positive across all the 18 *A. baumannii* strains.

3.3.2. Phenotyping and virulence traits

3.3.2.1. Biofilm formation

Biofilm formation was studied in the 18 representative strains of *A. baumannii*. These enteral feeding tube strains were selected according to their PFGE pulsotypes, hospital, date and birth of neonate. The ability of *A. baumannii* strains to form biofilms on plastic surfaces at different temperatures is shown in Figure 3-4. All *A. baumannii* strains demonstrated the ability to form high amounts of biofilms at 37 °C in infant formula when compared with un-inoculated infant formula used as negative control, however, some levels of variation were observed between the strains.

The lowest amount of biofilm formation was observed in strain 1595 (ST193) when compared to the other strains, in particular 1604, 1618, 1620 and 1628 (ST113), at 37 °C ($p < 0.05$). On the other hand, there was a greater amount of variation between strains in biofilm formation at 25°C, even amongst strains belonging to the same pulsotype, such as Ac1. The highest amount of biofilm formation was observed in strain 1618, which belongs to pulsotype Ac1 (ST113). On the other hand, the lowest amount of biofilm produced was noted in strain 1595, belonging to pulsotype Ac5 (ST193). In addition, significant differences ($p < 0.05$) were noted in most of the isolates across the same pulsotype (Ac1) when compared to strain 1618; most notably strains 1597, 1613, 1615, 1617, 1628 and 1630 produced less biofilm than strain 1618 at 25 °C. However, there was no statistically significant difference between strains, 1604, 1591, 1607, and 1636 compared to strain 1618, when analysed by means of one-way ANOVA followed by multiple comparisons with a Dennett's test. Overall, all strains showed statistically significant difference between 37 °C and 25 °C on infant formula by paired t-test, as summarised in Table 3-3.

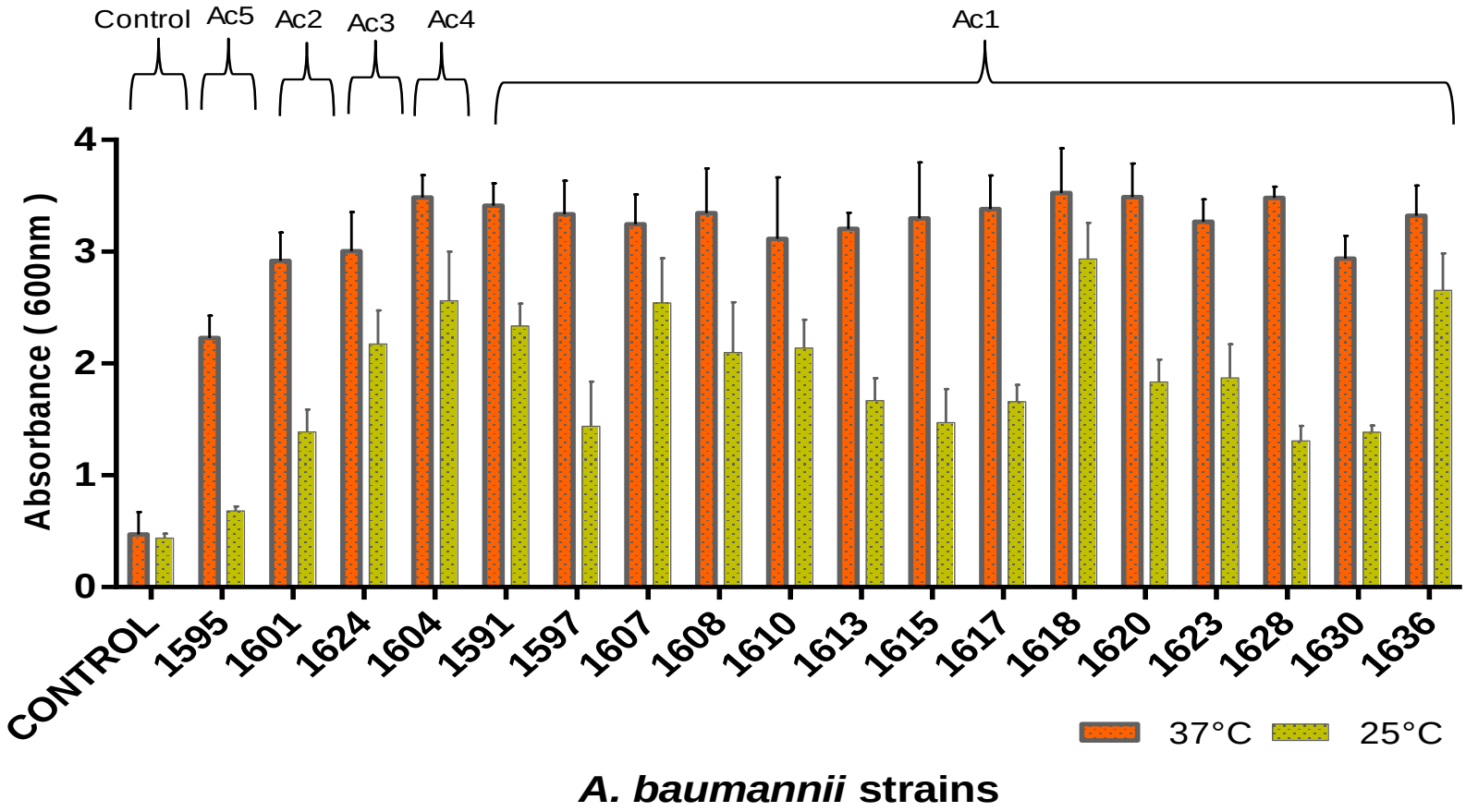


Figure 3-4 : Biofilm formation at 25 °C and 37 °C in infant formula using the crystal violet adsorption assay.

All strains showed an ability to form more biofilm at 37 °C than 25 °C in infant formula ($p < 0.05$). The lowest amount of biofilm formation was observed in strain 1595 (ST193) Un-inoculated infant formula was used as negative control.

Table 3-3: Summarises the biofilm formation level on infant formula by enteral feeding tube strains from two Jordanian hospitals in 2011. The lowest amount of biofilm produced was noted in strain 1595 at 25 °C in infant formula.

Isolate	P.T	Isolation date	Hospital	Infant age (d)	source	Biofilm formation	
						IF 25° C	IF 37° C
1591		15/07/2011	PRH	12	FM	H	H
1597		07/12/2011	KAH	5	FM	M	H
1607		10/12/2011	PRH	15	FM	H	H
1608		10/12/2011	PRH	8	FM	H	H
1610		12/12/2011	PRH	14	BM	H	H
1613		12/12/2011	PRH	11	FM	M	H
1615		12/12/2011	PRH	13	BM	M	H
1617	Ac1	15/12/2011	PRH	13	BM	M	H
1618		15/12/2011	PRH	14	BM	H	H
1620		15/12/2011	PRH	10	BM	M	H
1623		15/12/2011	PRH	10	FM	H	H
1628		21/12/2011	KAH	47	FM	M	H
1630		21/12/2011	KAH	16	BM	M	H
1636		20/12/2011	PRH	12	FM	H	H
1601	Ac2	10/12/2011	PRH	12	BM	M	H
1624	Ac3	15/12/2011	PRH	7	FM	H	H
1604	Ac4	10/12/2011	PRH	12	FM	H	H
1595	Ac5	01/12/2011	KAH	8	BM	L	H

PRH: Princesses Rahma Hospital, KAH: King Abdulla Hospital, FM: Flushed milk from neonatal nasogastric feeding tubes, BM: Biofilm materials from neonatal nasogastric feeding tubes, P.T: pulsotype, Ac: PFGE cluster groups, IF: infant formula, OD < 1 = Low biofilm formation, OD between 1&2 = moderate biofilm formation and OD >2 = High biofilm formation. OD, based on the OD values from the crystal violet assay.

3.3.2.2. Serum resistance

To determine serum sensitivity, bacterial viability counts were determined at four different time points (0 h, 1 h, 2 h and 3 h) in the presence of forty microliters of the bacterial culture were added to 360 µl of 50% human serum (Sigma, UK), as described in section 2.7.5.8. Figure 3-5 shows that there was robust survival of all *A. baumannii* strains in the presence of serum after incubation for 3 h with slight variations between the strains. For instance, strain 1615 demonstrated a medium level of resistance to the human serum when compared to all other strains. While strain 1618 and 1623 maintained the serum resistance. After the first hour of incubation with human serum, about 50% of *A. baumannii* strains showed an increase in their numbers. However, 61%

of these strains showed an increase in their viable count after the second hour of incubation. Strains 1595, 1601, 1624, 1597, 1607, 1613, 1620, 1628 and 1630 from different pulsotypes were able to persist in human serum and revealed increases in their initial numbers after the third hour of incubation, while strains 1610 and 1636 showed a slight decrease. *S.* Enteritidis was used as the serum resistant positive control strain and *E. coli* was used as the serum sensitive control strain.

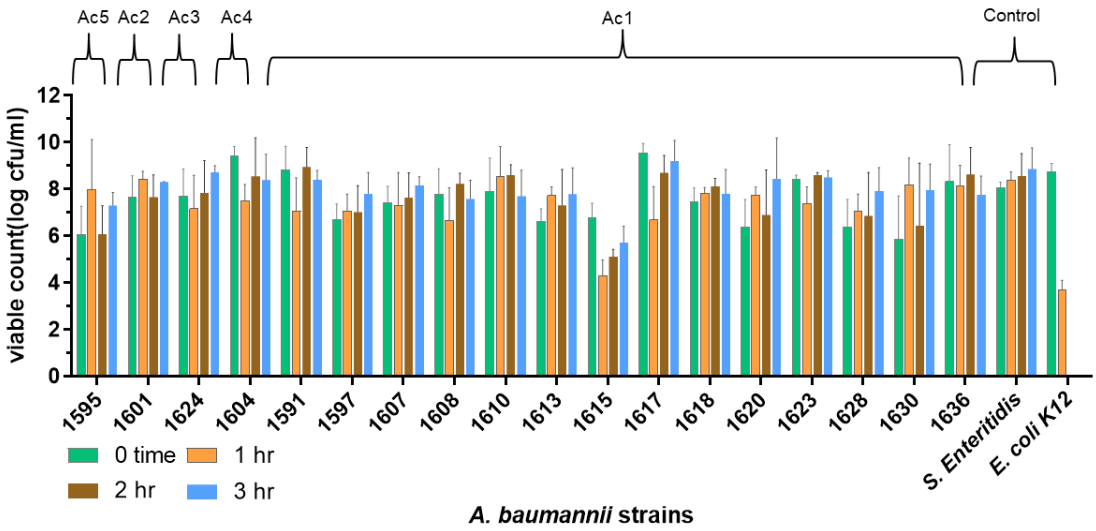


Figure 3-5: Sensitivity of *A. baumannii* (n=18) to human serum over 3 hours of incubation at 37 °C.

All *A. baumannii* strains (n=18) showed a high level of resistance to the human serum after 3 hours of incubation. All examined strains were significantly better at surviving serum compared to the *E. coli* K12 (1230) negative control. The assays were performed in duplicate in two independent experiments. Error bars represented using standard error. Presented data was showing viable count (log10cfu/ml) among the isolates.

3.3.2.3. Capsule production

Two different types of media were used in this study to detect the capsule production, which included soya and whey-based infant formula. As shown in Table 3-4 all *A. baumannii* strains were unable to produce capsular materials on both media.

3.3.2.4. Congo red morphotype (curli fimbriae).

Eighteen *A. baumannii* strains were cultured in LB agar media supplemented with Congo red to indicate the production of curli fimbriae. The morphology of the pigment produced on Congo red agar for each strain was defined according to the following

pigmentation: red indicates the ability of the bacterium presumed production of curli fimbria, while pink as a negative (Figure 3-6). The colony morphology were recorded after incubation at 37°C for 24 and 48 hours as an indication of the binding of the Congo red dye. All *A. baumannii* strains showed binding to the Congo red dye and considered as produce curli fimbriae according to their appearance on Congo red media as shown in Table 3-4.

Figure 3-6: Cell morphology of the pigment produced on Congo red agar, red indicates the ability of the bacterium to binding Congo red day, while pink is a negative control.

3.3.2.5. Cellulose formation

Cellulose production by eighteen *A. baumannii* strains was investigated by streaking on LBA medium supplemented by 25ml of Calcofluor. Cellulose production an indicated by the binding of the fluorescent dye calcofluor-white, were visualised using ultra-violet (366nm) fluorescence (Figure 3-7). All strains demonstrated high cellulose production, with the exception of 1591, 1608, and 1636 strains, which showed low levels of cellulose production, while strains 1595 and 1624 showed no fluorescence as shown in Table 3-4.

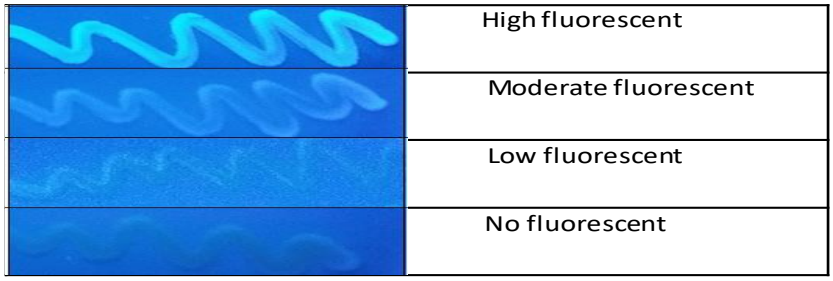


Figure 3-7: Cell morphology on Calcofluor media. Fluorescent signal indicated to cellulose production, whereas no fluorescent signal indicated to cellulose was not produced.

3.3.2.6. Siderophore production

Siderophores are considered as major virulence factors in the pathogenicity of organisms. Bacteria synthesise and secrete siderophores to chelate iron, which is an

essential element required by almost all bacteria. In this study, the ability of *18 A. baumannii* isolates to produce siderophores was analysed. Positive results were verified as a formation of halo around the colony growth (Figure 3-8). Table 3-4 shows that all strains examined were able to produce iron siderophores on chrome azurol sulphate (CAS) agar. Strains of *Y. enterocolitica* (1880) and and 520 *C. sakazaki* strain were used as positive and negative controls, which measures the ability of the compound to bind iron.

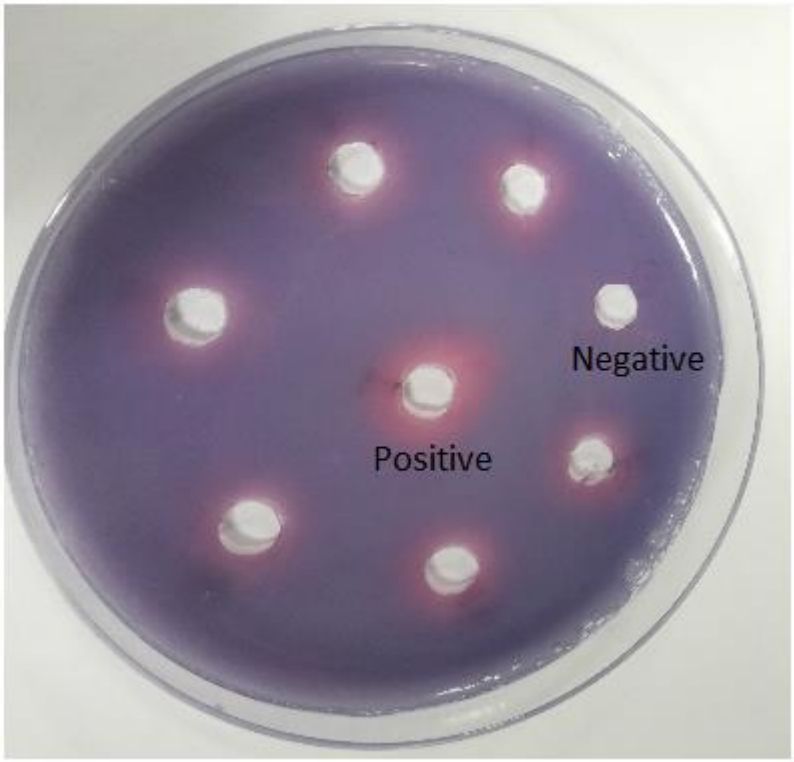


Figure 3-8: Siderophore activity using CASAD assay, all of *A. baumannii* strains have been able to produce iron sidrophores, CAS agar showing orange halo around the wells, however NTU 8081 *Y.* enterocolitica was positive control and 520 *C. sakazaki* strain was negative.

3.3.2.7. Haemolytic assay

Haemolysin, an extracellular protein produced by pathogenic bacteria, is one of the toxins that may play an essential role in causing disease. In the current study, diverse haemolytic activity was demonstrated by the 18 representative strains of *A. baumannii* on horse blood. On horse blood agar, β-haemolysis was demonstrated by 10 strains (55.5%) when compared to that exhibited by the *S. eureus* positive control, while 7 strains (38.8%) showed γ-haemolysis, and only strain (1623) was α-haemolytic (Table 3-5).

Table 3-4: A summary of the curli fimbria, cellulose, siderophore and capsule production, as well as haemolytic activity among the 18 representative strains of *A. baumannii*.

Isolate	P.T	Isolation date	Hospital	Infant age (d)	source	Capsule		Haemolysis	Curli fimbria	Siderophore	Celluolose
						IF1	IF2				
1591	Ac1	15/07/2011	PRH	14	FM	-	-	γ	+	+	+
1597	≠	07/12/2011	KAH	5	FM	-	-	γ	+	+	++
1607	≠	10/12/2011	PRH	15	FM	-	-	β	+	+	++
1608	≠	10/12/2011	PRH	8	FM	-	-	γ	+	+	+
1610	≠	12/12/2011	PRH	14	BM	-	-	β	+	+	++
1613	≠	12/12/2011	PRH	11	FM	-	-	β	+	+	++
1615	≠	12/12/2011	PRH	13	BM	-	-	β	+	+	++
1617	≠	15/12/2011	PRH	13	BM	-	-	β	+	+	++
1618	≠	15/12/2011	PRH	14	BM	-	-	β	+	+	++
1620	≠	15/12/2011	PRH	10	BM	-	-	β	+	+	++
1623	≠	12/12/2011	PRH	10	FM	-	-	α	+	+	++
1628	≠	21/12/2011	KAH	47	FM	-	-	γ	+	+	++
1630	≠	21/12/2011	KAH	16	BM	-	-	γ	+	+	++
1636	≠	20/12/2011	PRH	12	FM	-	-	β	+	+	+
1601	Ac2	10/12/2011	PRH	12	BM	-	-	γ	+	+	++
1624	Ac3	15/12/2011	PRH	7	FM	-	-	γ	+	+	-
1604	Ac4	10/12/2011	PRH	12	FM	-	-	β	+	+	++
1595	Ac5	01/12/2011	KAH	8	BM	-	-	β	+	+	-

PRH: Princesses Rahma Hospital, KAH: King Abdulla Hospital, FM: Flushed milk from neonatal nasogastric feeding tubes, BM: Biofilm materials from neonatal nasogastric feeding tubes, P.T: pulsotype, Ac: PFGE cluster groups. α: α -haemolysis, β: β-haemolysis γ: γ-haemolysis, F1: infant formula whey based, IF2: infant formula soya based, +: positive, -: negative.

3.3.2.8. Desiccation

Acinetobacter has been experimentally shown to survive desiccation and have demonstrated an ability to maintain their viability on inanimate surfaces in hospitals (Chapartegui-González et al., 2018). In this study, a total number of 18 *A. baumannii*

strains were evaluated for their desiccation survival in infant formula over a period of two weeks. The first 24 hours of desiccation caused the viability of the majority *Acinetobacter* strains to slightly decrease by 0.1 - 1.5 log cfu/ml. The viable cell counts for the majority of strains did not appear to be reduced by a significant amount, whereas strains 1623 and 1628 showed the lowest ability to persist under desiccation stress conditions for 24 hours. However, the reduction in viability observed over the next 14 days ranged between 0.6 and 4.1 log cfu/ml. There was some variation between strain in terms of their ability to persist under conditions of desiccation stress. Strains 1624, 1607, 1623 and 1628 showed low persistence compared with the other strains, in particular 1623. Strains 1608, 1610, 1613, 1615, 1618, and 1620 were able to persist for the longest time under desiccation stress conditions, but all strains remained recoverable until the last sampling point on day 14 (Figure 3-9).

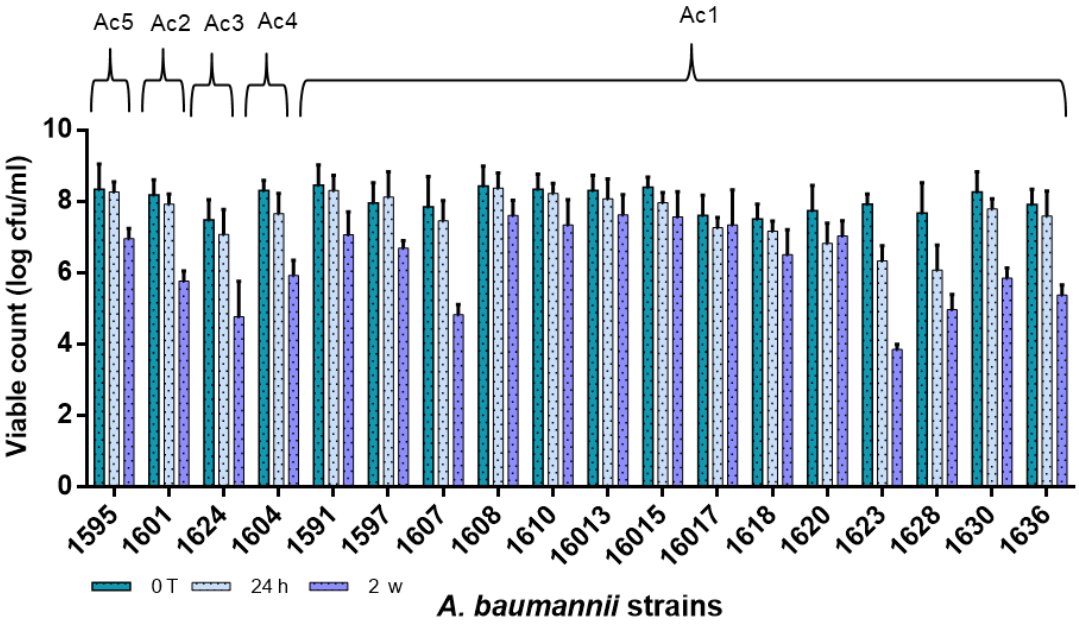


Figure 3-9: Survival of *A. baumannii* strains subjected to desiccation over a period of 2 weeks.

The figure shows a slight decrease in viability by 0.1-1.5 log cfu/ml, as a result of the first 24 h of desiccation, while the reduction observed after 2 weeks ranged between 0.6 and 4.1 log cfu/ml. Overall, it is clear that all strains were successfully able to persist during 2 weeks of desiccation stress conditions.

3.3.2.9. Acidity

Stomach acidity acts as the most important line of defence in protecting against infections through the route of ingestion. In this study, 18 strains of *A. baumannii* were challenged by acidic conditions and were assessed for their tolerance to withstand pH 3.5. The strains were exposed to pre-adjusted infant formula at pH 3.5 for two hours and the results are presented in Figure 3-10. In general, all *A. baumannii* strains were resistant to an acidic exposure of pH 3.5 for 2 hours. During the first 30 min of acid exposure, the viability of all 18 strains decreased by 0.4-1.4 log cycles. Sampling points at 60 min did not show a significant further reduction in cell counts for the 18 strains tested ($P > 0.05$). Two of the 18 strains (1595 and 1617) exhibited less than 1 log cycle decline over the 2 h incubation period. The most acid-sensitive strain was 1613, showing an approximate 2.5 log cycle decline. Although all 18 representative strains displayed a 0.8 to 2.5 log cycle decline during the 2 hours, these strains remained viable at pH 3.5 during this period, and were therefore regarded as acid-tolerant strains.

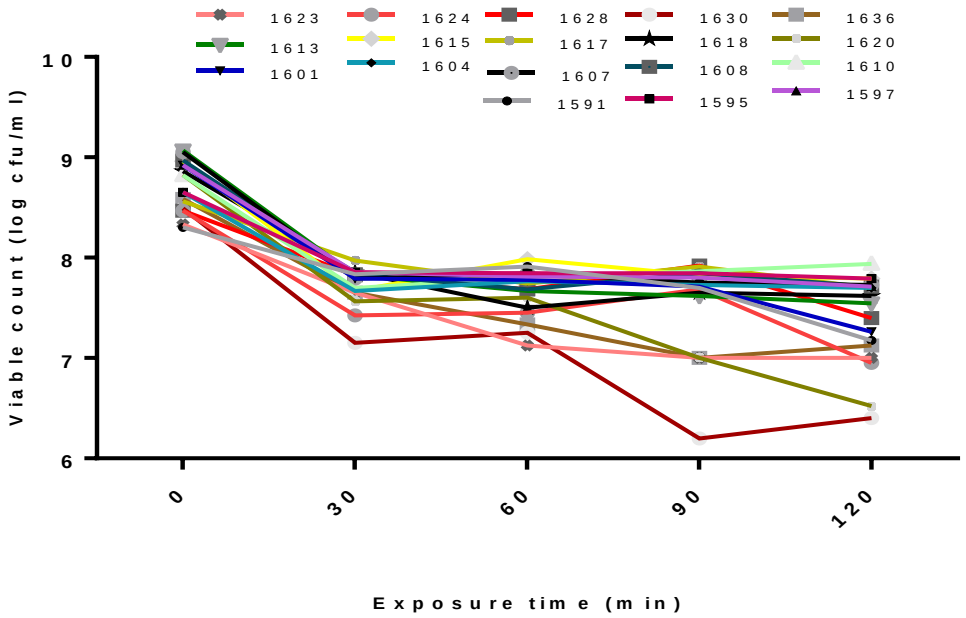


Figure 3-10 Survival curve of 18 *A. baumannii* strains in infant formula at PH 3.5.

Acinetobacter strains were assessed for their tolerance to pH 3.5 for two hours. The viable counts were determined by the Miles & Misra method. The figure shows the viability of all 18 strains decreased between 0.4-1.4 log cycles during the first 30 min of acid exposure, although, no significant further reduction was observed after the 60-min time-point. Overall, all *A. baumannii* strains showed an ability to resist pH 3.5 for the total 2 hours.

3.3.2.10. Antibiogram

Antibiotic susceptibility profiles were determined for the 18 *A. baumannii* strains, isolated from enteral feeding tubes from two Jordanian hospitals. This involved screening the sensitivity to six antibiotic groups: penicillins, aminoglycosides, carbapenems, cephalosporins, chloramphenicol, and trimethoprim. The antibiogram procedure was tested by the Kirby-Bauer disk diffusion method. As shown in Table 3-6, all *A. baumannii* strains belonging to pulsotypes Ac1 were generally highly resistant to all antibiotics with the exception of ciprofloxacin, and three strains (1607, 1613, and 1623) which also displayed susceptibility to trimethoprim/sulfamethoxazole. However, strain 1604, belonging to Ac4, showed resistance to all antibiotics but was susceptible to ciprofloxacin. Whereas strains 1601 and 1595 (pulsotypes Ac2 and Ac5, respectively) were resistant to at least two antibiotics, whilst being susceptible to the majority of antibiotics used in this study. Strain 1624, which belongs to Ac3, demonstrated resistance to the majority of antibiotics used in this study, whilst it was susceptible to the antibiotics meropenem, imipenem, chloramphenicol, and ciprofloxacin.

Table 3-5 Sensitivity for *A. baumannii* strains to Antibiotic agents

Isolate	Pulso type	Source	Hospital	Isolation date	Infant (d)	Sample Source	Penicillin's		Aminoglycosides	Carbapenems		Cephalosporin's				Chloramphenicol	Trimethoprim
							AUG	AP	GM	MEM	IMI	CXM	CTX	CRO	CIP	C	TS
1591	Ac1	FM	PRH	15/07/2011	14	F	R	R	R	R	R	R	R	R	S	R	R
1597	Ac1	FM	KAH	07/12/2011	5	F	R	R	R	R	R	R	R	R	S	R	R
1607	Ac1	FM	PRH	10/12/2011	15	F	R	R	R	R	R	R	R	R	S	R	S
1608	Ac1	FM	PRH	10/12/2011	8	F	R	R	R	R	R	R	R	R	S	R	R
1610	Ac1	BM	PRH	12/12/2011	14	B	R	R	R	R	R	R	R	R	S	R	R
1613	Ac1	FM	PRH	12/12/2011	13	F	R	R	R	R	R	R	R	R	S	R	S
1615	Ac1	BM	PRH	12/12/2011	13	B	R	R	R	R	R	R	R	R	S	R	R
1617	Ac1	BM	PRH	15/12/2011	13	B	R	R	R	R	R	R	R	R	S	R	R
1618	Ac1	BM	PRH	15/12/2011	13	B	R	R	R	R	R	R	R	R	S	R	R
1620	Ac1	BM	PRH	15/12/2011	10	B	R	R	R	R	R	R	R	R	S	R	R
1623	Ac1	FM	PRH	12/12/2011	10	F	R	R	R	R	R	R	R	R	S	R	S
1628	Ac1	FM	KAH	21/12/2011	47	F	R	R	R	R	R	R	R	R	S	R	R
1630	Ac1	BM	KAH	21/12/2011	16	B	R	R	R	R	R	R	R	R	S	R	R
1636	Ac1	FM	PRH	20/12/2011	12	F	R	R	R	R	R	R	R	R	S	R	R
1601	Ac2	BM	PRH	10/12/2011	12	B	R	S	S	S	S	S	S	S	S	R	S
1624	Ac3	FM	PRH	15/12/2011	7	F	R	R	R	S	S	R	R	R	S	S	R
1604	Ac4	FM	PRH	10/12/2011	12	F	R	R	R	R	R	R	R	R	S	R	R
1595	Ac5	BM	KAH	01/12/2011	8	B	S	S	S	S	S	S	R	I	S	R	S

S: susceptibility. R: resistance. I: intermediate resistance. PRH: Princesses Rahma Hospital. KAH: King Abdulla Hospital. FM: Flushed milk from neonatal nasogastric feeding tubes. BM: Biofilm materials from neonatal nasogastric feeding tubes. P.T: pulsotype. Ac: PFGE cluster groups. AUG: Augmentin. CIP: Ciprofloxacin. CXM: cefuroxime. CTX: Cefotaxime. MEM: Meropenem. C: Chloramphenicol. GM: Gentamicin. CRO: Ceftriaxone. AP: Ampicillin. TS: Trimethoprim Sulfametoxa. IMI: Imipenem.

3.3.3. Assessment of the pathogenicity of *A. baumannii* using tissue culture assays

Of the 18 strains examined in this study, 10 were selected as representative strains to evaluate their potential virulence using in vitro attachment, invasion and cytotoxicity assays with intestinal epithelial cells Caco-2. Macrophage survival were also examined by U937 cell line.

3.3.3.1. Bacterial attachment on Caco-2 human epithelial cells

The investigation sought to determine the ability of the *A. baumannii* strains to attach to human cell line Caco-2. To define the level attachment achieved by each isolate, the attachment efficiency (%) results were classified into three categories according to the observed level of attachment on the controls used in this study: high, medium, and low levels of attachment. Low attachment was considered to be less than 2% cfu/ml, while attachment efficiency of isolates between 2% and 4% cfu/ml referred to a medium level of attachment. Attachment efficiency greater than 4% of recovered cfu/ml was considered as a high level of attachment to the Caco-2 human epithelial cells. Statistical analysis using one-way ANOVA confirmed that attachment efficiency levels in the majority of isolates were significantly higher than that of the negative control strain *E. coli* 1230 ($p < 0.001$). Execept strain 1595, belonging to pulsotype Ac5 (ST193), had the lowest attachment levels among the representative strains analysed in this study. Strains 1601, 1613, and 1618 (all ST113) showed moderate levels of attachment, while the other strains, 1604, 1624, 1691, 1615, 1623 and 1630 (also ST113), demonstrated a high attachment level to the Caco-2 cell line. The highest attachment levels to Caco-2 was observed in strain 1604, which belongs to pulsotype Ac4 (ST113), as shown in Figure 3-11.

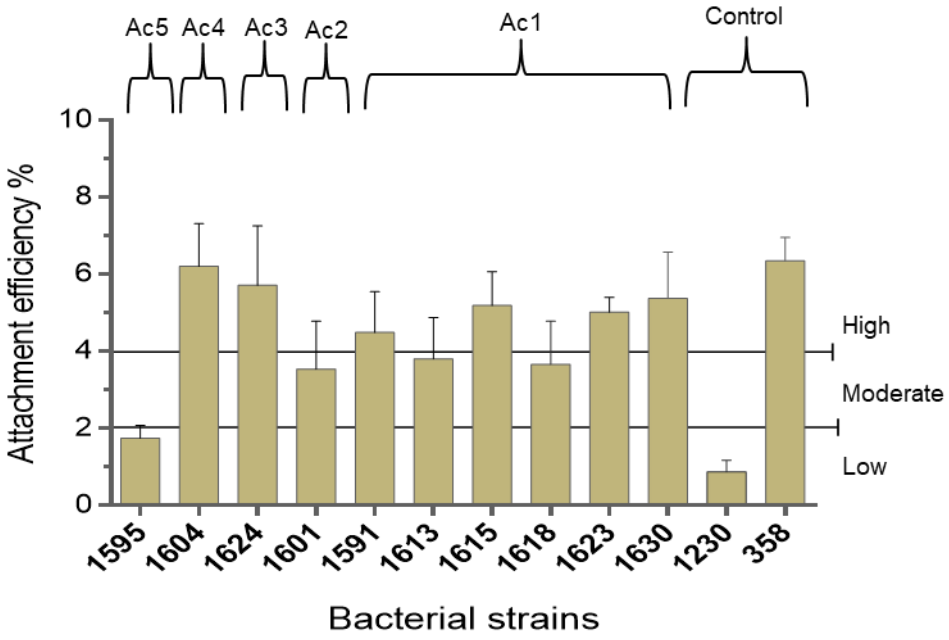


Figure 3-11 Attachment of *A. baumannii* strains to the human Caco-2 cell line.

The figure shows the differences in attachment levels among *A. baumannii* strains to human epithelial cell line Caco-2. All strains were able to attach to the epithelial cell line when compared to *E. coli* 1230 ($p < 0.001$), which was used as negative control, and. *S.* Enteritidis 358, which was used as a positive control. Error bars indicate the standard deviation of six data points taken from two independent experiments in triplicate.

3.3.3.2. Bacterial invasion of Caco-2 human epithelial cells

The gentamicin protection assay was used to determine the ability of *A. buamannii* strains to invade the Caco-2 human epithelial cells by killing all extracellular bacteria, allowing a quantification of intracellular bacteria. Results were classified into three categories according to the percentage of recovered cells after invasion. Less than 0.1% recovery was used to describe strains that were very poorly invasive (very low invasion), while 0.1 to 0.2% recovery was defined as a low level of invasion, 0.2 to 0.4% defined as a medium and greater than 0.4% recovery was defined as a high level of invasion. There was an observable amount of variation regarding the invasion rates of *A. baumannii* strains. Figure 3-12 shows that most of these strains were able to invade Caco-2 human epithelial cells at very low levels comparing with positive control 358, for example, strains 1591, 1595, 1601, 1613, 1615, 1623, 1624, and 1630 which all belonged to different pulsotypes while strain 1618 showed low invasion potential. When compared to the negative control strain, most strains showed no significant difference in invasion

levels when analysed by means of one-way ANOVA, followed by multiple comparisons with a Dennett's test. However, strains 1604, 1618, and 1624 showed significantly greater invasion levels than the negative control strain when using the same method of statistical analysis ($P < 0.001$). The highest percentage of invasion efficiency in the gentamicin protection assay was observed in strain 1604, which interestingly also displayed the highest level of attachment rate to the Caco-2 cell line.

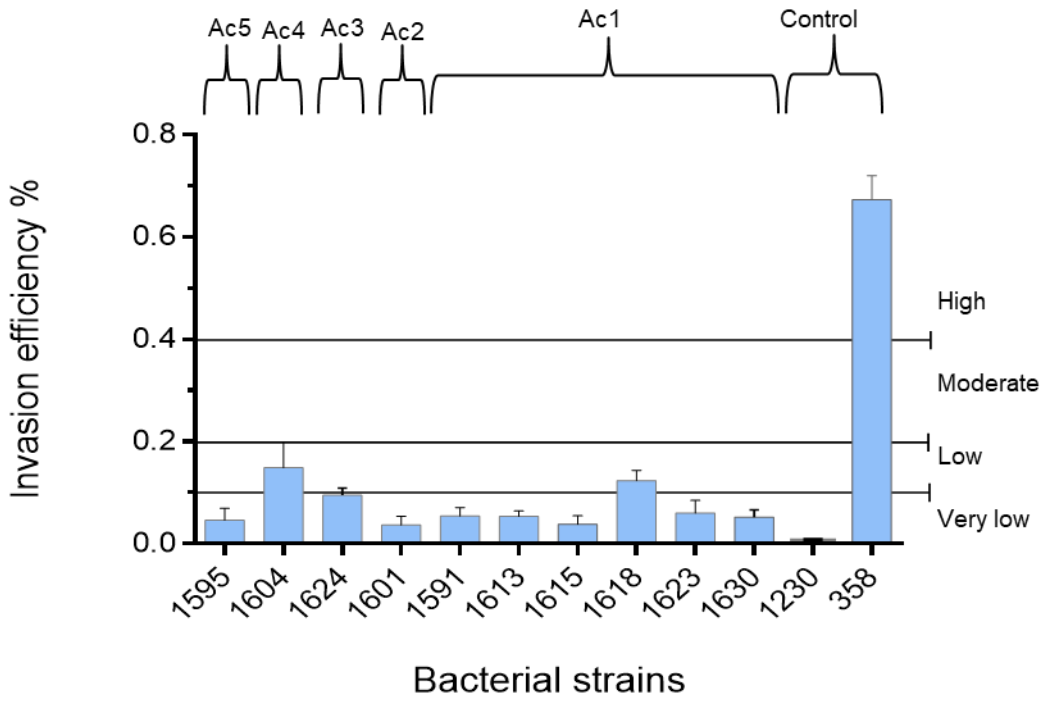


Figure 3-12: Invasion efficiency of *A. baumannii* strains to the mammalian Caco-2 cell line.

The figure shows the differences among *A. baumannii* strains with regards to their ability to invade the human epithelial cell line Caco-2, by performing the gentamicin protection assay. The majority of strains invaded Caco-2 human epithelial cells at very low levels. *S.* Enteritidis 358 and *E. coli* 1230 were used as positive and negative control strains, respectively.

3.3.3.3. 3.3.3.4 Macrophage survival assay

Macrophages are an important class of phagocytes that are commonly involved in the host response to pathogenic bacterial invasion. This response is known as the innate immune process, which involves the macrophages phagocytosing the invading microbes in order to protect the host body. Ten representative strains of *A. baumannii* were investigated for their ability to persist and replicate within human macrophages represented by the U937 cell line. The NTU collection strain *C. koseri* 48 was used as a

positive control, whilst *E. coli* K12 strain 1230 (also from the NTU strain collection) was used as a negative control.

Macrophage uptake of all *A. baumannii* strains was observed at different levels 60 mins after the initial inoculation period, except for strain 1618, which was not detected after 60 min, however the survival of this strain was evident after 24 h (Figure 3-13). After 24 hours of incubation, all of the strains showed an increase within the macrophages, particularly strains 1604 and 1624, which were able to multiply significantly when compared to the other strains ($P < 0.05$). Most of the strains displayed a relative increase in their numbers after 48 hours of incubation, whereas 1624 was the only strain to demonstrate a slight decline in numbers. All strains displayed a decrease in their intracellular numbers after 72 hours of incubation. Strain 1604 exhibited the greatest ability to persist and replicate inside the macrophages (Figure 3-13).

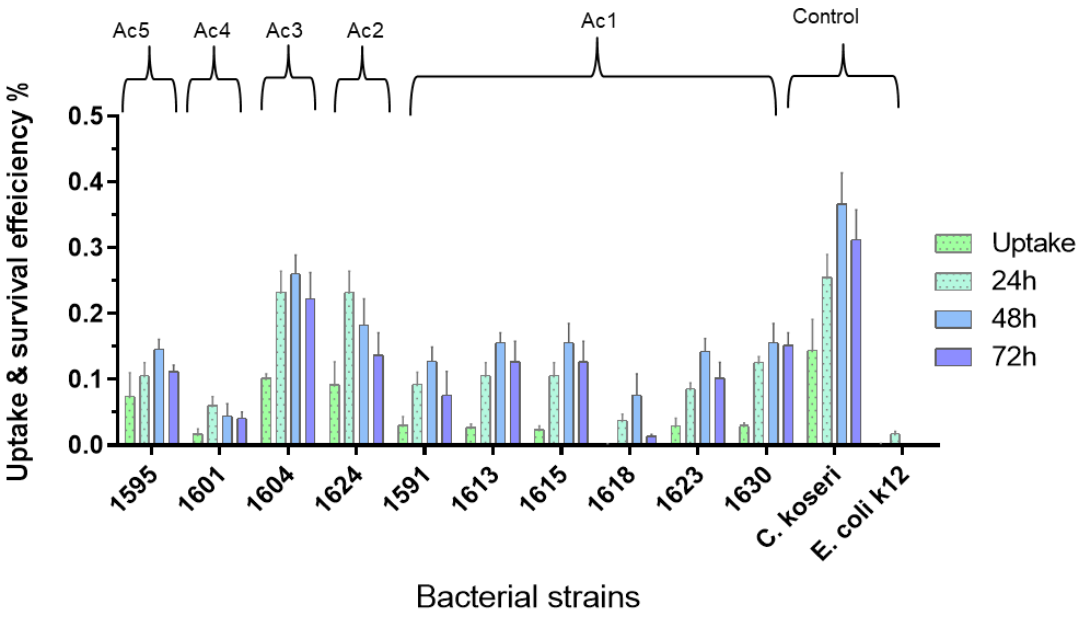


Figure 3-13: shows the differences in the ability of *A. baumannii* strains to persist in U937 macrophage cell lines for 72 hours.

The majority of the *A. baumannii* strains (n=10) demonstrated an ability to persist and replicate within the macrophage cell line U937 for up to 72 hours of incubation. The *C. koseri* 48 strain was used as positive control, while *E. coli* K12 strain 1230 was used as negative control (both from the NTU strain collection).

3.3.3.4. 3.3.3.5 Cytotoxicity of *Acinetobacter baumannii*

The MTT reduction assay was carried out to measure the effect of *A. baumannii* strains on the viability of the epithelial Caco-2 cells. Viable epithelial Caco-2 cells are able to reduce MTT to its insoluble purple form, known as formazan. An *S.* Enteritidis strain, which was used as positive control in this investigation, lowered the amount of MTT reduction, thus indicating their high cytotoxic ability. Conversely, Caco-2 cells in cell culture medium where is no bacteria been added to the cells was used as a negative control, which showed high levels of MTT reduction thus indicating low, or non-existent, cytotoxicity. The results were categorised as low cytotoxicity (OD = > 1), moderate cytotoxicity (OD 0.5 – 1), and high cytotoxicity (OD < 0.5). Most of the strains showed significant MTT reduction compared to the negative control after 1 hour of incubation, thus demonstrating moderate cytotoxicity, and the absorbance levels for all strains were relatively similar, as shown in Figure 3-14. The only exception, however, was strain 1623 which showed slightly higher cytotoxicity levels compared to the other the strains. All strains showed no significant difference in cytotoxicity levels ($P < 0.001$) compared with strain 1623 when analysed by means of one-way ANOVA, followed by multiple comparisons with a Dennett's test.

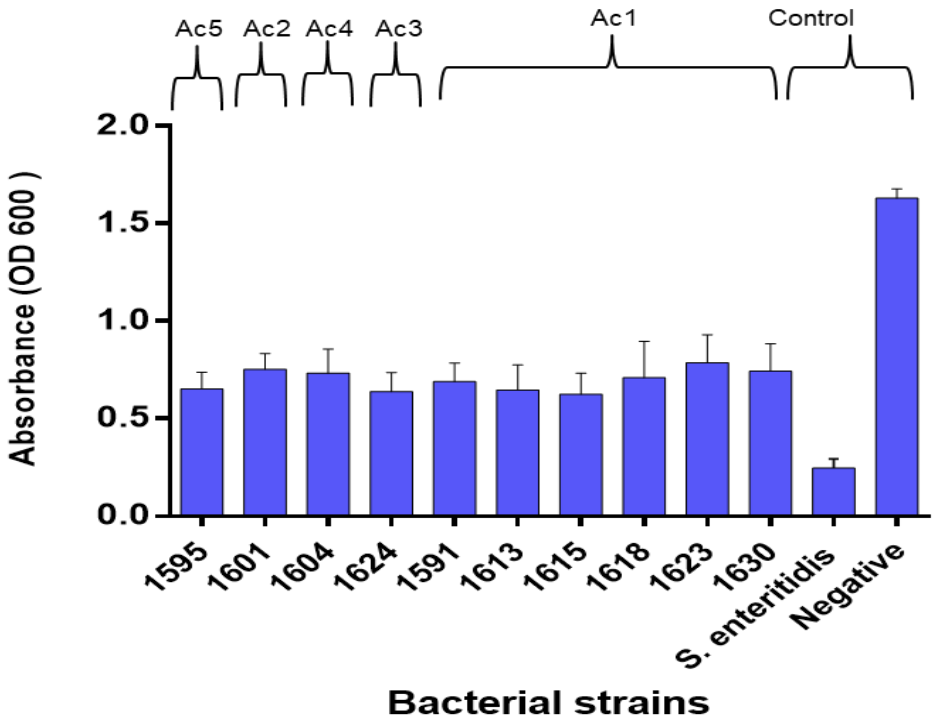


Figure 3-14: Cytotoxicity of *A. baumannii* strains for 1-hour incubation on the Caco-2 cell line.

Most of the strains showed moderate cytotoxicity and there was no significant difference between *A. baumannii* strains when analysed by means of one-way ANOVA, followed by multiple comparisons with a Dennett's test.

3.3.4. Screening of *A. baumannii* for virulence factors and antibiotic resistance genes

3.3.4.1. Virulence factors

A group of genes of interest in this study were chosen according to the *A. baumannii* virulence factors database (VFDB). These genes were classified into the major virulence factors of *Acinetobacter*, such as *Csu* fimbrial operon, which plays a vital role in the initial steps of biofilm formation and assists in attachment of the cell to biotic and abiotic surfaces. The *Csu* fimbrial operon (*csu ABCDE*) was noted among all the *A. baumannii* ST113 genomes analysed in this study, however, it was absent in the *A. baumannii* strain belonging to ST193. The biofilm-associated protein (Bap) and operon *pgaABCD* have also been associated with biofilm formation. Genomic analysis revealed that these genes were present in all *A. baumannii* ST113 and ST193 isolates. Other important putative virulence-associated genes (VAGs) included those which confer serum resistance (*pbpG*), immune evasion (*lpxMBCD*), phospholipase (*plcD*), genes associated with iron

acquisition (*bauABCDEF - basABCDEFJ –barAB*), outer membrane protein A (*ompA*), lipopolysaccharide (LPS) and regulator of a two-component regulatory system (*bfmRS*) were also investigated. The results of the genomic comparison of representative strains against the *Acinetobacter* virulence factors database indicated that all the strains belonging to ST113 and ST193 were positive for these virulence genes, as shown in Table 3-6 With regards to antimicrobial susceptibility and resistance of all representative strains, it was found that all strains share some common antibiotic resistance genes, such as *bla*$_{OXA}$-64, *bla*$_{ADC}$-25 and *bla*$_{OXA}$-51. However, only ST113 strains were found to harbour the β-lactam gene *bla*$_{OXA}$-23 and the aminoglycoside gene *aph(3')-Vla*. These strains were also found to be highly resistant to all the antibiotics tested, with the exception of ciprofloxacin. Among of these, only one strain (strain 1601, belonging to ST113) showed susceptibility to the majority of antibiotics tested, even though it was found to harbour multiple antibiotic resistance genes. In contrast, only strain 1595 (ST193) was lacking the antibiotic resistance genes *bla*OXA-23 and *aph(3')-Vla* and it was also noted that this strain was susceptible to the majority of antibiotics tested. The overall antibiotic resistance observed among *A. baumannii* strains was high and these strains encoded a number of different antibiotic resistance genes, as shown in Table 3-7. Further details regarding these genes are provided in the Discussion (3.4).

Table 3.8 shows the presence of biofilm associated protein *Csu* fimbriae genes and a correlation between the ability of these isolates to attach to epithelial cells and biofilm formation at the initial step of the colonisation process. There was a clear variation among the sequenced isolates analysed in the present study. The *csu* cluster, inclusive of *csuA, csuB, csuC, csuD,* and *csuE,* was detected in all of the ST113 isolates, with the only exception being strain 1595 (ST193), which evidently lacked this cluster. The genomic comparison study revealed that all of the sequenced isolates harbouring genes of the *csu* cluster were able to form biofilm and were also able to adhere to the Caco-2 cell line. Whereas the only non-ST113 strain showed an absence of the *csu* locus, and exhibited a very low amount of biofilm formation and low level of attachment to epithelial cells (Table 3-9). Moreover, the results indicate a positive correlation between epithelial cell adherence and amounts of biofilm formation (Statistical significance

($P \leq 0.037$) was calculated using two-tailed t-test), Figure 3-15 demonstrates this correlation and further details will be provided in the Discussion (3.4).

Table 3-6: Genomic comparison of representative isolates of ST113 and non-ST113 strains against the *Acinetobacter* virulence factors database (VFDB).

Isolate	PT	Hospital	ST	Adherence	Regulation				Enzyme	Immune evasion LPS	Serum resistance	Heme utilization	Iron uptake (Acinetobactine)				Biofilm formation			Resistance genes			
				ompA	Quorum sensing		component system		*plCD*	*lpxMBCD*	*pbpG*	*hemO*	*basA-J*	*bauA-F*	*barAB*	*entE*	Bap protein	Csu fimbriae	b-(1--)poly-N-acetyl-D-glucosamine (PANG)	A-glycoside	Beta-lactam		
					abaI	*abaR*	*bfmRS*	*bfmS*									*bapCD*	*csuABCDE*	*pgaABCD*	*aph(3')-VIa*	*blaOXA-23*	*blaOXA-64*	*blaADC-25*
1591	Ac1	PRH	113	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1613	Ac1	PRH	113	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1615	Ac1	PRH	113	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1618	Ac1	PRH	113	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1623	Ac1	PRH	113	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1630	Ac1	KAH	113	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1601	Ac2	PRH	113	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1624	Ac3	PRH	113	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1604	Ac4	PRH	113	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1595	Ac5	KAH	193	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	+	+

The results indicate that all isolates harboured a number of virulence and antibiotic resistance genes, whilst several genes were notably absent in *A. baumannii* strain 1595 belonging to ST193.

Table 3-7: comparison of phenotypic and genotypic analysis of *A. baumannii* strains of ST113 and St193.

Isolate	P.T	ST	Hospital	Infant age (d)	source	Aminoglycoside (gene)		Beta-lactam (gene)			Antibiogram										
						aph(3')-VIa	*blaOXA-23*	*blaOXA-64*	*blaADC-25*	*blaOXA-51*	AUG	AP	GM	MEM	IMI	CXM	CTX	CRO	CIP	C	TS
1591	Ac1	113	PRH	14	FM	+	+	+	+	+	R	R	R	R	R	R	R	R	S	R	S
1613	Ac1	113	PRH	11	FM	+	+	+	+	+	R	R	R	R	R	R	R	R	S	R	S
1615	Ac1	113	PRH	13	BM	+	+	+	+	+	R	R	R	R	R	R	R	R	S	R	R
1618	Ac1	113	PRH	14	BM	+	+	+	+	+	R	R	R	R	R	R	R	R	S	R	R
1623	Ac1	113	PRH	10	FM	+	+	+	+	+	R	R	R	R	R	R	R	R	S	R	S
1630	Ac1	113	KAH	16	BM	+	+	+	+	+	R	R	R	R	R	R	R	R	S	R	R
1601	Ac2	113	PRH	12	BM	+	+	+	+	+	R	S	S	S	S	S	S	S	S	R	S
1624	Ac3	113	PRH	7	FM	+	+	+	+	+	R	R	R	S	S	R	R	R	S	S	R
1604	Ac4	113	PRH	12	FM	+	+	+	+	+	R	R	R	R	R	R	R	R	S	R	R
1595	Ac5	193	KAH	8	FM	Not found	Not found	+	+	+	S	S	S	S	S	S	R	I	S	R	S

The non-ST113 strain 1595 lacked these genes and was found to be susceptible to the majority of antibiotics tested in this study, indicating a correlation between phenotype and genotype. Strain 1601 was the exception, belonging to ST113 and harbouring antibiotic resistance genes, but phenotypically sensitive to the majority of antibiotics tested in this study.

Table 3-8 *A. baumannii* strains of ST113 harboured the *Csu* fimbrial operon (*csu ABCDE*), which is associated with the ability of bacteria to form biofilm and attach to epithelial cells. The non-ST113 strain lacking this operon was a poor biofilm former and also had low attachment to epithelial cells. No correlation was observed between this operon and other traits.

Isolate	P.T	ST	Hospital	Infant age (d)	source	Csu fimbriae					Cellulose production	Biofilm IF 25° C	Caco-2		Macrophage response	MTT
						csuA	*csuB*	*csuC*	*csuD*	*csuE*			attachment	invasion		
1591	Ac1	113	PRH	14	FM	+	+	+	+	+	low	high	high	very low	persisted + replicated	moderate
1613	Ac1	113	PRH	11	FM	+	+	+	+	+	high	moderate	moderate	very low	persisted + replicated	moderate
1615	Ac1	113	PRH	13	BM	+	+	+	+	+	high	moderate	high	very low	persisted + replicated	moderate
1618	Ac1	113	PRH	13	BM	+	+	+	+	+	high	high	moderate	low	Low persisted	moderate
1623	Ac1	113	PRH	10	FM	+	+	+	+	+	high	High	high	very low	persisted + replicated	moderate
1630	Ac1	113	KAH	16	BM	+	+	+	+	+	high	moderate	high	very low	persisted + replicated	moderate
1601	Ac2	113	PRH	12	BM	+	+	+	+	+	high	moderate	moderate	very low	Persisted + killed at 48h.	moderate
1624	Ac3	113	PRH	7	FM	+	+	+	+	+	unable	high	high	low	persisted + replicated	moderate
1604	Ac4	113	PRH	12	FM	+	+	+	+	+	high	high	high	low	persisted + replicated	moderate
1595	Ac5	193	KAH	8	FM	-	-	-	-	-	unable	low	low	very low	persisted + replicated	moderate

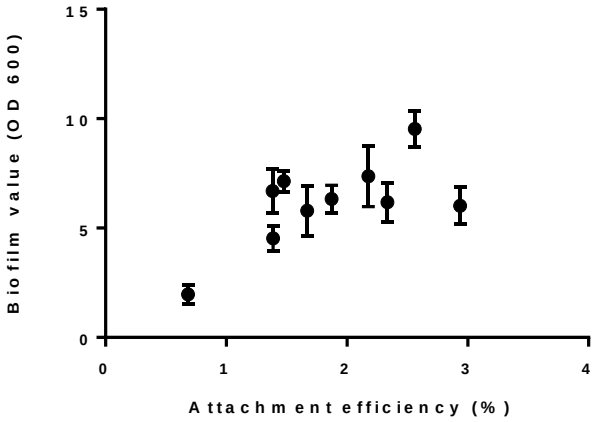


Figure 3-15: Correlation between amounts of biofilm formation and attachment ability among the representative strains of *A. baumannii.* Statistical significance ($P \leq 0.037$) was calculated using two-tailed t-test.

3.4. Discussion

Bacterial colonisation of infants starts as early as birth. Rapid detection and identification of routes of transmission of many bacterial pathogens is very important to control and prevent disease and outbreaks. NICUs are more exposed to nosocomial microorganisms during hospitalisation of neonates (Mohammed and El Seifi, 2014; Legeay et al., 2015; Ramasethu, 2017). Several molecular subtyping methods are important tools that allow rapid identification of these microorganisms and their sources. Molecular fingerprinting techniques such as PFGE are important in epidemiological investigations. PFGE has been applied in several bacterial outbreak studies, including *A. baumannii*, and exhibits high discriminatory power between pulsotypes (Villalon et al., 2011). In this study, PFGE has been used to assess the genetic diversity of strains and identify whether clonally related strains were isolated on different occasions. This technique was also used to determine whether any epidemiological relationship exists between pulsotype and clinical presentation.

Forty-three *Acinetobacter baumannii* strains were assessed in this project, which were isolated from nasogastric enteral feeding tubes from NICUs in Jordan. The strains had been collected from two hospitals between 15-07-2011 and 21-12-2011, and PFGE was performed in this study using the *ApaI* restriction enzyme. Representative strains were subsequently used to confirm the PFGE profiles using the *AscaI* restriction

enzyme. The combined PFGE profiles for both *ApaI* and *AscaI* restriction enzymes were then considered (Figure 3-2).

All *A. baumannii* strains were clustered into five different pulsotypes, with the majority of strains clustering with pulsotype Ac1, as showed in Figure 3-1. Interestingly, this pulsotype was found across enteral feeding tubes of different infants within the same hospital. Furthermore, the same pulsotype was prevalent among strains isolated from the two different hospitals (KAH and PRH in Jordan), which was quite unexpected. Of particular significance was pulsotype Ac1, which was composed of 38 indistinguishable strains from 21 neonates, over a six-month period. The first isolate was obtained from PRH hospital on 15/07/2011, then after a period of five months, strains of the same pulsotype (Ac1) were isolated from both hospitals (KAH and PRH). This indicates the ability of these strains to survive and persist for long period of time, possibly in both biotic and abiotic environments.

It is unknown whether there was transfer of neonates or hospital staff between the two hospitals. These strains had been isolated from neonates receiving different feeding milk, which may rule out the possibility of strains being acquired due to sharing the same feeding source. However, several studies have reported that *A. baumannii* isolates had demonstrated long-term persistence in desiccated infant formula (Juma et al., 2016; Chapartegui-González et al., 2018). A possible explanation for this observation could be contamination of the feeding tubes due to the surrounding environment or directly from carers within the NICU. The other possible sources might be from the hands of care workers when preparing PIF for infants in the NICU. Potential contamination of the fresh feed in the tube lumen may result in additional bacterial multiplication. It is therefore reasonable to suggest that inappropriate preparation, storage, and handling of powdered infant formula may pose a substantial risk to neonates with underdeveloped immune systems as well as preterm infants.

In addition, this study demonstrated that the pulsotypes Ac2, Ac3, and Ac4 comprised one unique strain, while pulsotype Ac5 was composed of two strains isolated from one neonate on the same day. Pulsotypes Ac2 - Ac5 were less persistent when

compared to pulsotype Ac1. This indicates a narrower range of these strains to colonise the enteral feeding tubes. Based on these results, one strain was selected as representative to study the virulence traits of *Acinetobacter* pulsotypes Ac2 - Ac5. Since the majority of strains clustered with pulsotype Ac1, it was decided to select representative strains according to hospital, neonatal sample source, and birth of neonate.

These isolates had been previously identified by the hospital with the API 20E test strip, however identification to the species level is often problematic within the *Acinetobacter* genus (Khosravi et al., 2015). Due the importance of these strains in hospital infections, precise identification to the species level is important to clarify the epidemiology and ecology of these species. Different encoding genes have been used to define species within the *Acinetobacter* genus, such as *gyrB, rpoB*, and *recA* (Scola et al., 2006; Higgins et al., 2007; T. L. Chen et al., 2014; Khosravi et al., 2015). Among these genes, the *rpoB* gene (350 bp) was used for the identification of *Acinetobacter* isolates due to the variability in its size (zone 1) and because it is highly discriminative among the different bacterial species (Gundi et al., 2009; Kamolvit et al., 2015). In the current study, the 18 representative strains were confirmed as *A. baumannii* by using whole genome sequencing (WGS). Agreement between the *rpoB* and WGS data in this study suggested that the partial *rpoB* sequence is likely to be a reliable and rapid identification method for *Acinetobacter* species. This is consistent with another study by Gundi et al. (2009) who reported that the method for identifying *Acinetobacter* spp. by *rpoB* sequencing is target for rapid molecular identification. An additional finding was that all isolates were carbapenem-resistant and carried the *bla*$_{OXA}$-51-like gene. This result is in agreement with another previous study which similarly showed that almost all *A. baumannii* strains possess the *bla*$_{OXA}$-51-like gene. This gene has generally been found to be chromosomally encoded and non-transferable. These results provide evidence that detection of *bla*$_{OXA}$-51 can be used as a simple and reliable method of identifying *A. baumannii* (Turton et al., 2006). Although detection of *blaOXA*-51 has been suggested to be highly specific for *A. baumannii*, which is in contrast with the results obtained by Teixeira et al. (2013) stated that this gene has also been found to be involved in *A. nosocomialis*. Another

study by Leski et al. (2013) showed that this gene can be also found in members of the *Enterobacteriaceae.*

MLST typing is a method that is often seen as a complementary typing method to PFGE (Tomaschek et al., 2016). This study therefore intended to apply the MLST scheme database (Institute of Pasteur) to 18 selected isolates among the collected strains of *Acinetobacter* spp. MLST analysis of *A. baumannii* strains revealed that all of them belonged to one of 2 different STs (ST193 and ST113). Seventeen strains belonging to pulsotypes Ac1, Ac2, Ac3, and Ac4 were sequence type (ST113). Amongst these fourteen belonging to the same pulsotype Ac1 found in both hospitals. However, the other single isolate belonging to pulsotype Ac5 had a unique genotype indicated by PFGE as well as MLST (ST193). The *A. baumannii* ST113 sequence type has been found to be the predominant sequence type that was recovered from a large number of feeding tubes from the two hospitals. The MLST database contains a total of 19 isolates belonging to ST113. It was important to note that all of the ST113 isolates in the MLST database originated from clinical sample sources (i.e. 22% from CSF, 11% from blood, 11% from sputum, 11% from upper respiratory tract, and 5.6 % from abdominal and IV catheters). Another interesting observation was the geographic spread of these isolates originating from three different countries: Brazil (72%), USA (11%), and China (17%), whereas our collection included isolates from two hospitals in Jordan, Middle East. A previous study conducted by Bonnin et al. (2013) reported that some *A. baumannii* isolates belonging to ST113 have been recovered from hospitals in Kuwait in 2013. More recently, several *A. baumannii* isolates belonging to ST113, recovered from a perineal swab in the intensive care unit of the Minas Gerais Hospital, Brazil, was found to be resistant to all β-lactams (Girlich et al., 2014). Based on MLST analysis, this strain was typed as ST113, the same ST which was recovered from the intensive care unit in Brazil. Considering the geographical separation between the two countries (Brazil and Jordan), the evidence indicates there might be a wide distribution of these strains throughout the world. In the present study, PFGE differentiated 18 isolates into 5 distinct patterns, 14 strains belonged to Ac1 were isolated from NICUs in 2 hospitals, over a 5-month period. One strain to each pulsotypes Ac2, Ac3 and Ac4 were isolated from the same hospital (PRH) and one

strain belonged to Ac5 isolated from KAH hospital. Whereas MLST identified two distinct STs among the 18 isolates, suggesting that PFGE is a more discriminatory method than MLST for typing these isolates. However, MLST is more efficient to improve our understanding of molecular epidemiology within a hospital environment or between hospitals and geographic locations (El-Shazly et al., 2015).

Medical devices, in particular enteral feeding tubes, are frequently used in NICUs for feeding the preterm infant or critically ill neonate. A previous study by Hurrell et al. (2009), revealed that an important source of bacteria entering neonates is the enteral feeding tube, which might act as a place for bacterial colonisation as a biofilm. In the last three decades, there has been a rise in the incidence of neonatal infections due to *A. baumannii* (Hsu and Tamma, 2014; Wei et al., 2015). It is widely distributed in clinical settings and it is an important problematic opportunistic pathogen. This organism has been found to be frequently associated with the surface of medical devices, such as catheters and feeding tubes (Tomaras et al., 2003; Pakharukova et al., 2018). It causes a wide range of infections, including meningitis, bacteraemia, wound infections, and UTIs (Antunes et al., 2014; Almasaudi, 2018). Moreover, it is well known for its ability to form biofilms and attach to biotic and abiotic surfaces. Therefore, biofilms play an important role in the colonisation of microorganisms, providing an opportunity for bacteria to be more resistant to environmental stresses.

In this study, all the representative strains of *A. baumannii* ST113 and ST193 showed the ability to form high amounts of biofilm when compared to the control. Additionally, the majority of these isolates were found to produce significantly higher amounts of biofilm at 37 °C than at 25 °C ($P < 0.05$). Only one strain, belonging to ST193, produced the lowest amount of biofilm at 25 ºC, which was similar to the control value. The effect of temperature on biofilm formation was evident based on this observation. The optimum temperature might be linked to the reaction rate of enzymes. Previous studies have revealed that several factors affect biofilm formation including, temperature, O_2 level and pH (Goller, 2008). It is widely accepted as a temperature-controlled process in living organisms. Thus, the temperature might be an important factor for biofilm formation in *A. baumannii*. Other studies reported that the curli fimbriae play an important role in biofilm formation in many species of bacteria, and

they help mediate host cell adhesion and invasion (Lasaro et al., 2009; Barnhart and Chapman, 2010; Kim et al., 2012; Sharma et al., 2016). In this study, representative strains of *A. baumannii* were characterised on Congo red media to evaluate the expression of curli fimbriae. All tested strains expressed red colonies, which indicates curli fimbriae as positive colonies for binding to the Congo red dye. However, genome comparisons using the Artemis genome browser were carried out to determine the presence or absence of the genes of interest, and BLAST search was carried out at the nucleotide level for curli fimbriae-associated genes. The investigation confirmed that all the analysed genomes of the representative isolates were found to lack the curli fimbriae region, thus suggesting that other proteins may be binding to the Congo red dye. Another extracellular material produced by bacteria for the formation of biofilm is cellulose. It has been shown to play an important role in biofilm formation in bacterial cells, structure, and protection in biofilms (Limoli et al., 2015). The same authors also reported that the cellulose was produced by many Enterobacteriaceae, including *S.* Typhimurium, *S.* Enteritidis, *Cronobacter* species, *K. pneumoniae* and *E. coli*. Similarly, Re and Ghigo (2006) revealed that cellulose is one of the most important components of the biofilm matrix In *Salmonella* and *E. coli*. Although the majority of the representative strains in this study produced cellulose in different quantities, even though ST113 strains showed a higher quantity of biofilm production at 37 °C, no correlation was observed between these strains to form biofilm and the amount of cellulose produced.

In addition, this study also revealed that there was no correlation between biofilm formation and capsular material produced by all representative strains. Bacterial capsule production was determined by colony appearance in two different growth media: soya and whey-based infant formula. The strains were unable to produce capsule material on both types of media; however, they were able to produce a high amount of biofilm on the same media. There are many studies that have found no correlation between capsule production and the ability to form biofilm. For example, Hurrell et al. (2009a) found that *C. sakazakii* strains were unable to produce any capsular materials, but nevertheless produced more biofilm. Although, the results indicate no obvious link between biofilm formation in this strains and capsule

production, capsules may provide resistance to desiccation, serum activity and have a significant role in avoiding phagocytic killing (Ogrodzki and Forsythe, 2015). Another study indicated that capsule production is essential for *A. baumannii* survival during growth in serum and infection (Russo et al., 2010). However, during the current study, it was identified that majority of *A. baumannii* isolated from enteral feeding tubes were strains with a high ability to form biofilm on abiotic surfaces. This may explain the possibility of biofilm formation inside neonatal feeding tubes, acting as a locus and increasing the risk of neonatal infection by the ingestion of these organisms.

Indeed, biofilm formation is controlled by complex regulatory systems in *A. baumannii*. A recent study by Selasi et al. (2016) showed that several genes are reported to be linked with biofilm formation of *A. baumannii*. For example, the *csu* locus encoding the chaperone usher pili assembly system is required for biofilm formation and attachment, and the *pga*ABCD operon is involved in the synthesis of poly-β-(1-6)-N-acetylglucosamine (PNAG) in *A. baumannii*. It has been described as a one of the major components of biofilms in *S. aureus*. Furthermore, the *bap* gene encoding the surface protein, and *ompA* encoding the major outer membrane protein, demonstrate a key role in biofilm formation (Gaddy et al., 2009; Brossard and Campagnari, 2012; Luo et al., 2015; Selasi et al., 2016). Analysing the genomes in the current work revealed that all previous genes have been detected in the genomes of the tested *A. baumannii* strains by using genome comparisons. However, only one strain (ST193), belonging to pulsotype Ac5, showed a lack of the *csu* cluster. This cluster consists of *csuA, csuB, csuC, csuD*, and *csuE* genes, which are essential genes involved in the adherence to and biofilm production on abiotic surfaces for *A. baumannii* strains (Bahreini et al., 2014; Luo et al., 2015). One of the most interesting observations is strain ST193, which also showed a very low amount of biofilm production at 25°C. These findings may suggest a significant correlation between the *csu* cluster and biofilm formation. These results are in agreement with the data obtained by Tomaraset al. (2003), who reported that the presence of pili-like structures on the surface of *A. baumannii* leads to the formation of biofilm. The disruption of *csuE* and *csuC* resulted in non-piliated cells and eliminated biofilm formation and cell attachment. This might be because genes that appear to be

required for pili formation, result in formation of biofilms and associated attachment to plastic surfaces by *A. baumannii* strains. However, the ability of strains of ST113 to form biofilm at 25°C varied, which indicates that some of the genes of the *csu* locus detected might be differentially expressed or influenced by other factors. Therefore, biofilm production of *A. baumannii* strains could enhance colonisation and persistence in enteral feeding tubes and also increase the probability of causing nosocomial infections and outbreaks.

Antimicrobial susceptibility testing in this study was performed for all the representative strains of *A. baumannii*. The results show that the majority of tested strains (ST113) were highly resistant to all the antibiotics tested, with the exception of ciprofloxacin. These strains were found to harbour β-lactamase genes, such as *bla*$_{OXA}$-23, *bla*$_{OXA}$-64, *bla*$_{ADC}$-25 and *bla*$_{OXA}$-51, as well as the aminoglycoside gene (aph (3')-Vla). In contrast, a single isolate of *A. baumannii* ST193 showed susceptibility to the majority of antibiotics tested, and additionally, the *bla*$_{OXA}$-23 and *aph(3')-Vla* genes were not detected. My study showed that the ST113 strains are defined as multidrug-resistant to different classes of agents and that the *bla*$_{OXA}$-23 gene was the most frequent carbapenem resistance gene among these strains. One previous study carried out in China reported that the most common carbapenem resistance gene is *bla*$_{OXA}$-23 among *A. baumannii* (Chang et al., 2015). Another study by Hou and Yang (2015) reported that most *A. baumannii* outbreak strains in ICUs are highly resistant to antibiotics. Also, *A. baumannii* have been reported as a cause of more severe diseases among critically ill patients (Almasaudi, 2018). The strains characterised in this study showed high resistance to antibiotics, and it can be considered as a risk factor to neonates in the ICU who are fed via a nasogastric tube, especially those born with low birth weight and undeveloped immune systems.

In addition, factors other than antibiotic resistance have led to the emergence of *A. baumannii* in the hospital settings as a significant pathogen, including the ability to resist desiccation. It has been reported that *A. baumannii* has the ability to colonise and persist in hospital environments for months, surviving in harsh and stressful conditions (Espinal et al., 2012; Ryu et al., 2017). A previous study reported that *A. baumannii* can survive for prolonged periods on dry surfaces, such as plastic, glass,

and other environmental surfaces implicated as a transmission route in infection and outbreaks (Espinall et al., 2012; Gallego et al., 2016). Representative *A. baumannii* strains in the current study were evaluated for their ability to survive desiccation. It was revealed that the majority of these organisms have the ability to maintain their viability during 14 days of desiccation in infant formula and are able to efficiently recover after this period of time. This recovery can be considered as a risk factor to the health of neonates in the ICU. Due to the ability of *A. baumannii* to resist desiccation, it is likely that this can contribute to the survival and persistence of this organism to maintain its viability under environmental pressures within the hospital. This may increase opportunities for transmission between neonates in the ICU, in particular those who often require feed via a nasogastric feeding tube. These strains also displayed multidrug resistance, which therefore makes them more difficult to treat. Consequently, these organisms can be considered as serious concerns and as major risk factors to neonates with immune systems that have not fully developed and thus, increases the likelihood of infection.

For pathogenic bacteria to cause infections they require the ability to survive in the bloodstream and overcome the defence mechanisms of the host. Resistance to human serum is an important factor in bacterial virulence (Russo et al., 2010). Previous studies reported that a common trait of organisms causing bacteraemia is the ability to resist antimicrobial factors in human serum (Williams et al., 2001). Results obtained in the current study demonstrate that all *A. baumannii* isolates displayed high resistance to human serum and revealed significant increases in their initial numbers after the third hour of incubation. Indeed, several recent studies have reported the involvement of some factors in serum resistance. For instance, Russo et al. (2009) reported that the *pbpG* gene is an encoded virulence factor in *A. baumannii* and the mutant shows reduced growth in human serum. Another report showed that phospholipase D (*PLD*) has been identified as a virulence factor in *A. baumannii*, and disrupting this gene also results in reduced resistance to human serum as well as decreased capacity for invading epithelial cells (Jacobs et al., 2010). Moreover, it was suggested by Kim et al. (2009) that the OmpA protein contributes to the survival of *A. baumannii* in human serum. OmpA has roles in adherence, invasion

of epithelial cells, and binds to factor H in human serum, which may allow *A. baumannii* to survive in blood by avoiding serum-mediated killing. However, *pbpG*, *PLD,* and *ompA* genes have been detected in the genomes of the *A. baumannii* strains analysed in this study. Such findings indicating the ability of these strains to survive in human serum would increase the clinical concern to neonates in the NICU, especially those with compromised immune systems, which are therefore highly susceptible to infections.

In addition to the survival of *A. baumannii* in human serum, acquisition of iron is essential in such environments to help bacteria survive and persist in the host. Iron is one of the most important nutrients for the host and pathogenic bacteria alike. However, in the human body, iron is rarely found as a free and unbound molecule, and is therefore one of the major limiting factors for pathogenic microorganisms. (Hasan et al., 2015). To counter this, pathogenic bacteria use several iron acquisition mechanisms to obtain iron from the host (Penwell et al., 2012). The most iron-chelating molecule found among *A. baumannii* clinical isolates is called siderophore acinetobactin (Mihara et al., 2004; Mortensen and Skaar, 2013). In this study, all *A. baumannii* strains were able to produce siderophores as shown in Table 3-5. This suggests that *A. baumannii* may have the ability of acquiring iron in iron-deficient environments. The biosynthesis of acinetobactin is encoded by the operon *basABCDFGHI* and secretion via a siderophore efflux system encoded by genes *barA* and *barB.* The transport system for acinetobactin is encoded by genes *bauABCDEF*, which are essential for translocation of ferric-acinetobactin complexes into bacterial cells, as well as putative binding sites for ferric uptake regulators such as *entE, basD*, and *bauF* (Penwell et al., 2012; Hasan et al ., 2015). In this study, screening for genes encoding iron transport systems and receptors carried out by BLAST searches against the virulence factor database (VFDB) confirmed their presence in all of the *A. baumannii* strains in this study. The presence of such genes might enable the bacteria to uptake sufficient amounts of iron and survive in the host, which could therefore contribute to bacterial pathogenesis.

Bacterial haemolysins and protease are considered as virulence factors that were detected among the tested *A. baumannii* strains. Such factors are important in

contributing to bacterial pathogenesis and disease. The present study showed that the majority of *A. baumannii* isolates were able to lyse the erythrocytes of horse blood. The protease enzyme was detected among all tested strains. A previous study by Antunes et al. (2011) also reported that clinical isolates of *A. baumannii* clinical demonstrated appreciable amounts of haemolysis in horse blood but not in sheep blood. The same authors also reported that the all strains displayed protease activity. It is possible that these activities might be an important part of the pathogenic mechanisms of these bacteria. These results therefore suggest that the *A. baumannii* strains have the ability to lyse the erythrocytes of horse blood through production of β-haemolysis and secreted proteases, which is recognised to interact with host systems to promote infection (Tilley et al., 2014). Consequently, the presence of such virulence traits is likely to increase the potential risk factors to neonatal health.

The FAO and WHO (2004 & 2006) risk assessment on the microbiological safety of infant formula, identified different microorganisms associated with powdered infant formula contamination including *Acinetobacter* spp. The ingestion of contaminated feed containing acid-tolerant bacteria may present the opportunity of bacterial colonisation and potentially cause infections in the patients. However, stomach acidity would normally provide an important front-line defence against pathogens when ingested with food (Martinsen et al., 2005). In this study, the tolerance of acidic conditions was determined by exposure of the *A. baumannii* strains for 2 hours at pH 3.5 in infant formula media. All tested strains were able to tolerate the 2 hour exposure time at pH 3.5. The protection of these strains from the low pH acidity could be because of their ability to form a biofilm. This is in agreement with a previous study by McNeill and Hamilton (2003) who showed that biofilm cells of *Streptococcus* are highly resistant to low pH. The survival of these strains in low pH acidity may allow them to succeed in causing infection through the route of ingestion, and particularly in neonates who feed via nasogastric feeding tubes. Neonates in NICUs are routinely fed through a feeding tube at regular period timed between 2 to 3 hours. In this study, majority of strains were capable of forming high amounts of biofilm, which also grew inside neonatal feeding tubes and were resistant to low pH. This therefore suggests that these strains could reach the intestines via ingestion and colonise the neonate's

stomach and cause infection. It's a potential health risk to neonates, particularly among premature babies in NICUs who lack a developed immune system, who are at greater risk.

Another important virulence factor would include the ability of the organisms to attach to human epithelial cells during the initial step of the colonisation process (Smani et al, 2012). In this study, attachment ability to human epithelial cells for the representative *A. baumannii* strains was assessed to determine the potential risk of infection to neonates in ICUs. Caco-2 monolayer cell line was used as a model of the small intestine. It was revealed that *A. baumannii* isolates possess the ability to adhere to the Caco-2 cell line. This shows that the strains might have a high virulence potential. Although the levels of adherence varied among the strains, all strains belonging to ST113 showed an ability to attach at high and moderate levels. However, several studies have shown involvement of the *csu* locus encoding the chaperone-usher pili assembly system in biofilm formation. It has also been involved in attachment to abiotic surfaces in *A. baumannii* strains (Tomaras et al., 2003; Luo et al., 2015; Selasi et al., 2016). Interestingly, the *csu* locus was identified among isolates of ST113 in this study, however, only one strain –1595, belonging to ST193 – was lacking the *csu* locus and demonstrated a very low amount of biofilm production and low levels of attachment. It can be suggested that the presence of this locus is most likely to be associated with the capacity of biofilm formation and attachment ability of this isolates. The results of this study agree with a previous study by Tomaras et al. (2003) which reported the disruption of the *csuE* and *csuC* resulted in non-piliated cells and eliminated biofilm formation and cell attachment. This has also has been characterised by Pakharukova et al., (2018) Jennifer suggesting that it plays an important role in bacterial attachment to biotic and abiotic surfaces and in biofilm formation. Similarly, another report suggested that a significant correlation exists between the capacity to form biofilms and the expression of the *csuC, csuD*, and *csuE* genes by carbapenem-resistant *A. baumannii* (CRAB) (Selasi et al., 2016). Outer membrane proteins, such as OmpA, have also been characterised in *Acinetobacter*, which is essential for bacterial attachment to epithelial cells and is involved in the development of biofilms on plastic surfaces. For example, Kim et al., (2016)

investigated the role of OmpA in the pathogenesis of *A. nosocomialis*. Their results indicated that *ompA* mutant displayed reduced attachment to A549 human alveolar epithelial cells and biofilm formation compared with the wild type. Consistent with this, the *ompA* gene has been detected in the genomes of all *A. baumannii* strains analysed in this study.

There are several studies which have reported a correlation between resistance to antibiotics and the ability to form biofilms. For instance, a recent study by Bardbari et al., (2017) found a significant correlation between multidrug-resistant (MDR) *A. baumannii* isolates and biofilm formation. But correlations between biofilm formation and cell adhesion among clinical isolates has been less well studied. The current study demonstrates interesting correlations between biofilm formation and the capacity of attachment, as shown in Figure 3-15. For example, strain 1595 (ST193) produced a very low amount of biofilm and low attachment while strains 1604, 1623, 1524 and 1591 (all ST113) showed higher levels of biofilm formation and higher levels of attachment. Such traits may not only confer persistence and survival of these strains in neonatal intensive care units, but also enables growth and colonisation in enteral feeding tubes. This could explain the ability of these isolates to successfully colonise neonatal feeding tubes and offers the opportunity for these isolates to survive and potentially cause subsequent infections in the neonate. It would appear that neonates born with low birth weights and undeveloped immune systems in NICUs are at high risk, especially those fed via nasogastric tubes. The feeding tube would therefore represent an important risk factor in NICU infections.

Attachment is a very specific process and the initial step in many infections. Once pathogenic bacteria adhere to a host surface, they may proceed to invade mammalian cells, in some cases. In this study, the gentamicin protection assay was performed to determine the ability of strains to invade Caco-2 human epithelial cells. As shown in Figure 3-12, most of these strains invaded Caco-2 human epithelial cells at very low levels, such as strains 1591, 1595, 1601, 1613, 1615, 1623, and 1630, which belonged to different pulsotypes and sequence types. However, strains 1604, 1618, and 1624 (ST113) showed significantly higher levels of invasion when compared to the other strains. When compared to the negative control strain, most of these strains showed

no significant difference when analysed by means of one-way ANOVA, followed by multiple comparisons with a Dennett's test. Nevertheless, it was notable that the majority of isolates belonging to ST113 revealed significant levels of attachment and low levels of invasion, while isolates of ST193 showed lower attachment and invasion abilities. This suggested that there was no correlation between the ability to adhere and invade for *A. baumannii*. This demonstrates the virulence potential of *A. baumannii* isolates for neonatal hosts. Although a low ability to invade intestinal cells was observed, it may still suggest a potential of pathogenicity in premature babies in NICUs who lack a developed immune system.

After invasion, many bacterial pathogens have the ability to use different strategies to survive and replicate in different types of host cells. On the other hand, the innate immune system constitutes the first line of host defence against infection. In this study, Figure 3-13 shows the *A. baumannii* strains (n=10) which were investigated for their ability to persist in human macrophages, using the U937 macrophage cell line. The majority of these strains were able to persist and replicate inside macrophages for up to 72 hours of incubation. This could possibly be due to all of these strains possessing the *pgaABCD* genes, which are involved in the synthesis of poly-β-(1-6)-N-acetylglucosamine (PNAG). PNAG is one of the important polysaccharides, which is reported as a virulence factor that protects bacteria against innate host defences, and is described as a major component of biofilm formation (Choi et al., 2009). Indeed, maintenance of the organism's viability within the macrophage suggests that these organisms may be reflective of the levels of pathogenicity in premature babies in NICUs. Additionally, these strains were confirmed to show a high level of resistance to human serum (Figure 3-5), and such characteristics might enhance the ability of *A. baumannii* strains to evade the host immune response and cause bacteraemia.

The effect of *A. baumannii* on the viability of the epithelial Caco-2 cells was also assessed by measuring MTT reduction. All strains were moderately cytotoxic to Caco-2 cells. Amongst the representative isolates tested, strain 1623 was slightly more cytotoxic than the other strains, but this difference did not reach statistical significance. Cell death has been described in *A. baumannii* by Choi et al. (2005) to be induced by apoptosis as the result of *A. baumannii* after 12 h, targeting the

mitochondria of the human laryngeal epithelial cells (HEp-2). In addition, *A. baumannii* was observed to cause the apoptotic death of A549 human alveolar epithelial cells (Gaddy et al., 2009). Cytotoxicity on intestinal epithelial cells might have significant implications for the evolution of infections. Due to the ability of these isolates to cause a cytotoxic effect and induce cell death, it can be expected that the pathogenic potential of *A. baumannii* could result in infection of neonates in NICUs.

As classically described, *A. baumannii* is an important nosocomial pathogen. It is also an important pathogen in neonates hospitalised in ICUs, particularly new-born babies with very low birth-weights and weakened immune systems (Von-Dolinger et al., 2005; Hsu et al., 2014). All isolates in this study were recovered from neonatal feeding tubes of 21 neonates in NICUs from two different hospitals. They were defined as multidrug-resistant and showed the ability to form significant amounts of biofilm. These strains also showed the ability to tolerate acidic conditions, desiccation, and survive in human serum. Furthermore, other important observations included their ability to attach and invade intestinal epithelial cells and persist inside macrophages. These features could allow them to survive and facilitate their persistence and transmission in the NICU. Additionally, colonised neonatal feeding tubes could serve as reservoirs for pathogenic *A. baumannii*. This strongly indicates a possibility of neonatal infections resulting from the persistent exposure by ingestion in hospitals where the neonates are at high risk of nosocomial infection in neonatal intensive care units.

The *A. baumannii* ST113 sequence type has been found to be the predominant sequence type that was recovered from a large number of feeding tubes from the two hospitals. Their presence confirms the colonisation of neonatal feeding tubes and causes a real concern over risk factors associated with neonatal feeding and infection sources. Of particular concern are new-borns with low birth weights who routinely feed via a feeding tube. As discussed earlier, the consumption of the feed with bacteria colonising the feeding tube could result in the ingestion of high numbers of bacteria. After ingestion, these bacteria have the potential to colonise the neonate's intestine and cause infection. This finding warrants due consideration because of the high risk of infection associated with premature babies in the NICU.

Chapter 4. Profiling a clinical collection of *Enterobacter* spp. strains, in particular *E. hormaechei*, isolated from neonatal sepsis cases, CSF and nasogastric enteral feeding tubes

4.1. Introduction

Neonates in neonatal intensive care units (NICUs) represent highly susceptible individuals within the paediatric group, particularly pre-term neonates with low birth-weight and weak immune systems. The risk of infection to compromised neonates increases with prolonged hospitalisation where a wide variety of medical devices are used, including enteral feeding tubes, ventilator tubes and catheters. The colonisation of nasogastric enteral feeding tubes by opportunistic pathogens, for example, has been recognised as a possible source of infectious organisms (Hurrell et al., 2009a).

The ability of bacteria to attach to medical devices is well known and these bacteria are often associated with hospital-acquired infections (Venkateswaran et al., 2016; Khelissa et al., 2017). The ability of opportunistic pathogens to form biofilms inside neonatal nasogastric feeding tubes has been a cause for concern. It has been reported in previous studies that neonatal enteral feeding tubes are prone to rapid colonisation by a wide variety of opportunistic pathogens of the Enterobacteriaceae, irrespective of feeding regime (Hurrell et al., 2009a; 2009b). In their study, 129 enteral feeding tubes collected from NICUs were examined and they showed that 76% of these tubes were colonised by microorganisms able to produce biofilms. The most common isolates included *E. hormaechei, E. cancerogenus, K. pneumoniae, S. marcescens* and *Escherichia coli*.

This colonisation of feeding tubes by pathogenic organisms may cause a serious health risk to neonates, particularly those with low birth-weight and premature in the NICU. For example, one quarter of all *E. hormaechei* isolated from neonatal enteral feeding tubes by Hurrell were resistant to the 3rd generation cephalosporins, cefotaxime and ceftazidime (Hurrell et al., 2009a). This was validated by another study which showed that *E. coli* K1 ST95 strains isolated from neonatal nasogastric feeding tubes possessed genes encoding numerous virulence traits associated with neonatal meningitis

(Alkeskas et al., 2015). The risk of infection by such bacterial strains is a concern in the neonatal unit.

Enterobacter spp. are one of the most serious ESKAPE pathogens and are a leading cause of nosocomial infections throughout the world (Santajit and Indrawattana, 2016). The ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* spp.) are important causes of nosocomial infection and dissemination of multidrug-resistant (MDR) strains (Santajit and Indrawattana, 2016; El-Mahallawy et al., 2016; Silva et al., 2017). Chen et al. (2014) reported that *Enterobacter* spp. were important pathogens of nosocomial bacteraemia in paediatric patients at Taipei General Hospital, during a 10-year study between 2001 and 2011 to review the clinical characteristics of bacteraemia. *Enterobacter* spp. have also been identified among Gram-negative microorganisms as a cause of neonatal sepsis in NICUs (Torkaman et al., 2009; Chen et al., 2009; Sharma al., 2013; Arowosegbe et al., 2017). Late-onset *Enterobacter cloacae* sepsis was described by Chen et al. (2009), an infection of very low birth-weight neonates admitted to the NICU. *E. cloacae* was also recognised by Weems et al. (2015) as a cause of late-onset sepsis in a preterm infant who was fed expressed mother's milk. During the past decade, outbreaks caused by *Enterobacter* spp. have been reported by several studies in hospital settings (Boban et al., 2011; Paauw et al., 2010; Stoesser et al., 2015).

4.1.1.*Enterobacter spp*.

Enterobacter spp. are facultative anaerobic Gram-negative rods, motile by means of peritrichous flagella. This genus is composed of saprophytic bacteria which are widely encountered in nature and commensal in the enteric flora in the human gastrointestinal tract. They can also be found in sewage and soil. Currently, six species of the genus *Enterobacter* have been assigned to the *E. cloacae* complex. These members are most frequently isolated from clinical samples (Paauw et al., 2008; Morand et al., 2009). The *Enterobacter cloacae* complex includes *E. hormaechei, E. cloacae, E. asburiae, E. ludwigii, E. rnimipressuralis* and *E. kobei*. The term *E. cloacae* complex is sometimes used because of difficulties in routine identification methods to

distinguish between clinically important species. Phenotypic identification to the species level within the complex group has been mostly unreliable. Although their identification by 16S rRNA gene is widely used, it is not highly discriminatory between members of the Enterobacter genus (Morand et al., 2009). Indeed, molecular methods based on single-locus typing sequence analysis of *hsp60, fusA, gyrB, or rpoB* resulted in distinct genetic clusters, but likely not all clusters are able to be assigned to a specific species. However, the taxonomy of this complex group is mainly based on DNA cross-hybridization (Paauw et al., 2008).

4.1.2. *Enterobacter hormaechei*

E. hormaechei was named as enteric group 75 and first described by the Centers for Disease Control and Prevention (CDC) on the basis of 23 isolates which were received for identification. The species *E. hormaechei* was suggested to be negative for esculin, melibiose, raffinose and d-sorbitol, and positive for dulcitol 86% of the time. Originally, these species were defined by O'Hara as a unique species in 1989 and they were found to be associated with bloodstream infections (Hoffmann et al., 2005; Morand et al., 2009). *E. hormaechei* species consists of three different subspecies: *E. hormaechei* subsp. *steigerwalti*, *E. hormaechei* subsp. *hormaechei* and *E. hormaechei* subsp. *oharae* (Morand et al., 2009). The three subspecies were specified based on DNA-DNA hybridization and differentiated on the basis of their biochemical tests and particular phenotypic properties (Hoffmann et al., 2005).

4.1.3. Incidences and Outbreaks

Over the last decade, members of the *Enterobacter cloacae* complex have emerged as nosocomial pathogens with clinical significance in intensive care units (Hoffmann et al., 2005). They can cause numerous infections such as meningitis, septicaemia, pneumonia, cerebral abscess, wound infection, urinary tract infection (UTI), as well as bloodstream infection. In the United States, between 1992 and 1997, the National Nosocomial Infections Surveillance System (NNISS) reported a frequent occurrence of *Enterobacter* infections in intensive care units (ICUs) (Richards et al., 1999). The National Healthcare Safety Network (NHSN) reported that *Enterobacter* spp. accounted for nearly 5% of hospital acquired infection cases between 2009 and 2010

(Sievert et al., 2013). Sligl et al. (2006) reported that *Enterobacter* spp. are one of the most commonly isolated among nosocomial pathogens.

The members of the *Enterobacter cloacae* complex can be considered as nosocomial pathogens, and *E. hormaechei* is generally the most isolated nosocomial pathogen within this group (Paauw et al., 2008). An outbreak of *E. hormaechei* infection and colonisation in year 1992 and 1993 was reported by Wenger et al. (1997). This outbreak occurred among low birth-weight, premature infants in an NICU at the Hospital of Pennsylvania. These organisms have also been reported by Paauw et al. (2009), as a result of a nationwide outbreak caused by an *E. hormaechei* outbreak strain in the Netherlands. The study by Paauw and co-authors showed that these isolates disseminated throughout hospitals and caused invasive infections in more than 100 patients. This species has also been reported in several outbreaks of sepsis in NICUs in the USA and in Brazil, where the outbreak originated from contaminated parenteral nutrition (Campos et al., 2007). Similarly, sepsis outbreaks in NICUs caused by *E. cloacae* have been reported among new-borns (Kose et al., 2016). Their study indicated the source of the sepsis outbreak of *E. cloacae* is more likely to be the contaminated parenteral nutrition solution.

4.1.4. Potential virulence-associated traits

E. cloacae complex are well known as human opportunistic pathogens and are most frequently isolated from clinical samples (Paauw et al., 2008; Davin-Regli and Pagès. 2015). They can cause numerous infections and outbreaks among patients who are hospitalised for a prolonged period (Paauw et al., 2008). Members of this complex are clinically significant, because they usually carry several potential virulence-associated genes (VAGs). For example, curli fimbrial genes, *csgABCD*, are generally known to be important for adhesion and binding to hosts tissues. These genes are highly conserved within the Enterobacteriaceae family and have a known role in biofilm formation (Zogaj et al., 2003; Kim et al., 2012; Smith et al., 2017). The *csg* operon was detected by Kim et al. (2012) in 11 of the 14 of clinical *E. cloacae* isolates from their study. Antimicrobial resistance genes have also been detected in *E. cloacae* complex strains. The fosfomycin gene (*fosA*), which confers fosfomycin resistance and β-lactam

resistance (ACT), was detected in the *E. cloacae* complex, recovered from the blood of a patient with peritonitis (Lin et al., 2017).

Moreover, multidrug efflux pumps can expel a broad range of antibiotics and also drive the acquisition of additional resistance mechanisms. The *acrA* and *tolC* efflux pump genes were detected among *Enterobacter cloacae* and were shown to be involved in resistance and virulence (Perez et al., 2012). The *E. cloacae* complex are intrinsically resistant to ampicillin, amoxicillin, amoxicillin–clavulanate and first generation cephalosporins (Davin-Regli and Pagès. 2015). Resistance to β-lactams is due to extended-spectrum β-lactamase production, such as TEM, SHV, CTX-M and VEB type enzymes, which can be found in *E. hormaechei* (Ho et al., 2005; Zhao and Hu, 2013; Carvalho-Assef et al., 2014). There is therefore a serious concern for increased frequency of infection, particularly in low birth-weight and premature infants receiving antimicrobial therapy in NICUs.

Another virulence factor is siderophores. Many pathogenic bacteria release these compounds with a high affinity to chelate iron from iron binding proteins. Enterobactin and other siderophores were reportedly produced by clinical isolates of *Enterobacter* spp. (Mokracka et al., 2004). Furthermore, attachment to and invasion of host cells are required for some bacterial strains to persist in the host and cause disease (Pizarro-Cerdá and Cossart, 2006). Krzymińska et al. (2010) reported the ability of *E. hormaechei* and *E. cloacae* strains isolated from human specimens to adhere and invade to human epithelial type 2 (HEp-2) cells. This can be considered as a risk factor to neonates in intensive care units, particularly in infants who are already at risk, such as those with low birth-weight and immune systems that have not fully developed.

4.1.5. Aim and objectives

In the neonatal intensive care unit, *Enterobacter* spp. have been recognised as a one of the most commonly isolated pathogens. Among this group, *E. hormaechei* is commonly isolated as a nosocomial pathogen of clinical significance. As mentioned earlier, it has been reported in several outbreaks of sepsis in NICUs. Of particular interest in this project were neonates in the intensive care unit which are highly susceptible to infection due to their prematurity, particularly of critically-ill neonates,

including those with low birth-weight, under-developed immune systems, or various other conditions. There are several risk factors to neonatal infection; the most important of which would include the use of medical devices such as nasogastric enteral feeding tubes (NEFTs). Neonates fed via a nasogastric tube are at increased risk with respect to neonatal infections in the NICU.

The overall aim of the study was to profile a clinical collection of *Enterobacter* spp., in particular *E. hormaechei* isolated from feeding tubes, CSF and blood samples (sepsis cases) of premature babies in NICUs. Additionally, to evaluate these isolates to determine important risk factors with respect to neonatal infections in NICUs. The aim was to also investigate any unique virulence factors, genetic relatedness, and source of these isolates, as the aetiology of neonatal infections is not yet well understood. The study of these organisms is very important to improve our understanding of how they invade and cause infection. This study also aimed to evaluate the possible associations between *E. hormaechei* isolated from neonatal sepsis cases and neonatal feeding tubes and the frequency of virulence genes found in these isolates. To our knowledge, there are no known studies investigating the possible correlations between *E. hormaechei* isolated from sepsis and neonatal feeding tubes.

Specific objectives of this study included:

- Determining the relatedness of isolated strains from the NICUs of QMC & Nottingham City Hospitals by genotyping (i.e. PFG and MLST) isolates over a period of time (7 years).
- Determining physiological attributes of the Enterobacter spp. with respect to biofilm formation, capsule production, desiccation tolerance, acid tolerance, and serum resistance.
- A range of phenotyping and genotyping methods and characterisation of virulence traits in order to determine the potential infection risk of these species to neonates.
- Evaluating the potential virulence of selected strains using in vitro attachment and invasion assays with intestinal and brain cells lines (Caco-2 and human brain microvascular endothelial cells; HBMEC) and macrophage survival.

- Whole-genome sequencing of selected isolates to detect a range of prospective virulence and other relevant genes of interest, such as those associated with fimbriae and antimicrobial resistance.

4.2. Materials and Methods

The methodology of this chapter is described in detail in Chapter 2 (Section 2). Twenty-six *Enterobacter* spp. were selected from the Nottingham Trent University culture collection. These strains were isolated from premature babies with sepsis cases and CSF at Queen Medical Centre (QMC) and Nottingham City Hospital (NCH). These clinical isolates provided by Dr Shiu. The collection were from sporadic cases at either NCH or QMC collected during 7 years period (Table 4-1).

This collection is very unique and has not been studied before. All these isolates were evaluated based on PFGE analysis and *fusA* gene sequence to identify the species identity of these *Enterobacter* isolates. An additional collection had been included in this study (2307, 2315, 2316, 2317, 2318, 2319 and 2320), which was recovered from sepsis, enteral feeding tubes and faecal samples of low-birth weight neonates from the same hospital (QMC). All isolates were subjected to physiological analysis such as capsule production, desiccation tolerance, acid tolerance, and serum resistance. Two strains were excluded from this investigation: strain 669 because it was identified as *C. sakazakii* by *fusA* gene sequences and strain 602, which was identified as *Serratia marcescens* by whole-genome sequencing.

Twenty-three strains were selected and subjected to whole-genome sequencing. Twenty-one were identified as *E. hormaechei and* one strain 602, which was identified as *Serratia marcescens*. While strain 2254 *fusA* allele 58 as *Enterobacter cloacae.* A maximum-likelihood phylogeny of these strains was constructed from a core genome alignment and their identification was confirmed. Six *E. hormaechei* strains were identified as *E. hormaechei* subsp. *oharae*, 1988, 668, 495, 663, 460 and 665, while the rest of the isolates were identified as *E. hormaechei* subsp. *steigerwaltii*, as shown in Figure 4-9. Pauline Ogrodzki, a member of our research group, carried out the whole-genome sequencing and SNP analysis. Twelve representative strains of the twenty-one *E. hormaechei* were selected based on genotype and phenotype, in order

to investigate their potential virulence using human intestinal cells Caco-2, brain barriers cells HBMEC, testing levels of cytotoxicity and macrophage survival.

Table 4-1: *Enterobacter* strains used in this study.

NTU culture	Organism	Source	Year	Hospital	Country	*A	*B	*C	*D
1448	*E. hormaechei*	Neonatal sepsis	2010	QMC	UK	X	X	X	
1977	*E. hormaechei*	CSF	2013	QMC	UK	X	X	X	X
1988	*E. hormaechei*	Neonatal sepsis	2012	QMC	UK	X	X	X	X
1439	*E. hormaechei*	Neonatal sepsis	2010	QMC	UK	X	X	X	X
450	*E. hormaechei*	Neonatal sepsis	NA	QMC/NCH	UK	X	X	X	X
667	*E. hormaechei*	Neonatal sepsis	NA	QMC/NCH	UK	X	X	X	
668	*E. hormaechei*	Neonatal sepsis	NA	QMC/NCH	UK	X	X	X	X
460	*E. hormaechei*	Neonatal sepsis	NA	QMC/NCH	UK	X	X	X	X
665	*E. hormaechei*	Neonatal sepsis	NA	QMC/NCH	UK	X	X	X	
462	*E. hormaechei*	Neonatal sepsis	NA	QMC/NCH	UK	X	X	X	X
495	*E. hormaechei*	Neonatal sepsis	NA	QMC/NCH	UK	X	X	X	X
664	*E. hormaechei*	Neonatal sepsis	NA	QMC/NCH	UK	X	X	X	
663	*E. hormaechei*	Neonatal sepsis	NA	QMC/NCH	UK	X	X	X	
660	*E. hormaechei*	Neonatal sepsis	NA	QMC/NCH	UK	X	X	X	
666	*E. aerogenes*	Neonatal sepsis	NA	QMC/NCH	UK	X	X		
1987	*E. aerogenes*	Neonatal sepsis	2012	QMC	UK	X	X		
451	*E. aerogenes*	Neonatal sepsis	NA	QMC/NCH	UK	X	X		
457	*E. aerogenes*	Neonatal sepsis	NA	QMC/NCH	UK	X	X		
2254	*E. cloacae*	Neonatal sepsis	2013	QMC	UK	X	X	X	
604	*E. cloacae*	Neonatal sepsis	NA	QMC/NCH	UK	X	X		
662	*E. cloacae*	Neonatal sepsis	NA	QMC/NCH	UK	X	X		
602	*S. marcescens*	Neonatal sepsis	NA	QMC/NCH	UK	X		X	
461	*E. cloacae*	Neonatal sepsis	NA	QMC/NCH	UK	X	X		
661	*E. cloacae*	Neonatal sepsis	NA	QMC/NCH	UK	X	X		
2215	*E. cloacae*	Neonatal sepsis	2013	QMC	UK	X	X		
669	*C. sakazakii*	Neonatal sepsis	NA	QMC/NCH	UK				
3215	*E. hormaechei*	Feeding Tube	2015	QMC	UK		X	X	X
2316	*E. hormaechei*	Faecal	2015	QMC	UK		X	X	
2317	*E. hormaechei*	Faecal	2015	QMC	UK		X	X	
2318	*E. hormaechei*	Feeding Tube	2015	QMC	UK		X	X	X
2319	*E. hormaechei*	Faecal	2015	QMC	UK		X	X	
2320	*E. hormaechei*	Feeding Tube	2015	QMC	UK		X	X	X
2307	*E. hormaechei*	Neonatal sepsis	2015	QMC	UK		X	X	X

NTU: Nottingham Trent University. *A: Pulsed Field Gel Electrophoresis was performed. *B: Physiological characterization was performed. *C: whole-genome sequencing was performed. *D: tissue culture was performed. QMC: Queen Medical Centre. NCH: Nottingham City Hospital. NA: Data not available.

4.3. Results

4.3.1. Genotyping Profiles

4.3.1.1. Partial *fusA* sequence analysis for *Enterobacter* genus identification

Twenty-six *Enterobacter* spp. were selected from the Nottingham Trent University culture collection. These strains were isolated from premature babies with sepsis cases at Queen Medical Centre (QMC) and Nottingham City Hospital (NCH). All isolates were evaluated based on the *fusA* gene sequence, to identify the species level identity of *Enterobacter* isolates. The phylogenetic tree, based on the *fusA* gene sequences, showed separation between the *Enterobacter* isolates. Fourteen strains were found to match *fusA* allele, 55, 56, 57, 75, 131 and 132 (Figure 4-1), identifying them as *E. hormaechei*. Whereas, four strains were found to match *fusA* allele 101, 130,133 and 134, and were identified as *E. aerogenes*. The other 7 strains matching allele 58 and 76 were not clearly identified and cannot be determined to the species level by *fusA*. Interestingly, one strain found to match *fusA* allele 1 was identified as *C. sakazakii*, which had been misidentified as *E. cloacae* by API testing. *E. hormaechei* was the predominant strain among the isolates analysed, as shown in Figure 4-1.

4.3.1.2. Pulsed Field Gel Electrophoresis (PFGE)

In this investigation, strain 669 was not included because it was identified as *C. sakazakii*. However, PFGE analysis of the remaining isolates was performed using *Xbal* restriction enzyme (Promega, UK). Twenty-five *Enterobacter* spp. isolates were digested with *Xbal* restriction enzyme and considered to be non-clonal when they have less than 95% band similarity (Tenover et al., 1995). The XbaI restriction enzyme separated the collection to unique pulsotypes, indistinguishable strains, and types containing bands ranging between 12 to 21 DNA fragments per strain, as shown in Figure 4-2.

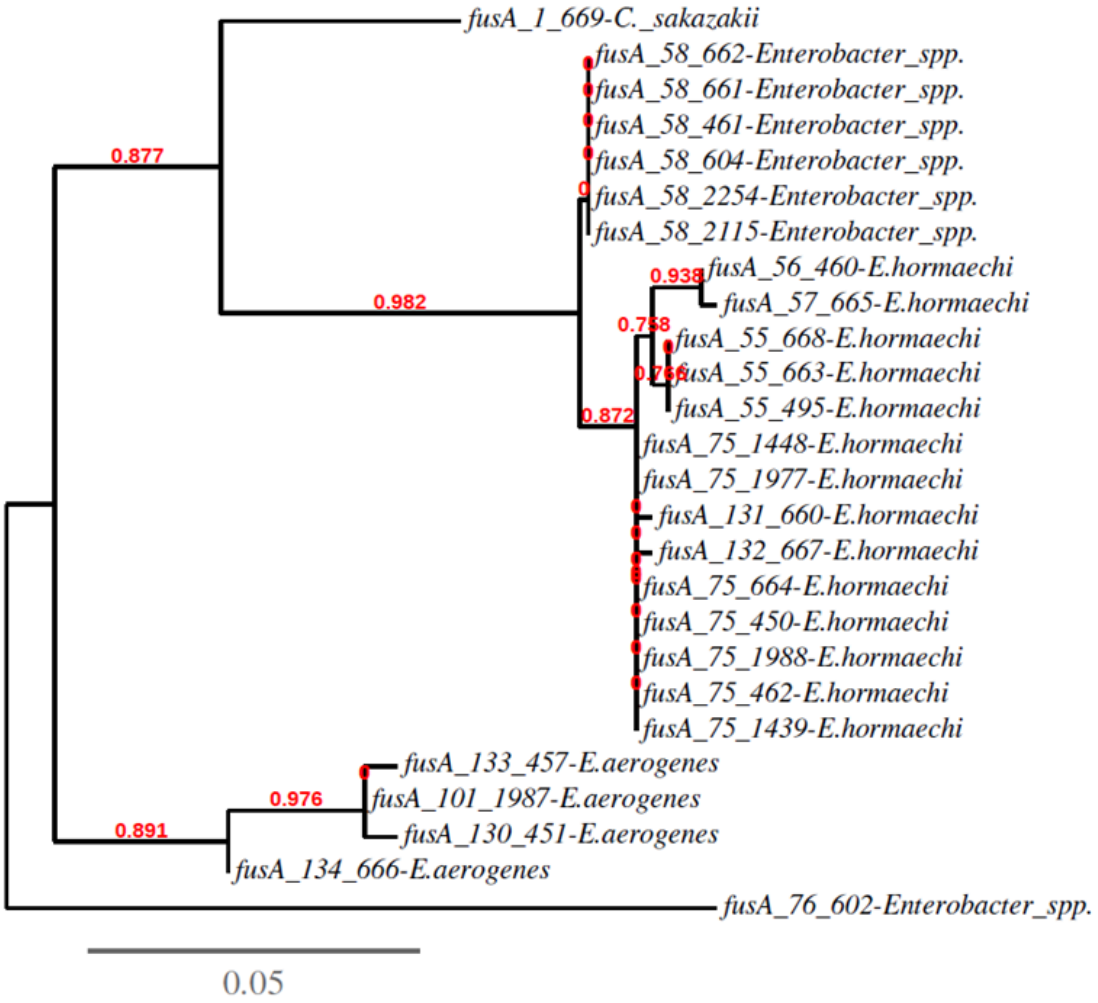


Figure 4-1: Phylogenetic relationships within different Enterobacter spp.

Twenty-Six *Enterobacter* spp. were selected from the Nottingham Trent University culture collection. Partial *fusA* gene sequence analysis was used to identify the isolates to species level. The tree was generated using MEGA7.

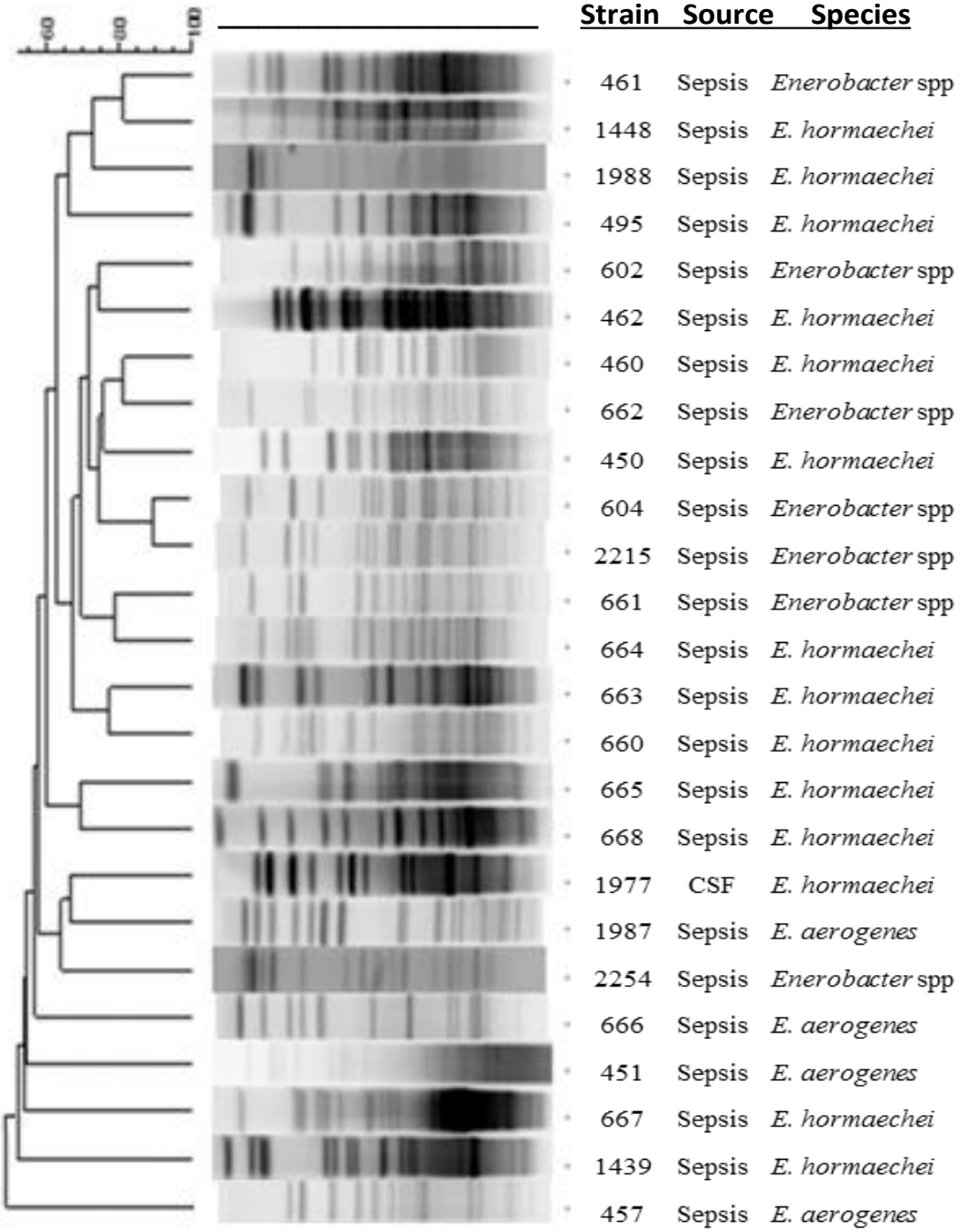


Figure 4-2: Xbal PFGE profiles of twenty-five Enterobacter spp. strains isolated from premature babies with sepsis at Queen Medical Centre (QMC) and Nottingham City Hospital (NCH)

Dendrogram construction and band assignment was achieved using BioNumerics software version 7.1. For cluster analysis, dice coefficient, un-weighted pair group method with arithmetic mean (UPGMA) was used. Band similarity of <95% was used to determine whether the isolates are non-clonal. The tolerance and optimisation of the bands was 1.5%.

4.3.2. Phenotyping and virulence traits

Among the twenty-six *Enterobacter* spp. isolates, strain 669 was not included in this investigation because it was identified as *C. sakazakii*. Also, strain 602 was not included and it was identified as *Serratia marcescens* after whole-genome sequencing, and was therefore not determined to the species level by *fusA*. An additional collection had been included in this study, which was recovered from sepsis, enteral feeding tubes, and faecal samples of low-birth weight neonates from the same hospital (QMC). This collection had been isolated by Pauline Ogrodzki, as part of a parallel PhD study, which includes strains numbers, 2307, 2315, 2316, 2317, 2318, 2319 and 2320, resulting in a total of 31 isolates.

4.3.2.1. Biofilm formation

All strains of *Enterobacter* spp. (n = 31) were tested for their ability to form a biofilm on plastic surfaces at temperatures of 25°C and 37°C and on two types of formula, TSB and infant formula (IF). Figure 4.3 and Figure 4.4 show the biofilm formation of the six strains of *E. cloacae*, four strains of *E. aerogenes*, six strains of *E. hormaechei* subsp. *oharae* and fifteen strains of *E. hormaechei* subsp. *steigerwaltii* isolated from sepsis cases, CFS and NEFTs. All *Enterobacter* strains showed the ability to produce more biofilm at 37 °C than at 25 °C on infant formula and TSB ($p < 0.001$) , as shown in Figure 4-5. While on TSB, some of the strains produced a higher amount of biofilm at 25 °C than at 37 °C, such as strains 663 and 668.

The highest values were seen by strains 662, 664, 462, 667, 1439, 1977, 2317, 2318 and 2320 in infant formula and at different temperatures, and majority among these strains were *E. hormaechei* subsp. *steigerwaltii*. Whereas the strains 604, 1987 and 2307 showed the lowest amount of biofilm production in same medium at 25 °C, when compared to the control. There was a clear variation between all species to form biofilm, in particular at 25 °C in TSB media. However, only two out of the thirty-one strains (666 and 664) were able produce a moderate amount of biofilm on TSB media at different temperatures. The majority of strains produced a low amount of biofilm, which was close to the control value in this medium, at different temperatures. Regardless of the

origin of these strains, most *E. hormaechei* subsp*. steigerwaltii* isolates showed higher biofilm than other strains when compared to the control.

Biofilm formation in IF was not significantly different between the *E. hormaechei* subsp*. steigerwaltii* strains 2315, 2318 and 2320 isolated from feeding tubes compared with the majority of strains isolated from sepsis and CFS. On the other hand, biofilm formation in infant formula was significantly different compared to TSA media, as well as higher biofilm formation was observed at 37 °C compared to 25 °C ($p < 0.001$) , as shown in Figure 4-5. Curli genes, *csgA, csgB, csgC*, and *csgD* were reported to have an important role in the formation of biofilms in clinical *E. cloacae* isolates (Sung et al., 2012). Genome screening in this study revealed that all of these genes are present in all representative *E. hormaechei* isolates, as explained later in section 4.3.3.

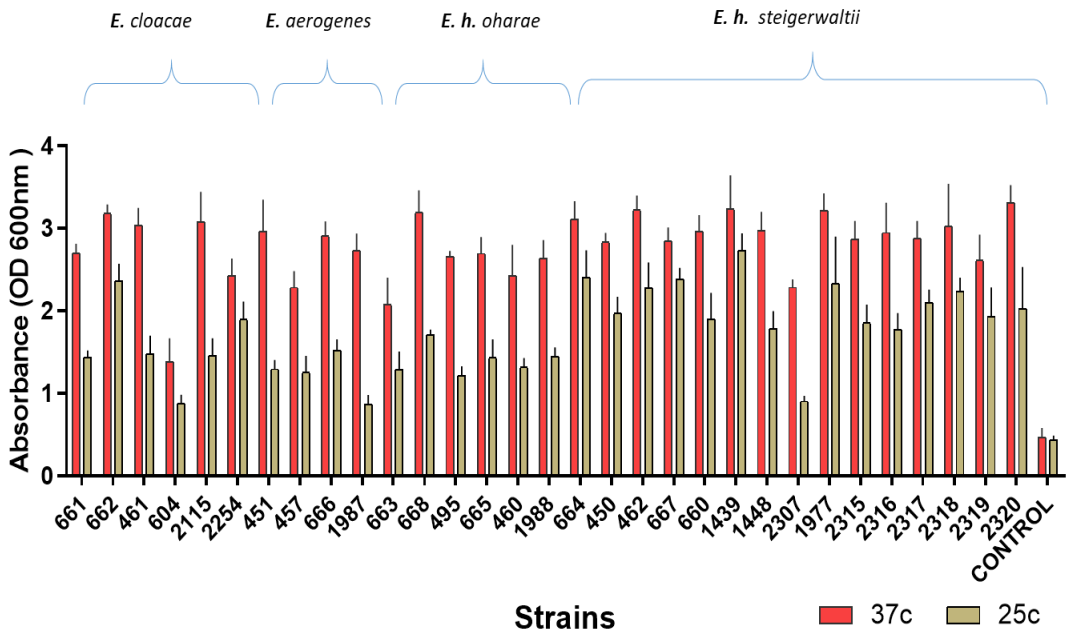


Figure 4-3: Biofilm formation at 25 °C and 37 °C in infant formula

All strains showed an ability to form more biofilm at 37 °C than at 25 °C on infant formula.Un-inoculated infant formula was used as a negative control. Error bars represent the standard deviation of at least two independent results.

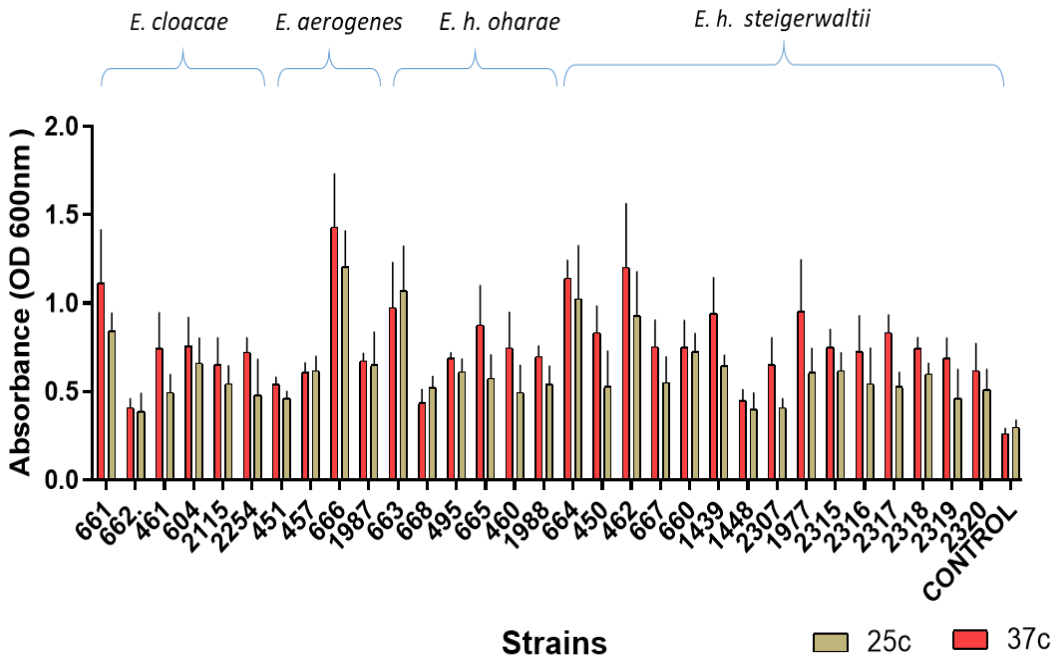


Figure 4-4 Biofilm formation at 25 °C and 37 °C in TSB

All strains showed an ability to form more biofilm at 37 °C than at 25 °C on TSA. Only strains 663 and 668 had produced a higher amount of biofilm at 25 °C than at 37 °C. Un-inoculated TSB was used as a negative control. Error bars represent the standard deviation of at least two independent results.

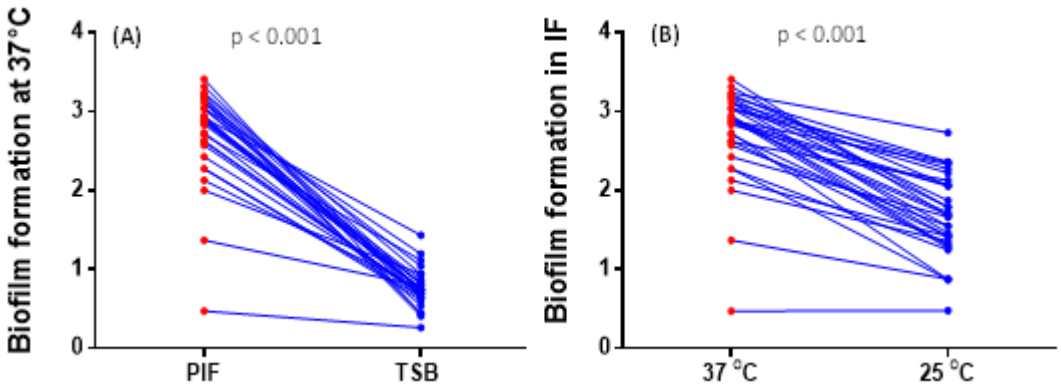


Figure 4-5 Paired t-test shows *E. hormaechei* strains form significant more biofilm in IF than in TSB ($p < 0.001$), (A) and also significant more biofilm in IF at 37°C than at 25°C ($p < 0.001$), (B).

4.3.2.2. Serum resistance

To determine serum sensitivity, viability was calculated at four different time points (0h, 1h, 2h and 3h), using log differentiation between the viable count of bacteria at time zero and after 3 hours (Figure 4-6). *Enterobacter* strains demonstrated a high level of resistance to human serum compared with negative and positive controls. Strains 604 demonstrated intermediate resistance to human serum exposure. While strain 1439 displayed the lowest serum tolerance with significantly poorer survival than other strains after the third hour of incubation ($P<0.05$). Strain 1977 *E. hormaechei* subsp. *steigerwaltii* isolated from CFS showed the highest serum tolerance. After the first hour of incubation up to 74% of strains revealed an increase in their viable count and 87% after the second hour of incubation. However, the majority of the strains from different species showed an ability to tolerate human serum for 3 hours.

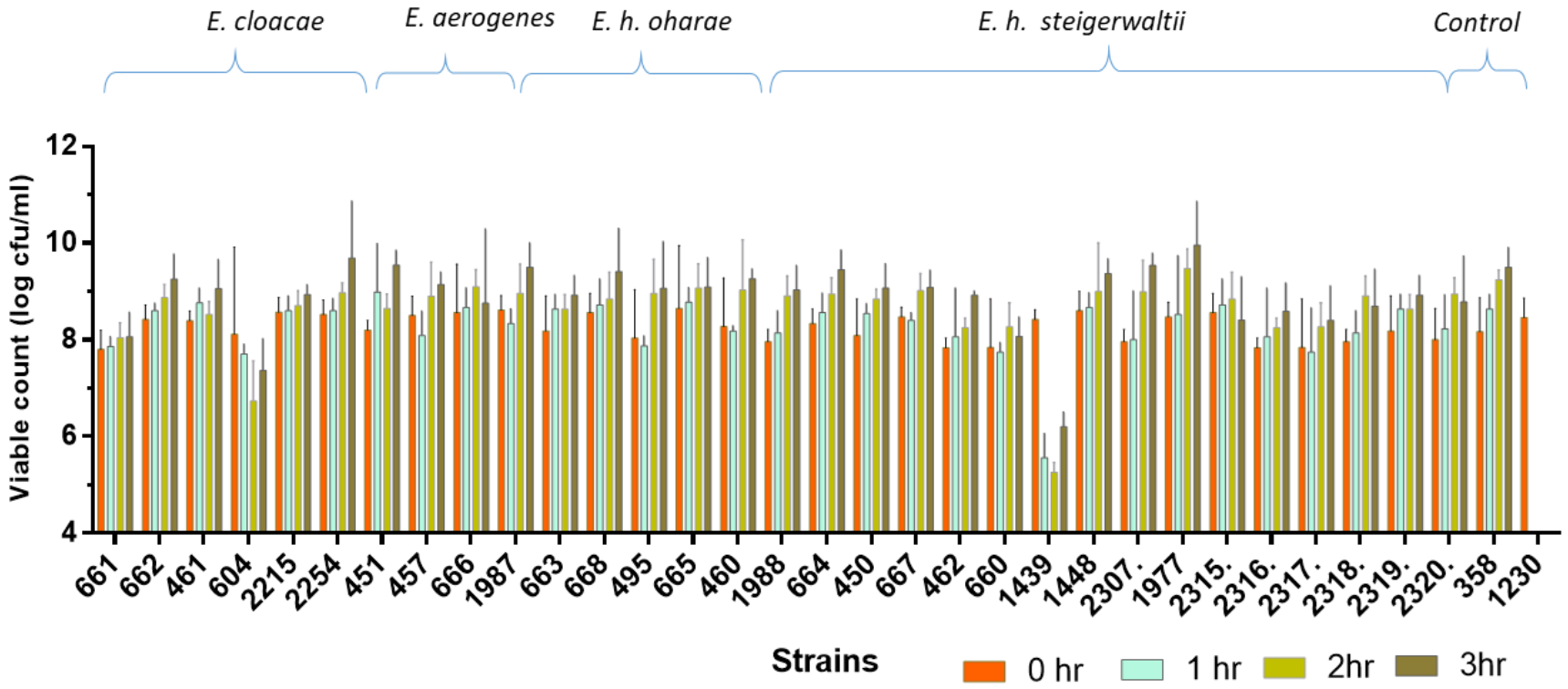


Figure 4-6 Sensitivity of *Enterobacter* strains (n = 31) to human serum over 3 hours of incubation at 37 °C.

Enterobacter strains demonstrated a high level of resistance to human serum after 3 hours of incubation. Only strains 1439 and 604 demonstrated intermediate resistance to human serum

4.3.2.3. The iron siderophores

Pathogenic bacteria require various iron acquisition systems to actively chelate iron from the host environment for a variety of metabolic processes. One of the systems is where the bacteria secrete siderophores, which are able to extract iron from the host. By using CAS agar diffusion assay, all strains examined in the study showed a formation of the orange zones around the wells. Production of siderophores was 100% visible in *E. cloacae, E. aerogenes, E. hormaechei* subsp*. oharae* and *E. hormaechei* subsp*. steigerwaltii*, as shown in Table 4-2. Most Enterobacteriaceae strains contain genes encoding iron uptake systems. *Enterobacter* has been reported to possess genes for enterobactin and aerobactin siderophore biosynthesis (Liu, et al., 2013). In this study, the iron siderophores, enterobactin biosynthesis genes (*entABCDE*) and aerobactin (*IucC*) were detected in all isolates recovered from sepsis cases, feeding tubes and CSF specimens.

4.3.2.4. Haemolysis on blood agar

Haemolytic activity was demonstrated by thirty-one strains of *Enterobacter* isolates on TSA agar supplemented with 5% horse blood. All *Enterobacter* strains produced β-haemolysis on horse blood agar plates by 29 strains (93.5%). Haemolysis was not detected in two strains (*E. cloacae* 661 and *E. hormaechei* subsp*. oharae* 460) on TSA horse blood agar (Table 4-2).

4.3.2.5. Cellulose formation

The expression of cellulose by *Enterobacter* strains was performed using the calcofluor binding assay and visualised by ultra-violet fluorescence. Table 4.2 shows the ability of cellulose production by the thirty-one strains of *Enterobacter* on calcofluor agar medium. In general, all strains showed an ability to produced cellulose. Results of this study indicated that the ability of cellulose production varies between *Enterobacter* strains. Seven strains, (2 *E. cloacae*, 3 *E. hormaechei* subsp*. oharae* and 2 *E. hormaechei* subsp*. steigerwaltii*) grown on calcofluor agar revealed a strong fluorescence, while 15 strains exhibited moderate fluorescence and the remaining 9 strains showed only weak fluorescence, in particular all *E. aerogenes* strains.

4.3.2.6. Capsule production

Bacterial capsule production was determined by colony appearance on two different infant formula agar media, soya-based infant formula (IF1) and whey-based infant formula (IF2). The results were classified into four categories according to the mucosity of the colonies: very high, high, medium, low and no capsule production, which are represented by ++++, +++, ++, + and – respectively. As shown in Table 4.2, there was a clear variation between all *Enterobacter* strains with regards to production of capsular material according to the milk agar composition. Only two strains of *E. cloacae* (2115 and 2254) formed high and medium capsular material in whey-based infant formula, respectively, whilst formed low capsule on soya-based infant formula. The rest of the strains were not able to produce capsular materials on either media (IF1 and IF2). With respect to *E. aerogenes*, all strains were able to produce capsules on two IF agar media used. In contrast, 3 out of 6 *E. hormaechei* subsp*. oharae* strains, 668, 663 and 1988 formed capsular material on IF2, but only strain 668 was able to form capsular material on IF1. The rest of the stains were not able to produce capsular materials on either media. The production of capsular materials by *E. hormaechei* subsp*. steigerwaltii* strains varied according the growth media. For example, 9 strains showed an ability to produce low to medium amounts of capsular material on IF1 and IF2, while strains, 1448 produced capsular material only on IF2. A very high level of capsular material was produced by *E. hormaechei* subsp*. oharae* strain 668 on IF2. In general, the majority of strains showed an ability to form capsules on whey-based infant formula more so than on soya- based infant formula.

4.3.2.7. Protease production

Investigations were conducted to determine the protease activity of *Enterobacter* strains. After 72 hours' incubation on skimmed milk agar at 37 °C, all strains of *E. cloacae, E. aerogenes, E. hormaechei* subsp*. oharae* and *E. hormaechei* subsp*. steigerwaltii* had revealed a positive result for production of protease activity (Table 4-2). Protease activity is defined as a clear circle on the skimmed milk agar. *B. cereus* was used as a positive control and *E. coli* NTU 407 was used as a negative control.

4.3.2.8. Congo red morphotype

Curli fimbriae was investigated by using LB agar media supplemented with Congo red. The colony morphology determined the formation of the Congo red phenotype. The expression of a red colony indicated the ability of the bacterium presumed production of curli fimbria, while pink was negative for curli fimbriae. The colony morphology of strains of *E. cloacae* (662, 461 and 2254), all *E. aerogenes* strains, strains of *E. hormaechei* subsp*. oharae* (495, 665 and 460), and *E. hormaechei* subsp*. steigerwaltii* (450, 462 and 1977) were considered as negative colonies for binding to the Congo red dye, while the rest of the strains formed red colonies and were therefore presumed positive for curli fimbriae (Table 4-2).

Table 4-2 Capsule production, biofilm formation, curli fimbria, cellulose, siderophore, protease and haemolytic activity among the 31 Enterobacter strains tested.

NTU No	Species	Source	Capsule		Biofilm				Curli fimbria	Cellulose	siderophore	Protease	Haemolysis
			IF 1	IF 2	IF 25C	IF 37C	TSB 25C	TSB 37C					H.B
661	*E. cloacae*	Sepsis	-	-	M	H	L	M	**Red**	++	+	+	ɣ
662	*E. cloacae*	Sepsis	-	-	H	H	VL	L	pink	++	+	+	β
461	*E. cloacae*	Sepsis	-	-	M	H	L	L	pink	+++	+	+	β
604	*E. cloacae*	Sepsis	-	-	L	H	L	L	**Red**	+++	+	+	β
2115	*E. cloacae*	Sepsis	+	+++	M	H	L	L	**Red**	+	+	+	β
2254	*E. cloacae*	Sepsis	+	++	M	H	VL	L	pink	+	+	+	β
451	*E. aerogenes*	Sepsis	++	++	M	H	VL	L	pink	+	+	+	β
457	*E. aerogenes*	Sepsis	+	+	M	H	L	L	pink	+	+	+	β
666	*E. aerogenes*	Sepsis	+++	++	M	H	M	M	pink	+	+	+	β
1987	*E. aerogenes*	Sepsis	++	++	L	H	L	L	pink	++	+	+	β
663	*E. h. oharae*	Sepsis	-	+	M	H	M	L	**Red**	++	+	+	β
668	*E. h. oharae*	Sepsis	++	+++	H	H	L	VL	**Red**	+++	+	+	β
495	*E. h. oharae*	Sepsis	-	-	M	H	L	L	pink	+	+	+	β
665	*E. h. oharae*	Sepsis	-	-	M	H	L	L	pink	++	+	+	β
460	*E. h. oharae*	Sepsis	-	-	M	H	L	M	pink	+++	+	+	ɣ
1988	*E. h. oharae*	Sepsis	-	++	M	H	L	L	**Red**	+++	+	+	β
664	*E. h. steigerwaltii*	Sepsis	-	-	H	H	M	M	**Red**	++	+	+	β
450	*E. h. steigerwaltii*	Sepsis	-	-	H	H	L	L	pink	+	+	+	β
462	*E. h. steigerwaltii*	Sepsis	+	++	H	H	M	L	pink	+++	+	+	β
667	*E. h. steigerwaltii*	Sepsis	+	+	M	H	L	L	**Red**	++	+	+	β
660	*E. h. steigerwaltii*	Sepsis	-	-	M	H	L	L	**Red**	++	+	+	β
1439	*E. h. steigerwaltii*	Sepsis	-	-	H	H	L	L	**Red**	+++	+	+	β
1448	*E. h. steigerwaltii*	Sepsis	-	++	M	H	L	L	**Red**	+	+	+	β
2307	*E. h. steigerwaltii*	Sepsis	+	+	L	H	VL	VL	**Red**	++	+	+	β
1977	*E. h. steigerwaltii*	CSF	-	-	H	H	L	L	pink	+	+	+	β
2315	*E. h. steigerwaltii*	F. tube	++	++	M	H	L	L	**Red**	++	+	+	β
2316	*E. h. steigerwaltii*	Faeces	++	++	M	H	L	L	**Red**	++	+	+	β
2317	*E. h. steigerwaltii*	Faeces	++	++	H	H	VL	L	**Red**	++	+	+	β
2318	*E. h. steigerwaltii*	F. tube	++	++	H	H	L	L	**Red**	++	+	+	β
2319	*E. h. steigerwaltii*	Faeces	++	++	M	H	L	L	**Red**	++	+	+	β
2320	*E. h. steigerwaltii*	F. tube	++	++	H	H	L	L	**Red**	++	+	+	β

β: β-haemolysis ɣ: ɣ-haemolysis H.B: horse blood, IF1: infant formula soya based, IF2: infant formula whey-based, +: positive, -: negative, VL= very Low biofilm formation L= Low biofilm formation, M = moderate biofilm formation and H = High biofilm formation.

4.3.2.9. Acidity

The ability of *E. cloacae, E. aerogenes, E. hormaechei* subsp*. oharae* and *E. hormaechei* subsp*. steigerwaltii* to tolerate acidic conditions at pH 3.5 was evaluated. They were exposed to pre-adjusted infant formula at pH 3.5 for two hours. Figure (4-7) displays the results for the different strains. In general, the initial viable count for all *Enterobacter* isolates was between 8.2 and 9.5 $\log_{10}$ CFU/ml. All strains were clearly resistant to exposure to pH 3.5 for 2 hours. During the first 30 mins of acid exposure the viability of all *Enterobacter* isolates decreased to 0.7-1.6 log cycles. After 60 minutes, the viable count cells for all strains tested did not show significant reduction. Two out of thirty-one strains showed the most acid sensitivity; strains 1448 and 664 showed approximately 1.4 to 2.5 log cycle decline over the 2-hour incubation period. However, these strains remained viable for 2 hours at pH 3.5 and were therefore regarded as acid tolerant.

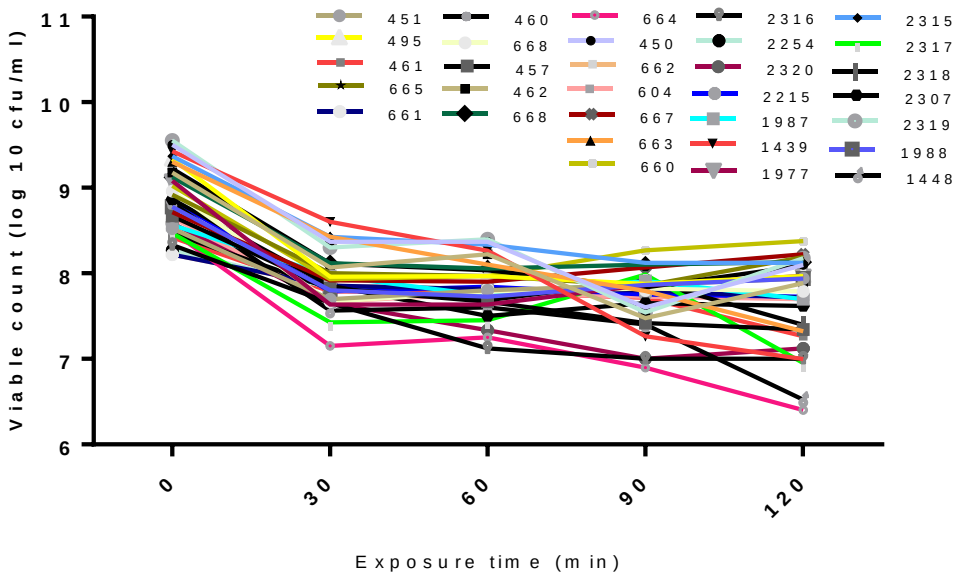


Figure 4-7 Survival curve of 31 *Enterobacter* strains in infant formula at pH 3.5.

Enterobacter strains were assessed for their tolerance to pH 3.5 for two hours. The viable counts were determined by the Miles & Misra method. Overall, all strains showed an ability to resist pH 3.5 for the total 2 hours. Two strains showed the most acid sensitivity; strains 1448 and 664 showed approximately 1.4 to 2.5 log cycle decline over the 2-hour incubation period. The number of survival cells in this assays were performed in duplicate in two independent experiments.

4.3.2.10. Desiccation

A total number of 31 *Enterobacter* strains were evaluated for their desiccation survival in infant formula. The first 24h of desiccation caused the viability of the majority of *Enterobacter* strains to slightly decrease by 0.3-1.5 log cfu/ml. The majority of viable cells of strains did not appear to be significantly reduced, while strains 450, 660 and 2315 were the lowest to persist under desiccated stress during 24h. The reduction observed over this time ranged between 1.7 and 2.1 5 log cfu/ml. However, the reduction observed after the next 14 days ranged between 0.9 and 3.2 log cfu/ml. There was some variation between all strains in their ability to persist under the stress of desiccation. Strains 457, 666, 665, 450, 1439, 660 and 2315 showed low persistence compared with the other strains, in particular strain 457. The reduction of viable cells observed for this strain was 3.2 log cfu/ml, followed by strain 666 and 665 at 3.1 log cfu/ml. Strain 663 had persisted the longer under desiccation stress. Overall, the majority of *Enterobacter* strains were resistant to desiccation in infant formula and remained detectable for the entire 2-week duration of the experiment (Figure 4-8).

4.3.2.11. Antimicrobial Susceptibility

Antimicrobial susceptibility was tested using the Kirby-Bauer disk diffusion method, as described by the BSAC Methods (2015). As shown in Table 4-3, all *Enterobacter* strains were resistant to ampicillin, cefotaxime, ceftizoxime, ceftriaxone and gentamicin. The majority of the isolates were also resistant to imipenem (58%), meropenem (19%), ceftazidime + clavulanic acid (51%), cefotaxime + clavulanic acid (29%). The effect of the antibiotics tested varied according to the species, as showed in Table 4-3. All of the screened strains were found susceptible to tobramycin and cefoperazone except strain 1977 was showed resistance to the same antibiotics. Also, it was found that ceftazidime + clavulanate (CAZ+CV) and cefotaxime, clavulanate (CTX+CV) had effect on the majority of isolates, in particular *E. hormaechei* subsp. *oharae*. Several studies have reported on the genes implicated in antibiotic resistance in *Enterobacter* spp. (Chollet et al., 2002; Davin-Regli and Pagès. 2015; Ghanavati et al., 2015). All *E. hormaechei* isolates possessed fosfomycin (*fosA*) and several *bla*ACT genes, including blaACT-15 (57%), blaACT-7(33%), blaACT-16 (9.5%) and only one

strain, 665, possessed *bla*TEM-1B. Only strain 1977 *E. hormaechei* subsp. *steigerwaltii* isolated from CFS had possessed aminoglycoside genes *aadA16, aadA2, aac(6')Ib-cr, aac(3)-IId,* macrolide *mph*(A), sulphonamide *sul1*(x2), rifampicin *ARR-3*, phenicol *catA2* and trimethoprim *dfrA2* (Table4-5).

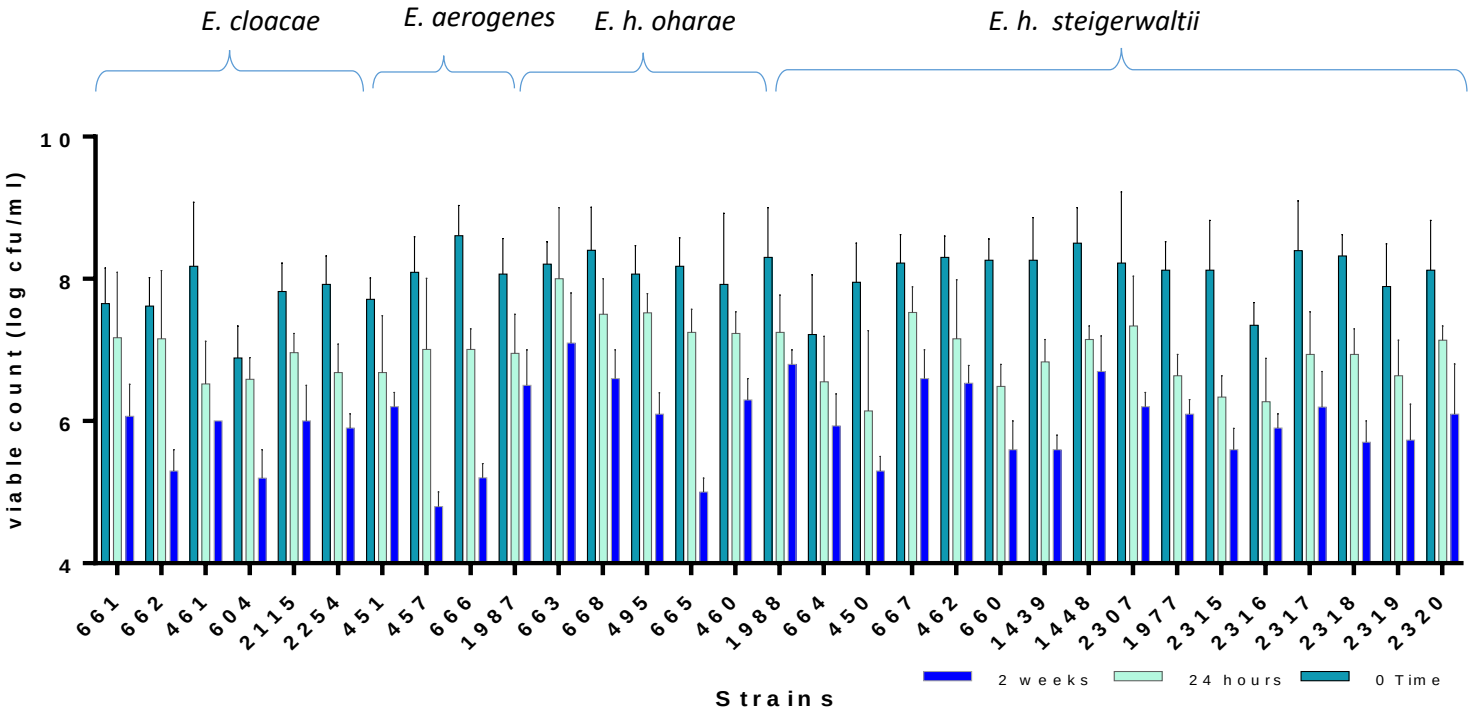


Figure 4-8 Survival of *Enterobacter* strains subjected to desiccation over a period of 2 weeks.

The figure shows a slight decrease in viability of 0.3-1.5 log cfu/ml, as a result of the first 24h of desiccation, while the reduction observed after 2 weeks ranged between 0.9 and 3.2 log cfu/ml. Overall, it is clear that the majority of strains were resistant to desiccation in infant formula and remained detectable for the entire duration of the 2-week experiment

Table 4-3 Sensitivity of Enterobacter strains to antibiotic agents.

NTU No	Species	Source	Hospital	Antibiogram											
				Carbapenems		Penicillins	Cephalosporins					ESBL family		Aminogly-cosides	
				IMI 10µg	MEM 10µg	AP 10µg	CFP 75µg	CTX 10µg	ZOX 30µg	CRO 30µg	CAZ 10µg	CTX+CV 30/10µg	CPD+CV 10/1µg	GM 10µg	TOP 10µg
661	*E. cloacae*	Sepsis	QMC	I	I	R	S	R	R	R	S	S	S	R	S
662	*E. cloacae*	Sepsis	QMC	I	S	R	S	R	R	R	R	S	R	R	S
461	*E. cloacae*	Sepsis	QMC	R	I	R	S	R	R	R	R	R	R	R	S
604	*E. cloacae*	Sepsis	QMC	R	R	R	S	R	R	R	R	R	R	R	S
2115	*E. cloacae*	Sepsis	QMC	S	S	R	S	R	R	I	S	S	S	R	S
2254	*E. cloacae*	Sepsis	QMC	S	I	R	S	R	R	I	S	S	S	R	S
451	*E. aerogenes*	Sepsis	QMC	R	I	R	S	R	R	R	R	R	R	R	S
457	*E. aerogenes*	Sepsis	QMC	R	R	R	S	R	R	R	R	R	R	R	S
666	*E. aerogenes*	Sepsis	QMC	S	I	R	S	R	R	R	R	S	S	R	S
1987	*E. aerogenes*	Sepsis	QMC	R	S	R	S	R	S	R	R	S	S	R	S
663	*E. h. oharae*	Sepsis	QMC	R	S	R	S	R	R	R	R	S	S	R	S
668	*E. h. oharae*	Sepsis	QMC	R	S	R	S	R	R	I	S	S	S	R	S
495	*E. h. oharae*	Sepsis	QMC	R	I	R	S	R	R	R	R	S	S	R	S
665	*E. h. oharae*	Sepsis	QMC	R	R	R	S	R	R	R	R	R	S	R	S
460	*E. h. oharae*	Sepsis	QMC	S	R	R	S	R	R	R	R	S	S	S	S
1988	*E. h. oharae*	Sepsis	QMC	S	S	R	S	R	R	R	R	S	S	R	S
664	*E. h. steigerwaltii*	Sepsis	QMC	I	I	R	S	R	R	R	R	S	R	R	S
450	*E. h. steigerwaltii*	Sepsis	QMC	I	I	R	S	R	R	R	R	R	R	R	S
462	*E. h. steigerwaltii*	Sepsis	QMC	S	I	R	S	R	R	R	R	S	S	R	S
667	*E. h. steigerwaltii*	Sepsis	QMC	S	I	R	S	R	R	R	S	S	S	R	S
660	*E. h. steigerwaltii*	Sepsis	QMC	R	S	R	S	R	R	R	S	R	R	R	S
1439	*E. h. steigerwaltii*	Sepsis	QMC	S	S	R	S	R	R	R	S	S	S	R	S
1448	*E. h. steigerwaltii*	Sepsis	QMC	S	S	R	S	R	R	R	S	S	S	R	S
2307	*E. h. steigerwaltii*	Sepsis	QMC	R	R	R	S	R	R	R	R	R	R	R	S
1977	*E. h. steigerwaltii*	CSF	QMC	R	R	R	R	R	R	R	R	R	R	R	R
2315	*E. h. steigerwaltii*	F. tube	QMC	R	I	R	S	R	R	R	R	S	R	R	S
2316	*E. h. steigerwaltii*	Faeces	QMC	R	I	R	S	R	R	R	R	S	R	R	S
2317	*E. h. steigerwaltii*	Faeces	QMC	R	I	R	S	R	R	R	R	S	R	R	S
2318	*E. h. steigerwaltii*	F. tube	QMC	R	I	R	S	R	R	R	R	S	R	R	S
2319	*E. h. steigerwaltii*	Faeces	QMC	R	I	R	S	R	R	R	R	S	R	R	S
2320	*E. h. steigerwaltii*	F. tube	QMC	R	I	R	S	R	R	R	R	S	R	R	S

S: susceptibility. R: resistance. I: intermediate resistance. QMC: Queens Medical Centre Hospital. NCH: Nottingham City Hospital. CTX: cefotaxime. MEM: meropenem. GM: gentamicin. CRO: ceftriaxone. AP: ampicillin. IMI: imipenem. ZOX: ceftizoxime. CAZ: ceftazidime. CFP: cefoperazone. TOB: tobramycin. CTX+CV: cefotaxime + clavulanate. CAZ+CV ceftazidime + clavulanate.

4.3.3. Genomic studies

4.3.3.1. Genome sequence and analysis

Genomic DNA was extracted from twenty-three strains. The Illumina MiSeq reagent kit was used for whole-genome sequencing. Strain 602 was identified as *Serratia marcescens* while strain 2254 *fusA* allele 58 as *Enterobacter cloacae.* However, Core genome alignments were produced for the *E. hormaechei* strains in order to confirm the species identity, and the CSI Phylogeny program were used to identify Single Nucleotide Polymorphisms (SNPs). This work was performed by Pauline Ogrodzki. Twenty-one *E. hormaechei* strains were selected and subjected to whole-genome sequencing. A maximum-likelihood phylogeny of these strains was constructed from a core genome alignment and the identification of these strains was confirmed by building a core genome phylogenetic tree. Six *E. hormaechei* strains were identified as *E. hormaechei* subsp. *oharae*, 1988, 668, 495, 663, 460 and 665, while, the rest of the isolates were identified as *E. hormaechei* subsp. *steigerwaltii*, as shown in Figure 4-9.

The three *E. hormaechei* subsp. *oharae* strains, 668, 495 and 663, formed a single cluster isolated from neonatal sepsis cases, and no other clinical details were available for these strains. These isolates belong to sequence type (ST) 108, and differed from each other by 183–265 SNPs (Figure 4-10). These samples were assumed linked in some way, could be the origin for these isolates hospital sources environment such as medical devices or carer's hands. Among the *E. hormaechei* subsp. s*teigerwaltii*, three strains, 2316, 2317 and 2319, were collected from faecal samples and three strains, 2315, 2318 and 2320, were from a feeding tube of a preterm infant in a NICU over a 1-month period. These isolates belong to ST106 and differed from each other by only 13–26 SNPs (Figure 4-10). It can be inferred that the small number of SNP differences suggest a common source of origin. On the other hand, the rest of the *E. hormaechei* subsp. *Steigerwaltii* differed from each other by very high numbers of SNPs, up to 66,582 SNPs.

All strains were obtained between 2009 and 2015. Following the aforementioned genomic analysis, twelve representative strains were chosen for further analyses to

assess the level of pathogenicity using tissue culture assays (Figure 4-9). This assay will be discussed in detail in section 4.3.4.

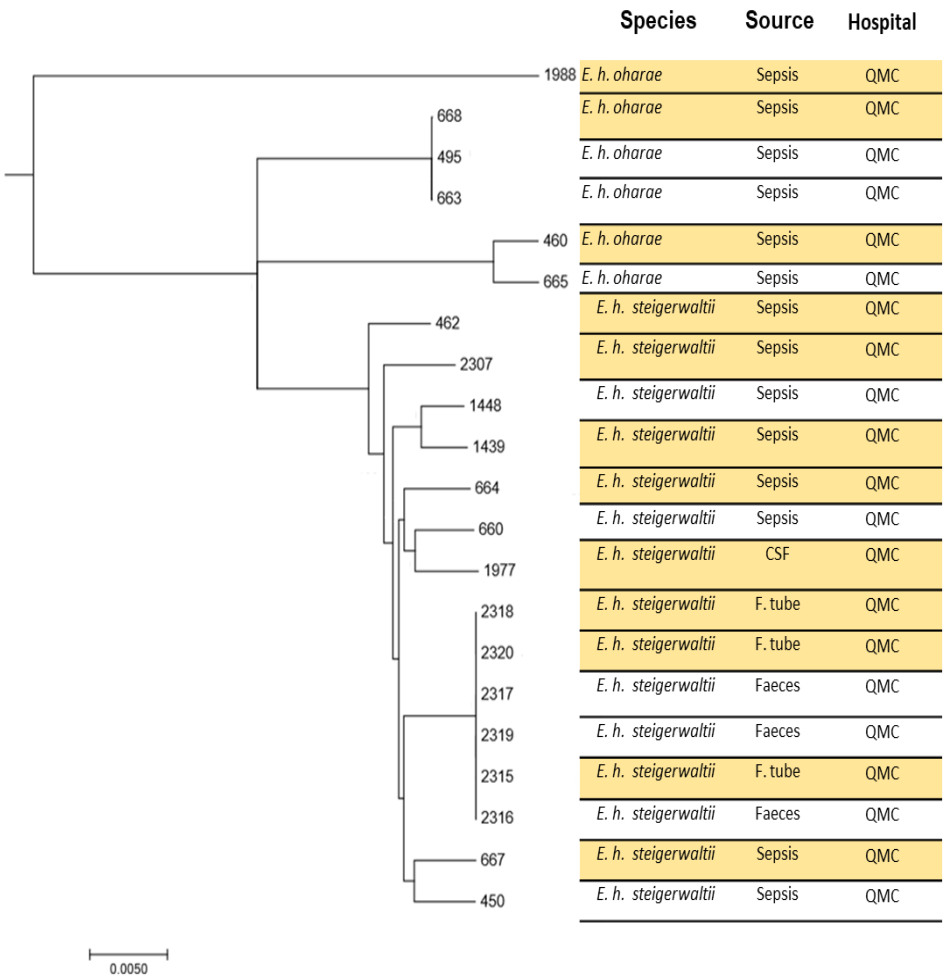


Figure 4-9 A phylogenetic tree of *E. hormaechei* strains based on core genome SNP analysis.

The highlighted twelve representative strains were chosen for further analysis to assess their pathogenicity using tissue culture assays.

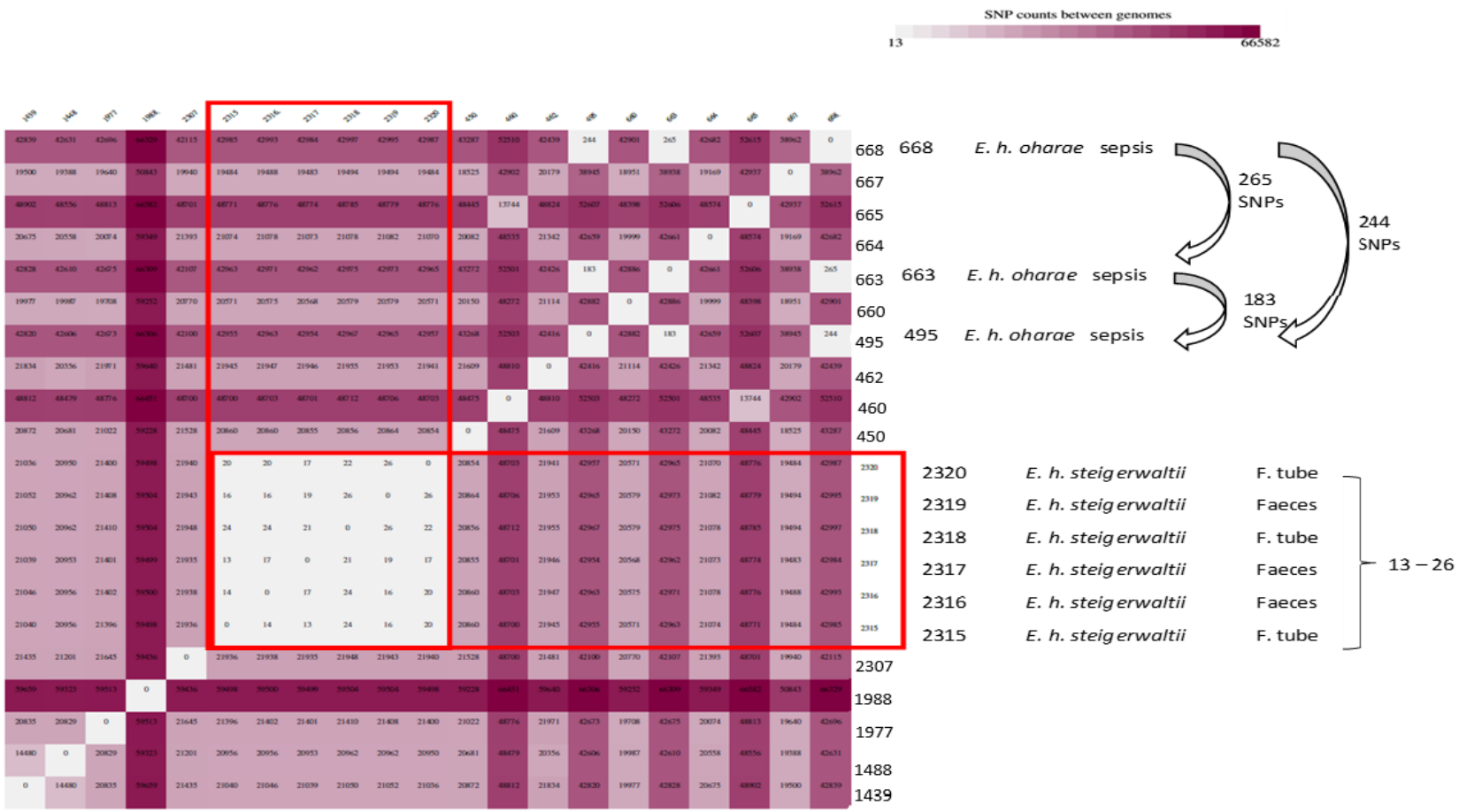


Figure 4-10 A heat map illustrating the difference of SNPs between each genome in the alignment.

The map was generated by Pauline Ogrodzki as part of a parallel PhD study, using the program CSI Phylogeny version 1.4.

4.3.3.2. Genome screening of *E. hormaechei* for virulence and physiological associated traits

Genome screening and comparative genomics work for twenty-one *E. hormaechei* strains was performed to examine the presence and absence of selected genes that may contribute to virulence or physiological features.

Table 4-4 shows a list of the proposed virulence genes classified into the major virulence factors of *E. hormaechei* strains, such as genes encoding Csg fimbriae or pili, which play a crucial role in adherence and biofilm formation in Gram-negative bacteria (Zogaj et al., 2003; Kikuchi et al., 2005; Ogasawara et al., 2010; Taylor et al., 2011). Flagellar biosynthesis plays a role in colonisation and adhesion, and in pathogenic microorganisms are considered as a virulence factor (Haiko and Wikström, 2013; De Maayer and Cowan, 2016). In addition, the role of *ompA* and *ompX* genes have been described by Mittal and colleagues (2009) in bacterial penetration of the blood-brain barrier (BBB). For example, *ompA* in *Cronobacter* plays an essential role in invasion of human brain microvascular epithelial cells (HBMEC) (Singamsetty et al., 2008). Furthermore, the *rpoS* sigma factor is the master regulator of response to various stress conditions in many Gram-negative bacteria (Stockwell and Loper, 2005; Battesti et al., 2011; Lago et al., 2017) and iron acquisition system which is mediated through siderophore, enterobactin (*entABCDE*) and aerobactin (*IucC*) biosynthesis genes Table 4-4. All these genes were noted among all *E. hormaechei* genomes analysed in this study.

E. hormaechei strains showed a variation in genome size between 4.45 and 5.16 Mb and only six strains possessed plasmids (633, 668, 660, 450, 667 and 1977). However, using the Center for Genomic Epidemiology database, genome analysis showed all *E. hormaechei* isolates encoded for fosfomycin (*fosA*), AmpC beta-lactamase and different *bla*ACT genes, such as *bla*ACT-15 (11/21), *bla*ACT-7(7/21), *bla*ACT-16 (2/21) and only strain 665 possessed *bla*TEM-1B. Only strain 1977 *E. hormaechei* subsp. *steigerwaltii* isolated from CFS possessed aminoglycoside genes *aadA16, aadA2, aac*(6')*Ib-cr, aac*(3)-*IId*, macrolide *mph*(A), sulphonamide *sul1*(x2), rifampicin ARR-3, phenicol *catA2* and trimethoprim *dfrA27*. With regards to the MLST analysis of 21 *E. hormaechei* isolates, it was revealed that a total of 11 STs were present, with 3 strains assigned to unknown STs. The most frequent STs of the feeding tube and faeces isolates was ST106 (28%),

followed by the sepsis isolates, which were ST108 (14%). However, the MLST sequences of the rest of the isolates were unique and were typed as ST134, ST740, ST45, ST133, ST102, S7175, ST346, ST50 and ST124. The sequence types obtained for the 21 *E. hormaechei* strains are given in Table 4-5.

Table 4-4 shows the results of the genomic comparison of *E. hormaechei* strains recovered from sepsis, feeding tube and CSF specimens.

NTU ID	Species	Source	curli fimbriae				enterobactn siderophore					aerobactin siderophore	flagellar biosynthesis		efflux pump	sigma factor	superoxide dismutase	adherence protein		hemolysin		Cellulose synthase		
			csgA	*csgB*	*csgC*	*csgD*	*entA*	*entB*	*entC*	*entD*	*entE*	*iucC*	*flhA*	*fliR*	*AcrAB*	*rpoS*	*sodA*	*ompA*	*ompX*	*hlyACD*	*hlyB*	*bcsA*	*bcsB*	*bcsC*
663	*E. h. oharae*	Sepsis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
668	*E. h. oharae*	Sepsis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
495	*E. h. oharae*	Sepsis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
665	*E. h. oharae*	Sepsis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
460	*E. h. oharae*	Sepsis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
1988	*E. h. oharae*	Sepsis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
660	*E. h. steigerwaltii*	Sepsis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
462	*E. h. steigerwaltii*	Sepsis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
664	*E. h. steigerwaltii*	Sepsis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
450	*E. h. steigerwaltii*	Sepsis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
667	*E. h. steigerwaltii*	Sepsis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
1439	*E. h. steigerwaltii*	Sepsis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
1448	*E. h. steigerwaltii*	Sepsis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
2307	*E. h. steigerwaltii*	Sepsis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
1977	*E. h. steigerwaltii*	CSF	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
2315	*E. h. steigerwaltii*	F. tube	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
2316	*E. h. steigerwaltii*	Faeces	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
2317	*E. h. steigerwaltii*	Faeces	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
2318	*E. h. steigerwaltii*	F. tube	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
2319	*E. h. steigerwaltii*	Faeces	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
2320	*E. h. steigerwaltii*	F. tube	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+

The results indicate that all isolates harboured a number of virulence-associated genes. (+) = present, (-) = absent.

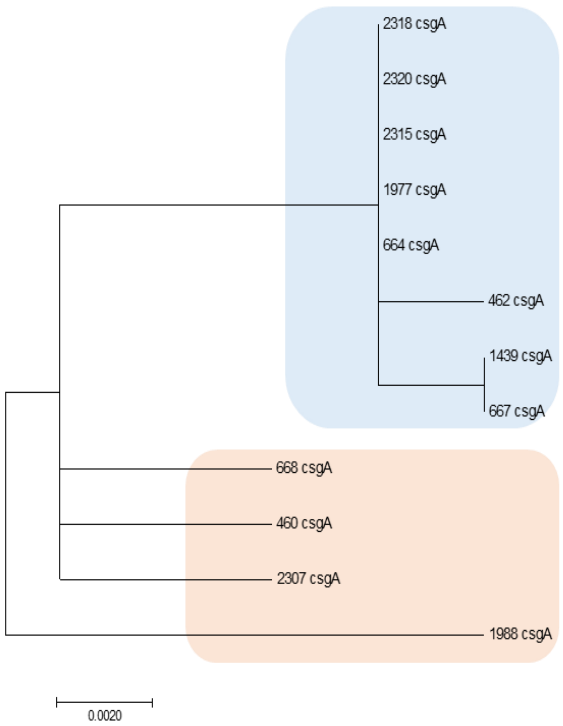
Table 4-5 shows the results of the genomic comparison of *E. hormaechei* strains recovered from sepsis cases, feeding tube and CSF specimens.

NTU ID	Species	Source	ST	Genome size (Mb)	Plasmids	Beta-lactam	Fosfomycin	Amino-glycoside	Macrolide	Phenicol	Rifampicin	Sulphonamide	Trimethoprim
663	*E. h. oharae*	Sepsis	108	4.51	IncR	blaACT-7	fosA	-					
668	*E. h. oharae*	Sepsis	108	4.75	IncFIB,IncFII	blaACT-7	fosA	-	-	-	-	-	-
495	*E. h. oharae*	Sepsis	108	4.51	Not found	blaACT-7	fosA	-	-	-	-	-	-
665	*E. h. oharae*	Sepsis	UN	4.60	Not found	blaTEM-1B, blaACT-16	fosA	-	-	-	-	-	-
460	*E. h. oharae*	Sepsis	UN	4.45	Not found	blaACT-16	fosA	-	-	-	-	-	-
1988	*E. h. oharae*	Sepsis	102	4.64	Not found	blaACT-5	fosA	-	-	-	-	-	-
660	*E. h. steigerwaltii*	Sepsis	45	4.45	ColpVC	blaACT-15	fosA	-	-	-	-	-	-
462	*E. h. steigerwaltii*	Sepsis	133	4.92	Not found	blaACT-7	fosA	-	-	-	-	-	-
664	*E. h. steigerwaltii*	Sepsis	134	4.60	Not found	blaACT-7	fosA	-	-	-	-	-	-
450	*E. h. steigerwaltii*	Sepsis	740	4.81	IncFII, IncFIB	blaACT-15	fosA	-	-	-	-	-	-
667	*E. h. steigerwaltii*	Sepsis	175	5.16	IncHI2A, IncN3, IncHI2	blaACT-7	fosA	-	-	-	-	-	-
1439	*E. h. steigerwaltii*	Sepsis	346	4.64	Not found	blaACT-15	fosA	-	-	-	-	-	-
1448	*E. h. steigerwaltii*	Sepsis	50	4.73	Not found	blaACT-15	fosA	-	-	-	-	-	-
2307	*E. h. steigerwaltii*	Sepsis	UN	4.78	Not found	blaACT-15	fosA	-	-	-	-	-	-
1977	*E. h. steigerwaltii*	CSF	124	4.81	IncR, IncFIB (x2)	blaACT-7	fosA	aadA16, aadA2, aac(6')Ib-cr, aac(3)-lld	mph(A)	catA2	ARR-3	sul1(x2)	dfrA27
2315	*E. h. steigerwaltii*	F. tube	106	4.72	Not found	blaACT-15	fosA	-	-	-	-	-	-
2316	*E. h. steigerwaltii*	Faeces	106	4.73	Not found	blaACT-15	fosA	-	-	-	-	-	-
2317	*E. h. steigerwaltii*	Faeces	106	4.73	Not found	blaACT-15	fosA	-	-	-	-	-	-
2318	*E. h. steigerwaltii*	F. tube	106	4.73	Not found	blaACT-15	fosA	-	-	-	-	-	-
2319	*E. h. steigerwaltii*	Faeces	106	4.72	Not found	blaACT-15	fosA	-	-	-	-	-	-
2320	*E. h. steigerwaltii*	F. tube	106	4.74	Not found	blaACT-15	fosA	-	-	-	-	-	-

The results indicate that all isolates harboured a number of virulence-associated and antibiotic resistance genes, (-) = absent.

4.3.3.3. Adhesion-associated traits

Bacterial attachment to the host cell is an important step towards bacterial pathogenesis as it facilitates colonisation, and thereby infection. Curli fimbriae play an important role in biofilm formation in *E. cloacae* isolates (Kim et al., 2012) and have been attributed a vital role in *E. coli* and *Salmonella* adhesion to host cells (Cordeiro et al., 2016; Tan et al., 2016). Curli fimbrial genes have been detected in the *E. hormaechei* strain in this study (Table 4-3). The CsgA protein is the major subunit of curlin, which has possibly been linked with the ability of *E. hormaechei* to attach to Caco2 cell lines. Figure 4-11 shows the correlation between CsgA and the adhesion ability of *E. hormaechei* strains. The cluster with high levels of adherence was highlighted in blue and the cluster of moderate level of attachment was highlighted in pink, except one strain (668) in this cluster which showed high attachment. This strain also showed an ability to produce high capsular material in the two infant formula agar media tested, soya-based infant formula (IF1) and whey-based infant formula (IF2). Furthermore, this strain produced high yields of cellulose, which for this reason, might result in high levels of attachment, though further details about this gene will be provided in the discussion section of this chapter.



Attachment Caco2	Capsule IF 1	Capsule IF 2	Cellulose	Source
high	++	++	++	F. tube
high	++	++	++	F. tube
high	++	++	++	F. tube
high	-	-	+	CSF
high	-	-	++	Sepsis
high	+	++	+++	Sepsis
high	-	-	+++	Sepsis
high	+	+	++	Sepsis
high	++	+++	+++	Sepsis
moderate	-	-	+++	Sepsis
moderate	+	+	++	Sepsis
moderate	-	++	+++	Sepsis

Figure 4-11 maximum-likelihood tree for *csgA* gene sequence.

Positive link between csgA gene and adhesion ability of *E. hormaechei* strains. The blue highlighted strains showed high attachment and pink showed moderate attachment except one strain (668) in this cluster, which showed high attachment. The phylogenetic tree was generated by using MEGA-7.

4.3.3.4. Stress response gene (rpoS)

The *rpoS* gene, which encodes the alternative sigma factor S, is the master regulator of responses to the various stress conditions in many Gram-negative bacteria (Lago et al., 2017). In this study, all *E. hormaechei* strains were screened for the presence of the *rpoS* gene. All *E. hormaechei* isolates were shown to harbour this gene, as expected. The sequences of the *rpoS* genes of strains 1448 and 664 were shown to cluster separately from other *rpoS* sequences of other strains, indicating the presence of difference in the nucleotide sequence. As shown in the phylogenetic tree of Figure 4-12, the blue highlighted strains clustered separately and were also the most acid sensitive strains over the 2-h incubation period.

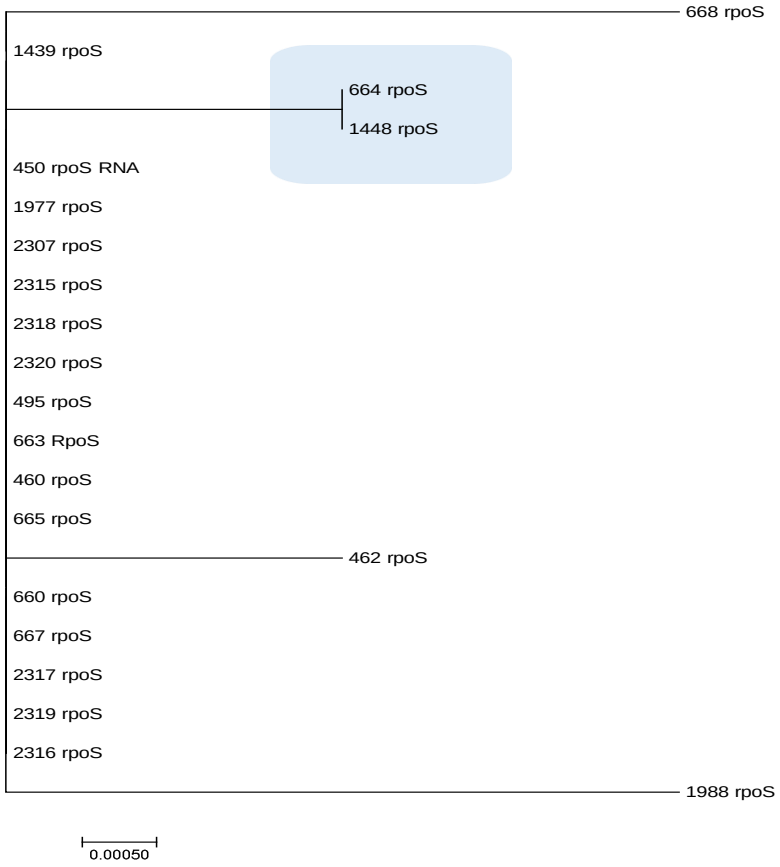


Figure 4-12 maximum-likelihood tree for rpoS sequence gene.

All *E. hormaechei* isolates were shown to harbour the *rpoS* gene. The blue highlighted strains clustered alone and were the most sensitive to acid at pH 3.5 compared with the other strains tested. The phylogenetic tree was generated using MEGA-7.

4.3.4. Assessment of the pathogenicity of *E. hormaechei* using tissue culture assays

This part of the study examined the twelve representative strains out of the twenty-one of *E. hormaechei* isolated from sepsis cases, CSF and neonatal feeding tubes. The representative strains were chosen based on genotype and phenotype, one strain chosen from each cluster based on sequence variation in the core genome as shown in the phylogenetic tree (Figure 4-9). Additionally, the strains were chosen based on phenotypic traits when they appeared to be more closely related (i.e. clustered together). For example, strain 1439 displayed the lowest serum tolerance and strain 664 showed the most acid sensitivity. Strain 668 showed an ability to produce high capsular material in the two infant formula and revealed high yields of cellulose. Also this strain produced a higher amount of biofilm on infant formula at temperatures of 25°C and 37°C among *E. hormaechei* subsp*. oharae.* Strains 667 was able to form capsular

material on both media compared with closely related strain 450 and only strain 460 showed non-haemolysis on horse blood among of *E. hormaechei* strains. However, strain 1977 E. hormaechei subsp. steigerwaltii was the only strain isolated from CFS and 2315, 2318 and 2320 strains were isolated from feeding tubes. These strains were evaluated for their potential virulence using Caco2 human intestinal cells and HBMEC brain endothelial cells to test for cytotoxicity and macrophage survival.

4.3.4.1. Bacterial attachment on Caco-2 human epithelial cells

An attachment assay was conducted to evaluate the adherence ability of twelve representative *E. hormaechei* strains to epithelial Caco-2 cells as shown in Figure 4.13. To define the level of attachment achieved by each isolate, the attachment efficiency (%) results were classified into three categories according to the observed level of attachment on the controls used in this study: high, medium, and low levels of attachment. Low attachment was considered to be less than 2% cfu/ml, while attachment efficiency of isolates between 2% and 4% cfu/ml was referred to a medium level of attachment. Attachment efficiency greater than 4% of recovered cfu/ml was considered as a high level of attachment to the Caco-2 human epithelial cells. It was observed that all strains from different sources were able to adhere to this cell line to different extents. For example, two strains, 1977 and 664, showed a higher attachment rate to Caco-2 than the other bacterial strains compared to the positive control when analysed by means of one-way ANOVA statistical testing, followed by multiple comparisons with a Dennett's test ($P < 0.001$). However, sepsis isolates 1988, 460 and 2307, exhibited the lowest attachment levels among representative strains when compared to the positive control.

On the other hand, feeding tube strains 2315 and 2320 had similar ability to adhere to Caco-2 cells as sepsis isolate 668, and also showed greater adherence to this cell line than the sepsis isolates 1988, 460 and 2307. Interestingly, this was also observed by the feeding tube strain 2318, as it displayed higher attachment levels compared with sepsis strains 668, 1988, 460 and 2307, and similar levels to sepsis isolates 462 and 1439, as shown in Figure 4.13.

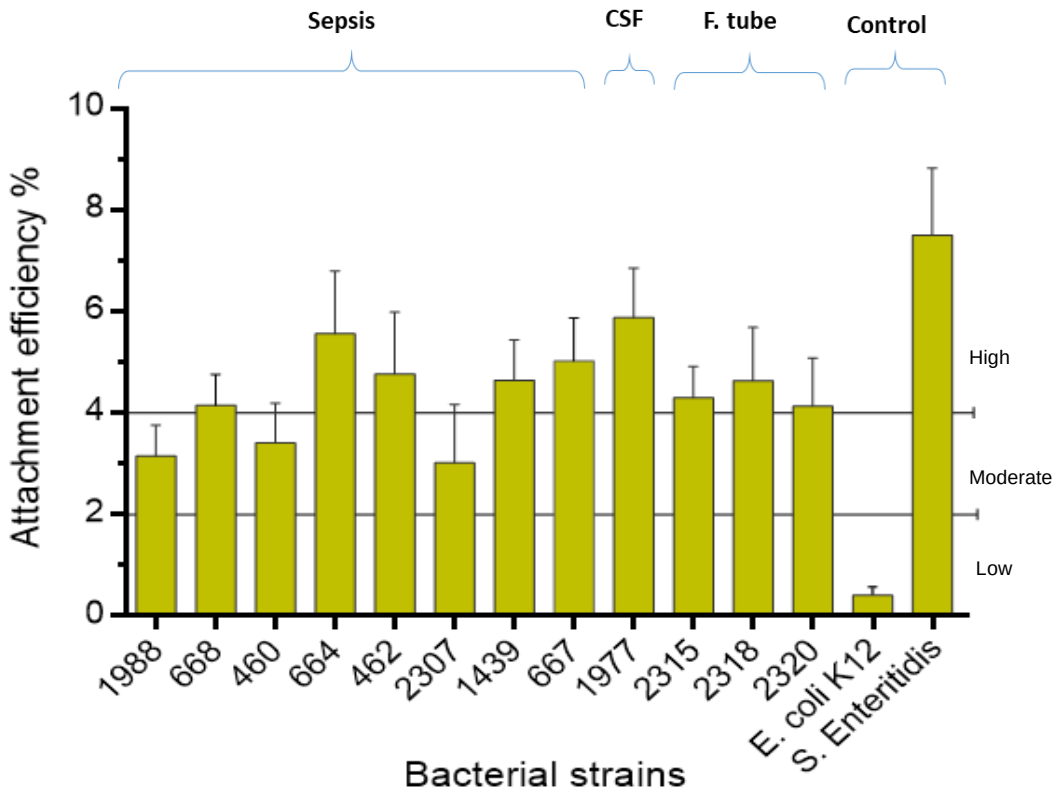


Figure 4-13 Attachment of *E. hormaechei* strains to the human Caco-2 cell-line.

The figure shows the differences in attachment levels among *E. hormaechei* strains to human epithelial cell line Caco-2. All strains were able to attach to the epithelial cell-line when compared to *E. coli* K12, which was used as negative control, and. *S*. Enteritidis, which was used as a positive control. Error bars indicate the standard deviation of six data points taken from two independent experiments in triplicate.

4.3.4.2. Bacterial invasion of Caco-2 human epithelial cells

The gentamicin protection assay was performed to investigate the capability of *E. hormaechei* strains isolated from different sources to invade the Caco-2 cells. Results were classified into three categories according to the percentage of recovered cells after invasion. Less than 0.2% recovery was defined as a low level of invasion, 0.2 to 0.4% was defined as a medium level, and greater than 0.4% recovery was defined as a high level of invasion. All isolates were able to invade human Caco-2 cells, however, the invasion rates varied among the examined *E. hormaechei* strains (Figure 4-14). Strains 1977 and 668 showed significantly a higher invasion rate than the other strains ($P<0.05$).

Strains 664 and 2315 both showed the lowest invasion levels of Caco-2 cells. With regard to feeding tube strain 2318, this strain showed the most significant invasion levels among the feeding tube strains compared to the negative control. This strain has the

ability to invade Caco-2 cells similar to sepsis isolate 462, which also showed an ability to invade this cell line more so than the sepsis isolates 664,1439 and 2307.

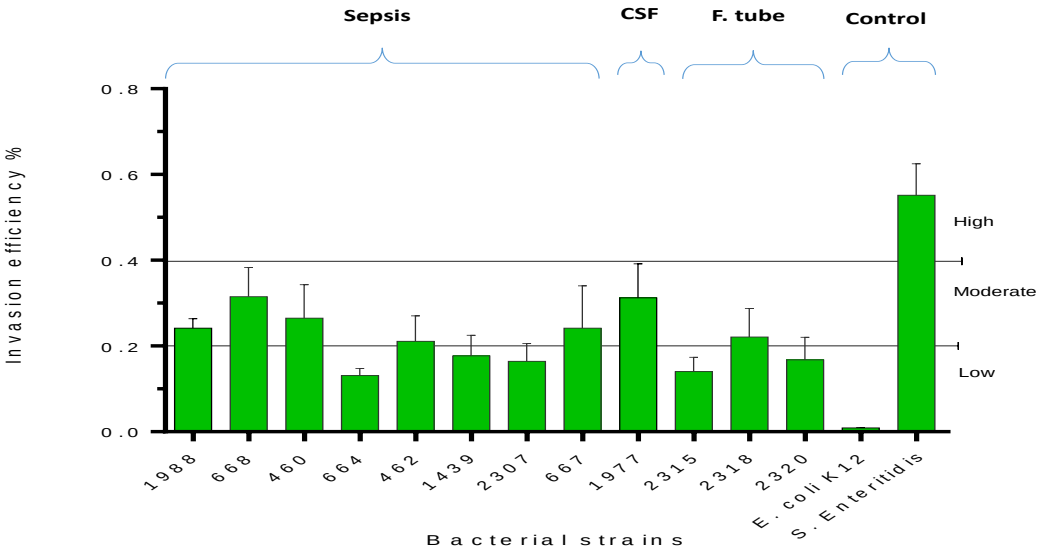


Figure 4-14 Invasion efficiency of *E. hormaechei* strains to the mammalian Caco-2 cell line.

The figure shows the differences among *E. hormaechei* strains with regards to their ability to invade the human epithelial cell line Caco-2, by performing the gentamicin protection assay. The majority of strains invaded Caco-2 human epithelial cells at moderate levels. *S.* Enteritidis and *E. coli* K12 were used as positive and negative control strains, respectively.

4.3.4.3. Attachment to human brain microvascular endothelial cells (HBMEC)

The ability of the selected *E. hormaechei* isolates to attach to human brain microvascular endothelial cells (HBMEC) are shown in Figure 4-15. In this assay, *E. coli* K12 was used as a negative control, while *C. koseri* was used as a positive control. All strains were able to adhere to this cell-line at different levels. *E. hormaechei-steigerwaltii* strain 1977 (ST124) isolated from CSF showed the highest adherence and invasion capabilities of the HBMEC and Caco-2 cell-lines among all of the tested strains. Sepsis strains 462 and 1439 revealed the ability to adhere to HBMEC cell lines more so than the other sepsis and feeding tube strains. On the other hand, feeding tube strain 2318 demonstrated a similar level of adhesion to some sepsis strains compared to the *C. koseri* positive control ($P < 0.001$), such as 668, 664 and 667, while displaying a higher adhesion level than other sepsis isolates, such as 2307 and 1988. The other feeding tube strains 2315 and 2320 also revealed an ability to adhere to this cell line, similar to sepsis strains 1988 and 460, but less than feeding tube strain 2318. Paired t-test shows no significant deferent

($p < 0.573$) between sepsis and feeding tube strains compared in the ability to adhere to HBMEC cell-line

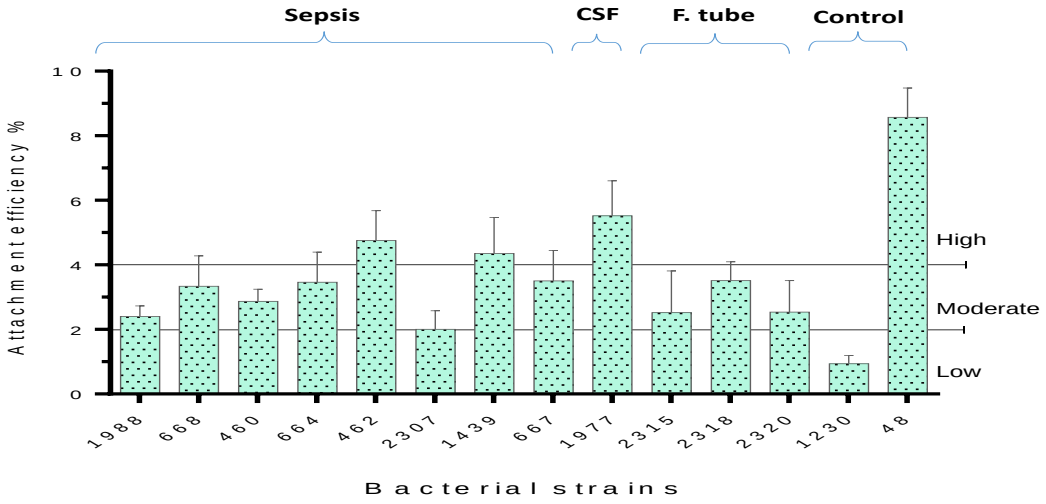


Figure 4-15 Attachment of *E. hormaechei* strains to HBMEC cell-line.

The figure shows the differences in attachment levels among *E. hormaechei* strains to human brain microvascular endothelial cells (HBMEC). All strains were able to attach to the HBMEC cell-line when compared to *E. coli* K12, which was used as negative control, and *C. koseri*, which was used as a positive control. Error bars indicate the standard deviation of six data points taken from two independent experiments in triplicate.

4.3.4.4. Invasion of the human brain microvascular endothelial cells (HBMEC)

With regards to the HBMEC cell line, most of these strains were able to invade the human blood brain barrier endothelial cells at low levels when compared to the negative control *E. coli* K12. CSF strain 1977 and sepsis strains 668, 664 and 667, displayed a higher invasion level among all of the tested strains ($P < 0.05$) when compared to the negative control. However, feeding tube strains showed a similar level of invasion to some sepsis strains compared to the negative control, such as 1439, 2307, 460 and 1988 (Figure 4-16). These results indicate no correlation observed between attachment and invasion with regards to the HBMEC cell line. For example, the sepsis isolate 1439 was one of the isolates demonstrating high levels of adhesion, but presented with the lowest level of invasion.

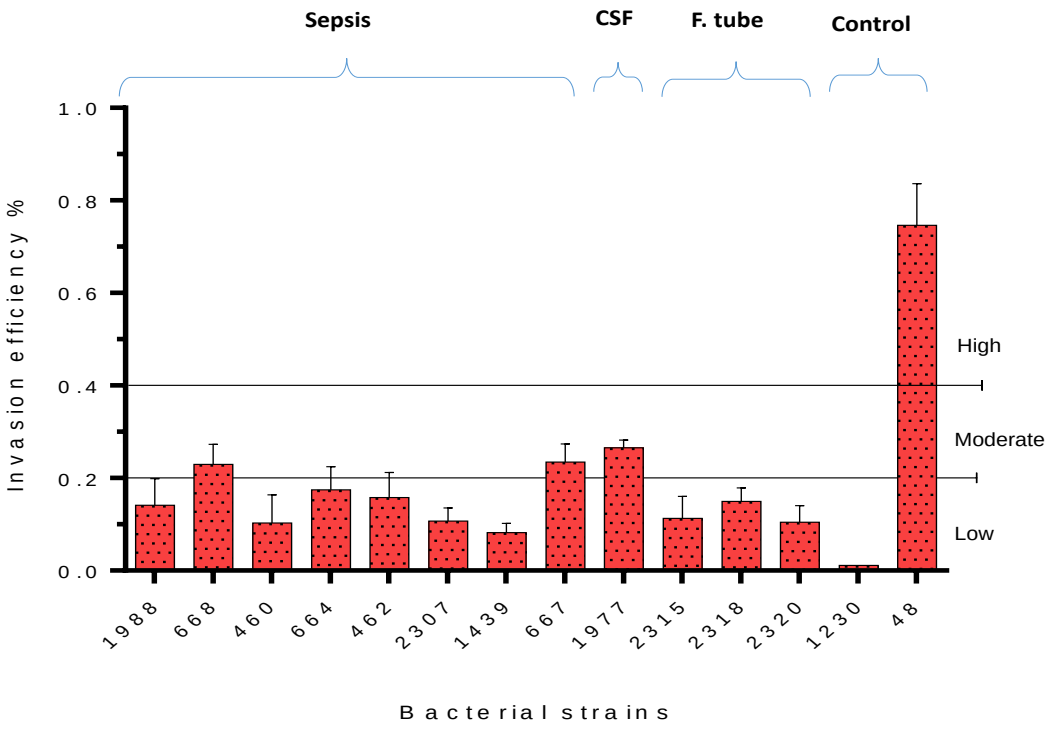


Figure 4-16 Invasion efficiency of *E. hormaechei* strains to HBMEC cell line.

The figure shows the differences among *E. hormaechei* strains in terms of their ability to invade the human blood brain barrier endothelial cells, by performing the gentamicin protection assay. The majority of strains invaded HBMECat only low levels. *K. koseri* and *E. coli* K12 were used as positive and negative control strains, respectively.

4.3.4.5. Macrophage survival assay

Twelve representative strains of *E. hormaechei* were investigated for their ability to persist and replicate within human macrophages, represented by the U937 cell-line. The NTU collection strain *C. koseri* 48 was used as a positive control, whilst *E. coli* K12 strain 1230 (also from the NTU strain collection) was used as a negative control. All tested strains were taken up by macrophage cells. After uptake, most strains persisted in macrophages for 72 hours. The majority of them were able to multiply significantly after 24 hours of incubation, including the positive control *C. koseri* ($P < 0.05$).

Two sepsis strains, 2307 and 1439, displayed a decline in their intracellular survival at 48 hours of incubation compared to other strains. All strains displayed a slight decrease in their intracellular numbers after 72 hours of incubation, whereas strains 460 and 1988 exhibited a fall in their numbers. CSF strain 1977, with the highest numbers, had exhibited the greatest ability to persist and replicate inside the macrophages. The

feeding tube strains showed a similar ability to survive and replicate within the U937 cells compared to some sepsis strains, such as 667 and 668 (Figure 4-17).

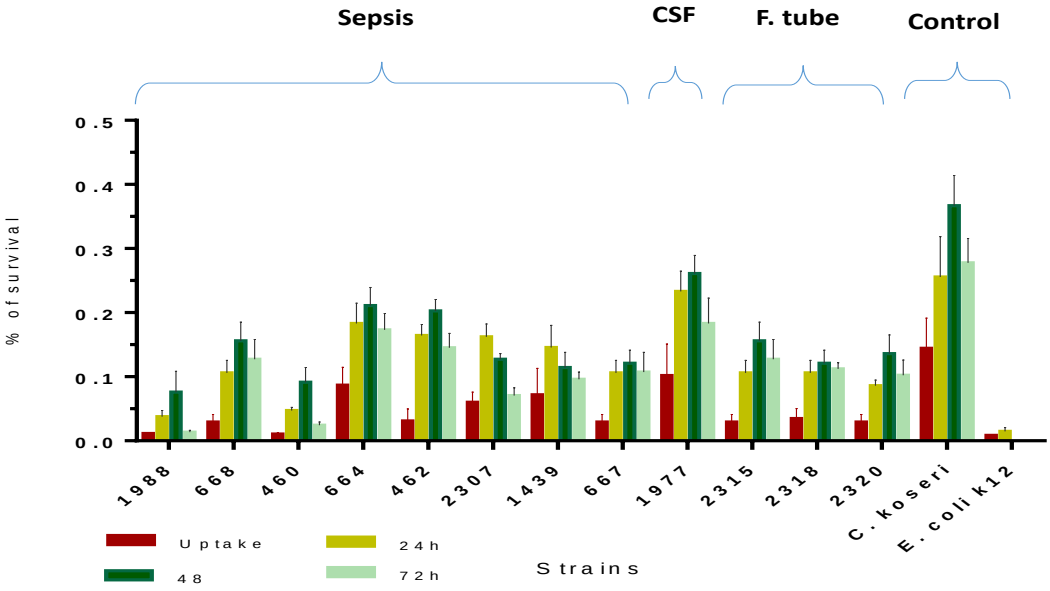


Figure 4-17 shows the differences in the ability of *E. hormaechei* strains to persist in U937 macrophage cell lines for 72 hours.

The majority of the *E. hormaechei* strains (n = 12) demonstrated an ability to persist and replicate within the macr ophage cell-line U937 for up to 72 hours of incubation. The *C. koseri* 48 strain was used as positive control, while *E. coli* K12 strain 1230 was used as a negative control (both were obtained from the NTU strain collection).

4.3.4.6. Cytotoxicity of *E. hormaechei*

The MTT reduction assay was carried out to measure the cytotoxic effect of selected *E. hormaechei* strains on the viability of the epithelial Caco-2 cells. Viable epithelial Caco-2 cells are able to reduce MTT to its insoluble purple form, known as formazan. An *S.* Enteritis strain, which was used as positive control in this investigation, lowered the amount of MTT reduction, thus indicating their high cytotoxic ability. Conversely, Caco-2 cells in cell culture medium with no bacteria added to the cells was used as a negative control, which showed high levels of MTT reduction thus indicating low or non-existent cytotoxicity and high cell viability. The results were categorised as low cytotoxicity (OD ≥ 1), moderate cytotoxicity (OD 0.5 – 1), and high cytotoxicity (OD < 0.5). The results from the assay indicated that all of the strains tested were highly cytotoxic. No significant difference between strains 1977, 667, 1988 and 462 and the positive control, when analysed by means of one-way ANOVA, followed by multiple comparisons with a

Dennett's test. Sepsis strain 462 was the most cytotoxic compared to the other strains. Interestingly, feeding tube strains showed a similar level of MTT reduction to some sepsis strains such as 2307 and 664 (Figure 4-18).

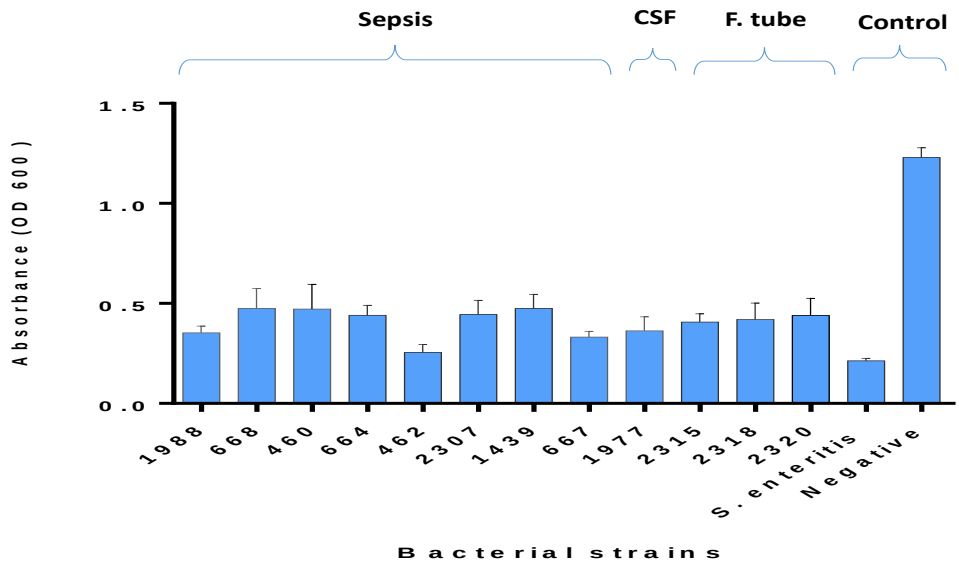


Figure 4-18 Cytotoxicity of *E. hormaechei* strains after 1-hour incubation with the Caco-2 cell line.

All strains showed high cytotoxicity and no significant difference was observed between strains 1977, 667, 1988 and 462 compared to the positive control, when analysed by means of one-way ANOVA, followed by multiple comparisons with a Dennett's test.

4.3.5.Summary of virulence factors for *E. Hormaechei*

This part of the study examined the twelve representative strains out of twenty-one *E. hormaechei* isolates from sepsis, CSF and neonatal feeding tube samples. These strains were evaluated for their potential virulence using human intestinal cells Caco-2, brain endothelial cells HBMEC, as well as an evaluation of cytotoxicity and macrophage survival. All results are summarised in Table 4-6.

Table 4-6 Results summary of attachment, invasion Caco-2, HBMEC and phagocytosis survival of U937 cell lines and cytotoxicity Caco-2 cells.

Strain	Species	Source	Caco-2		HBMEC		Survival in macrophages	MTT
			Attachment	Invasion	Attachment	Invasion		
1988	*E. h. oharae*	Sepsis	moderate	moderate	moderate	low	low persisted	high
668	*E. h. oharae*	Sepsis	high	moderate	moderate	moderate	persisted	high
460	*E. h. oharae*	Sepsis	moderate	moderate	moderate	low	low persisted	high
664	*E. h. steigerwaltii*	Sepsis	high	moderate	moderate	low	persisted	high
462	*E. h. steigerwaltii*	Sepsis	high	moderate	high	low	persisted	high
2307	*E. h. steigerwaltii*	Sepsis	moderate	low	moderate	low	persisted	high
1439	*E. h. steigerwaltii*	Sepsis	high	low	high	low	persisted	high
667	*E. h. steigerwaltii*	Sepsis	high	moderate	moderate	moderate	persisted	high
1977	*E. h. steigerwaltii*	CSF	high	moderate	high	moderate	persisted	high
2315	*E. h. steigerwaltii*	F. tube	high	low	moderate	low	persisted	high
2318	*E. h. steigerwaltii*	F. tube	high	moderate	moderate	low	persisted	high
2320	*E. h. steigerwaltii*	F. tube	high	low	moderate	low	persisted	high

4.4. Discussion

Over the last decade, the incidence of neonatal infections has been increasing due to members of the Enterobacteriaceae, including species of the genus *Enterobacter*, which are considered one of the most common opportunistic pathogens (Davin-Regli and Pages, 2015). They can cause numerous infections such as meningitis, septicaemia, pneumonia, cerebral abscess, wound infection, urinary tract infection, and are also associated with bloodstream infections. However, neonates are at high risk of nosocomial infection in neonatal intensive care units (NICUs) (Joseph et al., 2012; Basiri et al., 2015; Rameshwarnath and Naidoo, 2018). This project concerns the risk assessment to neonates, whose immune systems have not fully developed, have low birth-weights and are fed via nasogastric feeding tubes. Therefore, this research work aimed to profile a diverse clinical collection of *Enterobacter* species, in particular *E. hormaechei*, by applying a range of phenotyping and genotyping methods to determine the potential risk of these species to neonates. Furthermore, this study aimed to evaluate the possible correlations between *E. hormaechei* isolated from sepsis and neonatal feeding tubes and the frequency of virulence genes. It is important to improve our understanding of the potential risk of *E. hormaechei* isolated from neonatal feeding tubes to neonate in NICUs. Twenty-six *Enterobacter* spp. were selected from the Nottingham Trent University culture collection, which has not previously been studied. These strains were isolated from premature babies with sepsis cases and CSF specimens.

In addition, 7 strains from sepsis, nasogastric enteral feeding tubes and faeces samples from Queen Medical Centre (QMC) in Nottingham were also included in the collection.

A variety of rapid typing methods include molecular techniques which have been developed for the identification of bacterial strains isolated from clinical samples, such as PCR-based typing methods, pulsed-field gel electrophoresis (PFGE), and multilocus sequence typing (MLST). Such molecular fingerprinting techniques are important techniques in epidemiological investigations. These methods are widely used for the molecular subtyping of bacteria, including *Enterobacter* spp. This would assist in identifying if the same strains were isolated on different occasions and to determine if there is any epidemiological relationship between pulsotype and clinical presentation. PFGE analysis was performed for twenty-five strains isolated from premature babies with sepsis by using *XbaI* restriction enzyme to confirm their clonal origin. PFGE profiles indicates that all of the *Enterobacter* strains formed unique pulsotypes of distinguishable strains. These sporadic strains were isolated from an NICU during a 7-year period and were unlikely to be related.

The similarity of phenotypic of *Enterobacter*, in particular *E. cloacae* genomic groups, resulted in several problems with their identification. Although phenotypic identification of this genus is usually difficult, molecular methods are often used to achieve an accurate definition. Multilocus sequence analysis (MLSA), including *fusA*, was shown as an important tool for taxonomic analysis of the Enterobacteriaceae. The *fusA* allele has been reported to be more reliable for identification of *Cronobacter* from non-*Cronobacter* such as *E. hormaechei* (Jackson et al., 2015; Ogrodzki and Forsythe, 2015).

All isolates in this study was evaluated based on the *fusA* gene sequence to identify the species level identity of *Enterobacter* spp. According to the phylogenetic tree, the *fusA* gene sequences showed separation between the *Enterobacter* isolates. Fourteen strains were identified as *E. hormaechei*, four strains identified as *E. aerogenes*, one strain identified as *C. sakazakii*, and the other 7 strains were not clearly identified and clustered within the *Enterobacter* genus. Interestingly, strain 669 was found to match *fusA* allele 1, identified as *C. sakazakii*, and as originally identified as *E. cloacae* by API

biochemical profiling in the hospital. Previous studies by Townsend et al. (2008b) also reported that the misidentification of *E. hormaechei* as *C. sakazakii* isolated from neonates during a nosocomial outbreak in a Californian hospital. Similarly, false identifications was reported by Jackson (2015), where patient isolates in a hospital outbreak investigation were misidentified as *C. sakazakii*, then later characterised as *Enterobacter hormaechei* and a species of *Enterobacter* by using multilocus sequence typing.

Misidentification is problematic issue during clinical infection, particularly in NICUs, because it may result in inappropriate treatment of the disease and mislead the investigation and attribution of the source. This is also one problem in developed countries were most clinical laboratories routinely identify Enterobacteriaceae by semi-automated systems or commercial kits which limit discrimination of isolates to within this group (Jesumirhewe et al., 2016). However, the results of *fusA* typing did not discriminate between the subspecies of *E. hormaechei*. While the rest of strains clustered within the *Enterobacter* genus through the aforementioned methods were strains that did not match or showed ambiguous taxonomy. Similarly, previous Studies reported that the sequencing of 16S rRNA and other housekeeping genes such as *rpoB*, *hsp60* and *gyrB* alone is not an appropriate method for determining to the species level of the *E. cloacae* strains (Paauw et al., 2008; Pavlovic et al., 2012). In this study, these strains were subjected to whole-genome sequencing to identify the correct species of *Enterobacter* and discriminate between the subspecies of *E. hormaechei* which isolated from several neonatal in QMC.

Strains had clustered within the *Enterobacter* genus with deferent alleles and fourteen *E. hormaechei* strains were subjected to whole-genome sequencing. An additional collection recovered from sepsis, enteral feeding tubes, and faecal samples of low-birth weight neonates from the QMC hospital were also subjected to whole-genome sequencing. These selected strains were sequenced using the Illumina MiSeq and it was revealed that the majority of strains were identified as *E. hormaechei*, in particular subsp. *E h. ssteigerwaltii*. However, strain 602 was identified as *Serratia marcescens*, and thus, was not included in this study. In addition, strain 669 also was not included because it

was confirmed by *fusA* gene sequences to be *C. sakazakii*. All *Enterobacter* spp. were described using a range of phenotyping methods, in order to determine the potential infection risk of these species to neonates. However, having evaluated the importance of both organisms, I decided to focus on *E. hormaechei* for genomic and further analyses. The reason for this was that although *E. hormaechei* is associated with severe neonatal infections, there have been few in-depth studies in the literature, whereas *E. aerogenes* and *E cloacae* have been studied by many research groups. Furthermore, *E. hormacechei* was predominant among the strains collected in this study.

Formation of biofilms is a one of the important virulence factors, which is a critical feature for the development of clinical infection (Wu et al., 2015). It plays a significant role in colonisation by microorganisms, providing an opportunity for bacteria to become resistant to environmental stresses. In this study, I investigated the biofilm-forming potential of *Enterobacter* strains (n = 31) on plastic surfaces. The two variables used were incubation temperature and formula composition. These investigations found that the quantity of biofilm was significantly higher ($p < 0.05$) at 37 °C compared to 25 °C among the majority of *Enterobacter* isolates. It was also noted that the quantity of biofilm was higher in infant formula when compared to standard TSB medium under similar conditions. These observations indicate that the composition of the medium had an influence on the ability of *Enterobacter* strains to produce biofilm. These findings are in agreement with another study by Kadam et al. (2013), who assessed biofilm formation of *Listeria monocytogenes*. Their study showed that biofilm formation was higher in nutrient poor medium when compared to nutrient rich medium. Similarly, Allan et al. (2002) showed that biofilm formation had been reduced under nutrient limitation.

The present study also compared the two different incubation temperatures and observed a higher quantity of biofilm production at 37 °C compared to 25 °C. This might refer to the fast growth rate of the strains tested in this study at higher temperatures. Thus, temperature is an important factor for biofilm formation, also the type of nutrient is vital for enhancement of biofilm formation. The most notable result was that the same quantity of biofilm was formed by *E. hormaechei* subsp. *steigerwaltii* isolated from feeding tubes when grown in infant formula at 37 °C compared to biofilm formed by *E.*

hormaechei subsp*. steigerwaltii* isolated from CFS and sepsis cases (Figure 4-3). This finding probably indicates the potential risk of neonatal infection by *E. hormaechei* subsp*. steigerwaltii* isolates from feeding tubes in NICUs. It is well known that biofilm protects bacteria during infections (Römling and Balsalobre, 2012). Jamal (2018) reported that according to the National Institute of Health, "more than 65% of all microbial infections are caused by biofilms". Another study indicated that the most common pathogens in a healthcare setting are found to be biofilm producers, such as the ESKAPE pathogen (Bales et al., 2013; Santajit and Indrawattana, 2016; Navidinia, 2016).

Previous studies have reported that the curli fimbriae play an important role in biofilm formation. For example, a study by Kim et al. (2012) has suggested an association between expression of curli genes (*csgA* & *csgD*) and the ability of biofilm formation in *E. cloaca* isolates. Although the ability of biofilm formation varies among *Enterobacter* strains, the genome screening shows that these genes are present in all representative isolates. This indicates that some of the identified genes may not adequately express biofilm or might be influenced by other factors. Another study was carried out by Kim et al. (2012), who examined the expression of curli genes and biofilm formation in clinical isolates of *Enterobacter cloacae* by using RT-PCR. They found that curli fimbriae played a significant role in biofilm formation in clinical isolates that were tested. Such techniques could be used in further studies to determine or confirm whether the same phenomenon can be observed in our isolates.

Curli fimbriae have also been described to contribute to biofilm formation in many species of bacteria, and are responsible for mediating host cell adhesion and invasion (Barnhart and Chapman, 2006; Kim et al., 2012). Curli fimbriae expression by *Salmonella* strains is also indicated by binding of Congo red dye (Gophna et al., 2001; Uhlich and Solomon, 2006; Reichhardt et al., 2015). In this study, *Enterobacter* isolates were characterised on Congo red media to evaluate the expression of curli fimbriae. Red colonies would be an indicator presumed of production of curli fimbria as positive colonies indicate binding to the Congo red dye. As shown in Table 4-2, not all *Enterobacter* strains were able to bind the Congo red dye. In contrast, all these strains

were confirmed to have *csgA-G* genes. This finding indicated no association between the morphology of *Enterobacter* isolates on Congo red and presumed Curli fimbriae expression. Indeed, some bacterial traits can affect biofilm formation, such as capsular materials and production of cellulose (Van Houdt and Michiels, 2010; Liu, et al., 2014; Wang et al., 2015). In this study, the above traits were investigated to find any correlation between these traits and biofilm formation among *Enterobacter* strains.

Bacterial capsule production was determined by colony appearance on two types of infant formula agar (whey and soya). It is apparent in this study that some strains are not able to produce capsular material in both infant formula, while capsules were produced by all *E. aerogenes* strains in both formula used (Tables 4.2). However, in general, the majority of *Enterobacter* species are able to produce capsular material on whey rather than soya-based infant formula. This finding may indicate the difference in composition of infant formula used possibly affects the production of capsular material. It was also noted that there is no correlation between biofilm formation and capsular material production. For instance, *E. hormaechei* subsp*. steigerwaltii* 1977 and 660 produced high biofilm in infant formula, as shown in Table 4-2, whilst unable to produce capsular materials in both types of infant formula used. Similarly, a previous study by Hurrell et al. (2009a) found that *C. sakazakii* strains were unable to produce any capsular materials, but nevertheless produced more biofilm. Although, the results indicate no obvious link between biofilm formation and capsule production in these strains, capsules may provide resistance to desiccation, serum activity and have a significant role in avoiding phagocytic killing (Ogrodzki and Forsythe, 2015).

Cellulose is another extracellular material identified as one of the major components of the biofilm matrix. Its production is a widespread phenomenon by members of the Enterobacteriaceae family, including *Enterobacter* spp. It has been shown to be a crucial cause of biofilm formation and protect cells from various environmental stressors (Re and Ghigo, 2006, Vu, 2009). Cellulose production was evaluated among *Enterobacter* strains in this study by comparing the visualised fluorescent colonies under UV light. All isolates showed differing amounts of cellulose production, although all strains showed high quantities of biofilm production. However, there is no obvious correlation

of this phenotype with cellulose production. For example, strains 1448, 450 and 495 produce high biofilm, however these strains had produced lower amounts of cellulose. In contrast, strains 988, 460 and 1439 produce high biofilm as well as high amounts of cellulose, as shown in Table 4.2. In addition, bcs operon have been reported to be associated with the production of cellulose synthase in *Enterobacter* (Römling and Galperin, 2015; Ji et al., 2016). Similarly, cellulose-associated genes *bcsA, bcsB* and *bcsD* were investigated by Hu et al. (2015) and confirmed that *bcsA* and *bcsB* were necessary to express cellulose and are involved in biofilm formation in *Cronobacter* spp. Indeed, the BLAST searches were performed to check the presence of cellulose-associated genes in the genomes of *E. hormaechei* strains. It was revealed that all analysed genomes possess all of these genes.

Although biofilm formation has a significant role in the pathogenesis of bacterial infection, there are a number of supposed virulence factors that are involved in the ability of a bacterial pathogen to cause disease. For example, protease and haemolysins were detected among the tested *Enterobacter* strains. Proteases have a number of key roles in the ability of several bacteria to infect the host and cause disease (Gellatly and Hancock, 2013; Koziel and Potempa, 2013; Culp and Wright, 2017). Bacterial haemolysins are enzymes that destroy red blood cells and they are classified into different types of haemolysins including α-haemolysin, β-haemolysin and γ-haemolysin (Kong et al., 2016). In the present study, all *Enterobacter* strains produced β-haemolysis on horse blood agar plates, with the exception of two strains, 460 and 661. Previous studies have revealed that the β-haemolysin plays a crucial role in the result of group B streptococcal sepsis by contributing to liver failure and high mortality (Ring et al., 2002). The presence of such virulence traits among the examined *Enterobacter* strains indicates a potentially higher risk to neonatal health in NICUs.

Other virulence factors would include siderophores, where many pathogenic bacteria release these compounds with a high affinity to chelate iron from iron-binding proteins (Caza and Kronstad, 2013; Raymond et al., 2015). It is important for these bacteria to obtain nutrients such as iron, which is essential for the growth, replication, and survival in the host environment and could assist in virulence. The production of siderophores

was detected in all tested strains by using the CAS agar diffusion assay. Apart from siderophore production, molecular characterisation was also performed. Most Enterobacteriaceae strains contain genes encoding iron uptake systems including *Enterobacter* spp., which has been reported to possess genes for enterobactin and aerobactin siderophore biosynthesis (Mokracka et al., 2004). In this study, the iron siderophores, enterobactin biosynthesis genes (*entABCDE*) and aerobactin (*IucC*) were detected in all isolates recovered from sepsis, feeding tube and CSF specimens. These results suggest that the production of enterobactin and aerobactin siderophores were not related to the source of the isolate. However, the detection of *entABCDE* and *IucC* genes may enhance the ability of pathogenic bacteria to grow in the host in conditions of low-level iron and facilitate replication during infections. Therefore, the presence of such virulence traits is likely to increase the potential risk factors to neonatal health.

Resistance to human serum is an important factor in bacterial virulence (Webb and Kahler, 2008; Miajlovic and Smith, 2014; Sanchez-Larrayoz et al., 2017). Results obtained from the present study demonstrate that all the sporadic isolates of *Enterobacter* demonstrated a high level of resistance to human serum. This finding suggests that human serum resistance might be an important determinant of virulence in *Enterobacter* species. A previous study by Townsend et al. (2008b) stated that *E. hormaechei* strains isolated from neonates during a nosocomial outbreak in a California hospital were serum-resistant. Figueroa and Densen (1991) reported that a common feature of meningococci isolated from CSF or blood is serum-resistant. Similarly, *E. hormaechei* strains were confirmed to be serum-resistant. Such findings may indicate the ability of these strains to cause bacteraemia by avoiding or escaping human serum killing. Indeed, several studies reported that OmpA contributes to serum resistance and mutants of the *ompA* gene reduce the ability of bacteria to survive in human serum (Weiser and Gotschlich, 1991; Lindholm and Oscarsson, 2017). However, it was revealed that the *ompA* gene was detected in all *E. hormaechei* strains. On the other hand, capsular material production could also have a role in serum resistance and macrophage evasion (Ogrodzki and Forsythe, 2015). My results showed that not all strains were able to produce capsular materials on both media used in this study, however, all strains were serum-resistant, therefore suggesting that these strains might

be able to escape human serum killing by using different strategies. Indeed, such findings suggest that the ability of these strains to survive in human serum would increase the clinical concern to neonates in the NICU, especially those with compromised immune systems, making them highly susceptible to infections.

In addition to the survival in human serum, the antibiotic sensitivity test revealed that the isolates of *Enterobacter* were almost 100% resistant to ceftazidime, ampicillin, cefotaxime and gentamicin (Table 4-3). Also, some strains were resistant to imipenem, meropenem and 3rd generation cephalosporins. These results are consistent with Hurrell et al. (2009), who reported that many *E. hormaechei* strains were found to be resistant to the 3rd generation cephalosporins. Torkaman et al. (2009) also suggest that *Enterobacter* spp. is the most common pathogen causing neonatal sepsis in NICUs, especially by multiple drug-resistant strains. Antimicrobial resistance genes were predicted among all isolates tested, such as fosfomycin (*fosA*) conferring fosfomycin resistance and *bla*ACT β-lactamase. The majority of these isolates investigated in this study were resistant to cephalosporins. However, the resistance might be due to the presence of the multidrug efflux pump AcrAB-TolC, which was detected among examined genomes. It is one of the most important antimicrobial resistance mechanisms. Efflux pumps can expel a broad range of antibiotics and also drive the acquisition of additional resistance mechanisms. The multidrug efflux system AcrAB has been linked with the ability of *E. coli* to resist ampicillin, chloramphenicol, nalidixic and tetracycline (Scatamburlo et al., 2004; Yasufuku et al., 2011; Amábile-Cuevas et al., 2010; Kafilzadeh and Farsimadan, 2016). The study by Pérez et al. (2012) found that both *acrA* and *tolC* genes are key to the fitness of clinical *E. cloacae* isolates. Similarly, another study by Guérin et al. (2016) confirmed the essential role of the AcrAB-TolC efflux pump in both virulence and antimicrobial resistance of *E. cloacae*. Thus, these results indicate that there could be serious risk associated with neonates, especially those born with low birth-weight. Five strains of *E. hormaechei* isolates have been found to harbour at least one plasmid (Table 4.5), the majority of which belonged to Inc groups, for example, strain 667 harboured 3 plasmids IncHI2A, IncN3, IncHI2. Such plasmids may able to harbour a variety of resistance genes and additional virulence factors. A study by Chen et al. (2016) has shown that IncHI2 was the major plasmid, predominant in

antibiotic-resistant *Salmonella* isolates and contribute to the spread of antibiotic resistance among these isolates.

Among these sporadic isolates, only *E. hormaechei* subsp*. steigerwaltii* 1977 isolated from CSF showed resistance to all antibiotics used in this study. It had possessed more antimicrobial resistance genes, such as macrolide *mph*(A), sulphonamide *sul1*, rifampicin *arr-3*, trimethoprim *dfrA27* and aminoglycoside genes *aadA16, aadA2, aac(6')Ib-cr* and *aac(3)-lld*. This strain harboured four aminoglycoside genes, which might be a serious issue because these resistance genes are generally found on mobile genetic elements such as plasmids, integrons or transposons. They can easily be spread among other bacteria in the hospital setting (Vaziri et al., 2011). Such isolates raise alarms, suggesting that more attention should be given to neonates in NICUs to control the spread of these MDR isolates, particularly among preterm and low birth-weight infants. Indeed, there are many factors contributing to *Enterobacter* outbreaks. It was noted that human milk can also be involved in outbreaks of infection through milk sharing and contamination of equipment used in feed preparation, bottle warmers and milk collection equipment. A previous study reported that sepsis outbreaks in NICUs caused by *Enterobacter cloaceae* among new-borns was reported by Kose et al. (2016). Their study indicated that the source of the sepsis outbreak of *E. cloacae* is more likely to be the contaminated parenteral nutrition solution. Pulsed-field gel electrophoresis (PFGE) analysis revealed the same profile among the isolates identified in the blood cultures and samples of the parenteral solutions. Another study by Jackson (2015), indicated the first report of an *Enterobacter* species transmission from powdered infant formula (PIF) to patient. These strains were shown to belong to the same pulsotype and were characterised using multilocus sequence typing (MLST).

However, once the pathogenic bacteria are ingested, they are exposed to the acidity of the stomach. The ability of bacteria to persist in the human stomach is an opportunity that could allow these bacteria to invade the intestine, and consequently this can lead to human illness. In this study, all strains were tested under acidic pH conditions similar to those present in the gastrointestinal tract. The results showed the survival of these isolates and they were clearly resistant to exposure to pH 3.5 for 2 hours. The RpoS

sigma factor is the master regulator of the various stress responses in many Gram-negative bacteria (Lago et al., 2017). It is described by Stasic et al. (2012) to be essential to desiccation tolerance among *E. coli* strains. Similarly, Álvarez-Ordóñez et al. (2012) showed that RpoS is required for the tolerance of *Cronobacter* to osmotic, acidic and oxidative stresses. In this work, all *E. hormaechei* isolates were found to harbour this gene. Strain 664 and 1448 were shown to be the most acid-sensitive strains over the 2-h incubation period. Interestingly, the sequence of *rpoS* gene for these strains was different from other *rpoS* sequence of *E. hormaechei* strains. They clustered separately, as shown in Figure 4-12, indicating the differences within the *rpoS* gene. The analysis suggested a possibly positive correlation between bacterial acid tolerance and rpoS activity. These strains were regarded as acid-tolerant, and this feature will enhance such bacteria to pass through the stomach and into the intestines of the neonates. It could potentially contribute to an increased risk of these bacteria to colonise and infect the infants through toxin productions. As previously mentioned, these bacteria proved to have virulence factors such as bacterial haemolysins, protease and serum resistance, which may confer a higher potential risk to neonatal health.

Other factors may contribute to the ability of pathogens to establish persistent infections, such as environmental stresses. One of the most significant environmental stresses encountered by microorganisms is desiccation. A total number of 31 *Enterobacter* strains were evaluated for their desiccation survival in infant formula. The majority of these organisms have the ability to maintain their viability during 14 days of desiccation in infant formula and are efficiently able to recover after this period of time. Resistance to desiccation may contribute to the survival and persistence of this organism to maintain its viability under environmental pressure. This can be considered as a risk factor to neonates in intensive care units, particularly infants who are already at risk, such as those with low birth-weights and immune systems that have not fully developed. A previous observation by Barron and Forsythe (2007) showed that the *E. cloacae* strain ATCC 13047^{T} was recoverable after an 8-month survival period of desiccation. This result support the potential risk of infecting the neonates through the feeding of milk contaminated by *Enterobacter* strains, as originally proposed by FAO-WHO (2006).

Virulence factors such as attachment and invasion are also required for pathogenic strains of bacteria to cause disease, which enable the bacteria to colonise and persist in host cells (Pizarro-Cerdá and Cossart, 2006; Stones and Krachler, 2016). *E. hormaechei* is an opportunistic pathogen involved in nationwide outbreaks. It has been reported in several outbreaks of sepsis in NICUs (Townsend et al., 2008b; Paauw et al., 2009; Ohad et al., 2014). Therefore, I have decided to focus on *E. hormaechei* among strains collected as I mentioned previously, although *E. hormaechei* is associated with severe neonatal infections, there have been few in-depth studies published in the literature. Furthermore, it is a domain among strains collected in this study. For this reason, 12 of 21 *E. hormaechei* isolates from sepsis, CSF and neonatal feeding tubes were assessed for their virulence potential using human intestinal and brain barrier cell-lines, Caco-2 and HBMEC. This part of the study will lead to the discovery of the potential infection risk of *E. hormaechei* isolated from NEFTs in NICUs, when compared to CSF and sepsis *E. hormaechei* isolates.

All *E. hormaechei* strains showed an ability to attach to both Caco-2 and HBMEC cells at high and moderate levels indicating their virulence potential. This observation is in agreement with the results found by Krzymińska et al. (2010), who reported the ability of *E. hormaechei* and *E. cloacae* strains isolated from human specimens, which were able to adhere to and invade human epithelial type 2 (HEp-2) cells. Another study by a previous PhD student at NTU found that *E. hormaechei* isolated from feeding tubes were able to attach to Caco-2, HBMEC and rBCEC4 cells lines (Abudalla, 2014, unpublished data). Although some variations were observed among *E. hormaechei* in this study, it was interesting to note that feeding tube isolates showed no significant difference when compared with sepsis isolates for their ability to attach to Caco-2 ($P < 0.05$). Furthermore, these strains also showed no significant difference for their ability to attach to HBMEC when compared with some sepsis isolates, such as strains 2307, 460 and 1988.

Adhesion is commonly mediated by fimbriae. Bacterial fimbriae or pili are filamentous surface proteins that play a crucial role in the attachment to biotic and abiotic surfaces in both Gram-positive and Gram-negative bacteria (Chagnot et al., 2013; Berne et al., 2015). For example, curli fimbriae have been described to play a vital role in *E. coli* and

Salmonella for adhesion to the host cells. Their presence is linked with severe human infections (Cordeiro et al., 2016). In addition, flagellar biosynthesis-related proteins are generally recognised to play a role in colonisation and adhesion in pathogenic bacteria and are considered to be an important virulence factor (De Maayer and Cowan, 2016). A previous study by Du et al. (2016) reported the involvement of two flagellar biosynthesis genes (*flhA* and *fliR*) in the adhesion of *C. sakazakii*. These genes have also been detected in the genomes of examined *E. hormaechei* strains in this study.

Curli fimbriae have been reported to play a vital role in biofilm formation in *E. cloacae* (Kim et al., 2012). Although the role of curli fimbriae among *E. hormaechei* strains remains unclear, it has been suggested that may be have an important role in adhesion to host surfaces. Curli fimbrial genes *csgABCD* have been detected in the assessed *E. hormaechei* strains in this study (Figure 4-11). The CsgA protein is the major subunit of curlin, which has been linked to the ability of *E. hormaechei* to attach to Caco2 cell-lines. As shown in Figure 4-13, a positive correlation between *csgA* and adhesion ability of *E. hormaechei* strains was noted. The sequence analysis of this gene presented 2 clusters among *E. hormaechei* strains; a cluster with high levels of adherence and a cluster of moderate levels of attachment. One exception was strain 668 within the cluster of moderate attachment level which displayed high attachment. This might be because of the ability of this strain to produce high capsular material and high yields of cellulose, and thus result in high levels of attachment. However, the possibility of variations among these strains may explain the other bacterial traits were involved with the adhesion activity, or suggest that the expression of fimbrial genes were not completely expressed during the adhesion function. This requires further work to confirm whether these genes are differentially expressed by transcriptomic analysis studies.

Following attachment, invasion of mammalian cells can be induced by many pathogenic bacteria (Ribet and Cossart, 2015). Indeed, the ability of pathogenic bacteria for invasion of host cells such as brain endothelial cells is a concern (Townsend et al., 2008a), particularly if *E. hormaechei* strains are found to colonise NEFTs. Therefore, the virulence potential of *E. hormaechei* strains was investigated for their ability to invade

the Caco-2 and HBME cell-lines to better understand the pathogenic potential for these isolates.

The gentamicin protection assay showed that the *E. hormaechei* strains were able to invade the two cell-lines, indicating their virulence potential. However, outer membrane proteins have been reported to play a vital role in *C. sakazakii* to invade host eukaryotic cells. For instance, deletion mutants of *ompX* and *ompA* of *C. sakazakii* revealed significantly reduced invasion levels of Caco-2 cells (Kim et al., 2010). Another study had reported that the *ompX* deletion mutant in *E. coli* showed decreased adhesion to pulmonary epithelial A549 cells (Kim et al., 2010). These genes were also detected in the 12 *E. hormaechei* strains in this study and they may contribute to the invasion ability of these isolates.

Most of the strains tested showed moderate invasion of Caco-2. It was notable that the *E. hormaechei* strains shows higher invasion levels of Caco-2 than HBME cells, suggesting that the strains have the tendency to invade epithelial cells (Caco-2) more so than endothelial cells (HBMEC). This may indicate why *E. hormaechei* isolates are frequently isolated from blood and associated with sepsis instead of the CSF. The invasion results in this research showed lower levels of invasion of Caco-2 cell-lines compared with results of the previous study by Townsend et al. (2008b), particularly the *E. hormaechei-steigerwaltii* strain isolated from neonatal blood samples. The differences of the invasion experiments may be due to the variances in the growth conditions of the strains or the passage number of the cell-line used. This variation is not only due to experimental differences, but also possibility due to the variability of the demonstration and interpretation of the data analysis.

Some strains, such as 2315 and 664 showed high attachment to Caco-2 cells whilst strain 1439 showed high attachment to HBMEC cells. However, they demonstrated low invasion capabilities of these cell lines. These results suggest that there was no correlation between invasion and adhesion among *E. hormaechei* strains. *E. hormaechei* subsp. *steigerwaltii* strain 1977 (ST124) isolated from CSF showed the highest adherence and invasion of Caco-2 and HBMEC cell-lines among all strains. It may be that this strain associated with CSF is expected to have a high virulence potential

based on the location of isolation. However, it was notable that strain 2318 from feeding tubes revealed high invasion levels to Caco-2 when compared to sepsis isolates, such as strains 664, 2307 and 1439. Similarly, this strain also showed high invasion level to HBMEC in comparison with sepsis isolate 460 and 1439. Indeed, the ability of *E. hormaechei* subsp. *steigerwaltii* strain isolated from low birth-weight neonatal feeding tubes to adhere to and invade Caco-2 and HBMEC cell-lines may be a cause for concern. Particularly among neonates with low birth-weights representing a potential path for neonatal infections of the bloodstream following oral ingestion. Certainly, the interaction of feeding tube isolates with epithelial cells is an important initial signal for neonatal infections. It is believed that the ability of microbes to attach to host epithelial and endothelial cells leads to disease in their hosts (Ribet and Cossart, 2015).

This work has shown that feeding tube strains exhibited a similar behaviour to that observed in isolates from CSF and sepsis cases, since they have the ability to adhere and invade Caco-2 and HBMEC cell-lines and also share the same virulence genes. It is clear that they would have the ability to cause infections of neonates in NICUs. It is believed that sepsis is associated with *Enterobacter* spp., for instance, Chen et al. (2014) reported that *Enterobacter* species have appeared as significant pathogens of nosocomial bacteraemia in paediatric patients.

Adhesion is an important step to facilitate pathogens to colonise and cause infection (Soto and Hultgren, 1999). In order to get access to the bloodstream, bacteria must overcome the host defence system, including macrophages. In the human immune system, macrophages are the body's first line of defence against pathogenic bacteria. After invasion, many bacterial pathogens have the ability to use different strategies to survive and replicate in host cells (Ribet and Cossart, 2015). In this study, *E. hormaechei* strains (n = 12) were investigated for their ability to persist in human macrophages, using the U937 macrophage cell-line. The majority of these strains were able to persist and replicate inside macrophages for up to 72 hours of incubation. This could possibly be due to all strains possessing the *sodA* gene. This gene codes for a stress protein, which is involved in the protection of bacteria against phagocytosis. For example, in *C. sakazakii* this gene has been reported to be associated with macrophage

survival and encodes for superoxide dismutase (Townsend et al., 2007a). Similarly, another study revealed that *sodA* plays a role in the protection and survival of *Streptococcus agalactiae* in murine macrophages (Poyart et al., 2001).

Another phagocytosis resistance-associated gene *mazG* has been suggested to be essential for the survival of bacteria in macrophages. For example, deletion mutations of the *mazG* gene have reduced survival of *Mycobacterium tuberculosis* in macrophages, as described in a previous study (Lyu et al., 2013). Furthermore, the betaine aldehyde dehydrogenase (*betB*) gene, has also been suggested to affect the phagocytic pathway in human phagocytes, which is highly expressed during infections, (Lee et al., 2014). BLAST searching indicated that these genes were present in all tested isolates and could have a role in persistence and replication inside macrophages. However, in this study virulence-associated genes were notably shared by *E. hormaechei* isolated from sepsis, CSF and neonatal feeding tubes. Indeed, maintenance of the organism's viability within the macrophage suggests that these organisms may be reflective of the levels of pathogenicity in premature babies in NICUs. Although these strains were able to persist and replicate inside macrophages, this might induce apoptosis and may be considered as a contributing factor in disease development. The ability to survive inside macrophages can help bacteria to disseminate through the patient's body and giving the bacteria the opportunity to infect other organs. Thus, the effect of their ability on the viability of the epithelial Caco-2 cells was also assessed through mitochondrial activity, using the MTT assay.

This analysis revealed that all strains had demonstrated high cytotoxicity to Caco-2 cells. Interestingly, feeding tube strains showed a similar level of MTT reduction to some sepsis strains, such as 2307 and 664 (Figure 4-18). Cytotoxicity of intestinal epithelial cells may have significant implications for the evolution of infections. Due to the ability of feeding tube isolates to cause a cytotoxic effect, it can be expected that the pathogenic potential of these isolates would result in infection of neonates. Infants in the NICU are known to be at high risk of acquiring sepsis (Joseph et al., 2012; Soni and English, 2015; Dobrić et al., 2016). Neonatal sepsis is one of the important causes of mortality and morbidity among infants, particularly among low birth-weight and

preterm neonates (Cortese et al., 2016). *Enterobacter* spp. are one of the Gram-negative bacteria which play an important role in neonatal sepsis in NICUs (Spironello et al., 2015). *E. hormaechei*, in particular, has been shown to be of clinical significance within the genus *Enterobacter* by causing nosocomial infections, including sepsis (Townsend et al., 2008b). Furthemore, *E. hormaechei* is commonly the most isolated nosocomial pathogen among *Enterobacter* spp. (Paauw et al., 2008). In 1996, an outbreak of *E. hormaechei* infection and colonisation was reported by Wenger et al., (1997) where the outbreak occurred among low birth-weight premature infants in an NICU at the hospital of Pennsylvania. It was also reported by Paauw et al. (2010) that a nationwide outbreak occurred with due to an *E. hormaechei* outbreak strain in The Netherlands. This strain disseminated throughout hospitals and caused invasive infections in more than 100 patients.

This part of the study was undertaken to investigate the possible correlation between *E. hormaechei* isolated from sepsis and neonatal feeding tubes and how closely related they are. Also, it was essential to evaluate the potential risk to neonates acquiring organisms from enteral feeding tubes through ingestion of contaminated feed. This work has shown that feeding tube strains exhibited a similar behaviour to that observed in isolates from CSF and sepsis cases, as they have the ability to adhere and invade Caco-2 and HBMEC cell lines. It was interesting to note that feeding tube isolates showed no significant difference when compared with sepsis isolates for their ability to attach Caco-2. Also, these strains were able to persist and replicate inside macrophages for up to 72 hours of incubation and showed a similar level of MTT reduction to some sepsis strains. Other important observations included their ability to attach to and invade intestinal epithelial cells and persist inside macrophages. Feeding tube strains also shared the same virulence genes despite differing from each other by a very high number of SNPs (up to 66582 SNPs). It is clear that these strains would have the ability to cause infections of neonates in NICUs. In addition, the isolates in this study that were recovered from neonatal feeding tubes were defined as multidrug-resistant and showed an ability to form significant amounts of biofilm. These strains also showed the ability to tolerate acidic conditions, desiccation, and survive in human serum. It can be concluded these features could allow them to survive and facilitate their persistence and transmission

within the NICU. The results of this study indicate that these bacteria are potentially virulent and could present a significant risk to the health of premature neonates in the NICU.

Chapters 3 and 4 provided comprehensive details on the characterisation of the strains obtained from different hospitals at Nottingham, UK and Jordan. Different assays performed indicated the pathogenic potential of these isolates. The strains were linked with sepsis and NEFTs cases, however, these strains only provided the "snapshot" of single samples lacking descriptive metadata such as patient information. Furthermore, no follow up samples for these isolates were available, and no information regarding the persistence of these isolates whether in the NEFTs or intestinal tract of the babies, was available. The lack of this information made it extremely difficult to determine whether the strains that infected the babies originated from NEFTs. Therefore, in the next chapter, bacterial strains have been collected in paired neonatal feeding tubes and faeces. Chapter 5 describes a longitudinal study of twin babies with different feeding regimes, to compare potentially pathogenic isolates within and between twin pairs over time

Chapter 5. Longitudinal study in twin babies to compare *E. faecium* isolates within and between the feeding tubes and faeces over time.

5.1. Introduction

Preterm infants kept in intensive care units (ICUs), are considered as high-risk patients due to their increased susceptibility to infections whilst hospitalised (Ramasethu, 2017). Early exposure to harmful pathogens during the infant's hospitalisation is likely to influence the microbial colonisation in their gut. Commensal bacteria are derived from the surrounding environment such as nursing staff, other infants and medical equipment (Guaraldi and Salvator, 2012). The use of medical devices is one of the main clinical risk factors in neonatal infection, which includes nasogastric enteral feeding tubes (NEFTs). These tubes, which are often required to feed new-borns, may also contribute to colonisation by pathogenic microorganisms. (Hurrell et al., 2009a; Gómez et al., 2016).

In addition to contaminated medical equipment, infant feed such as reconstituted powdered infant formula (PIF) can also be considered as unsterile and a source of infection (Drudy et al., 2006). An example of such outbreaks was reported by Acker and colleagues (2001) in an NICU in Belgium, where preterm infants with low birth-weights were fed infant formula contaminated with *Cronobacter* spp. Recently, another outbreak was reported by the European Centre for Disease Prevention (ECDC) and Control and European Food Safety (EFSA) in 2018. This French outbreak affected 39 neonates and was associated with consumption of infant formula contaminated with *Salmonella* Agona.

Human milk may also be associated with contamination, although human milk provides complete enteral nutrition and contains phagocytes, secretory antibodies, and prebiotics which improve gastrointestinal function and host defence. However, human milk may also be associated with outbreaks of infection in NICUs through collecting, storing and handling or contamination of equipment (Engür et al., 2014; Kato et al., 2018). Indeed, the environmental contamination issue may pose a greater challenge in the NICU. Equipment and external surfaces in the NICU environment are

more likely to harbour a high prevalence of bacteria associated with nosocomial infection in neonates (Hewitt et al., 2013). For example, one of the most frequent nosocomial infections is neonatal sepsis, which has an increasing incidence in neonatal intensive care units, due to long periods of hospitalisation of infants (Polin and Saiman, 2003). Cross-transmission among neonates in the NICU may potentially occur due to prolonged hospitalisation. Transmission via the contaminated equipment play an important role in dissemination of the pathogen. For example. In 2005, Barwolff and co-workers demonstrated that the transmission rates of enterococcal isolates are high among nosocomial patients in ICUs, even higher than for *E. coli* and *S. aureus*. The colonisation of the enteral feeding tubes by nosocomial bacteria and their impact on earlier gut colonisation of the premature newborn babies was investigated in a recent study (Gomez et al., 2016). Their study indicated that *E. coli, K. pneumoniae, S. aureus, S. epidermidis, Serratia marcescens, E. faecium* and *E. faecalis* were the predominant isolates in faeces and milk samples amongst 4,000 bacterial strains recovered (Gomez et al., 2016). Another study by Alkeskas *et al*. (2015) revealed that meningitic *E. coli* K1 were recovered from multiple neonatal enteral feeding tubes from two local hospitals on NICUs, UK.

Nosocomial infections are caused by many microbes. Bacteria from the ESKAPE group are responsible for the majority of nosocomial infections (Navidinia, 2016). The ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species) are important causes of nosocomial and multidrug-resistant infections. *Enterococcus*, in particular, have been recognised among the most frequent causes of nosocomial infection (Santajit and Indrawattana, 2016; El-Mahallawy et al., 2016; Zhang et al., 2017a).

5.1.1. Characteristics of *Enterococcus* Genus

Enterococcus faecium and *Enterococcus faecalis* were split from the genus *Streptococcus* in 1984. The reclassification of this genus resulted in these species being assigned to a new genus and renamed to *Enterococcus faecium* and *Enterococcus faecalis* (Schleifer and Kilpper-Bälz, 1984). Subsequently, more than

forty species have been included and classified within the genus *Enterococcus* after the continuous use of new molecular technologies (Lebreton et al., 2014).

The *Enterococcus* species, which belong to the family Enterococcaceae, are facultatively anaerobic, Gram-positive cocci which grow as single, paired, or grouped cocci, or in short chains. They are catalase-negative bacteria that are non-spore forming and have a fermentative metabolism. They generally grow at temperatures of enterococci between 10 °C and 45 °C, and 37 °C is the optimum growth temperature. These bacteria are able to survive when exposed to a high pH, and they are highly tolerant to desiccation and can persist on dry surfaces for months (Lebreton et al., 2017). *Enterococcus* have been found to survive in a variety of environments and have been isolated from food, water, soil and plants. (Byappanahalli et al., 2012; Graves and Weaver, 2010). The *Enterococcus* genus are also commensal bacteria that inhabit the mammalian gut. They have also adapted to the oral cavity and vagina of humans (Fisher and Phillips, 2009).

Enterococcus species also belong to a group of lactic acid bacteria (LAB), which produce bacteriocins (Araújo and Ferreira, 2013). Some species have been used as probiotics in food and also used as supplement for poultry feed to replace the use of sub-lethal antibiotics. For example, Ghareeb et al. (2012) reported that use of probiotics, including *E. faecium*, reduced the colonisation of *C. jejuni* in broiler chickens via drinking water. Because of this, they were thought to be harmless to humans for a long time and were initially believed to be medically unimportant (Foulquie et al., 2006). Recently however, *E. faecium* and *E. faecalis* became the most common nosocomial pathogens reported to be associated with many infections and also carry antibiotic resistance factors. Various serious diseases can be attributed to enterococci such as neonatal infection, bacteraemia and urinary tract infection (O'Driscoll and Crank, 2015)

5.1.2. Clinical importance of *Enterococcus faecium*

Enterococcus species are widely spread in various environments and can be isolated from different sources, although they are most common among human-clinical and food isolates. Generally, enterococci are considered to be part of the

commensal flora within the gastrointestinal tract of humans and many animals (Santagati et al., 2012). Enterococci have emerged as important nosocomial pathogens within the past few decades. *E. faecium* and *E. faecalis* are recognised as important human pathogens and the most predominant enterococcal species in humans. These organisms are usually associated with diversity of clinical infections including urinary tract infection, intra-abdominal, pelvic, soft tissue infections and bacteraemia (Higuita and Huycke, 2014). The pathogenesis of enterococci has been attributed to a high degree of multidrug resistance, as well as various virulence factors such as adhesion, secreted agents and biofilm formation (Saffari et al., 2017).

The emergence of multidrug-resistant enterococci poses a problem with regards to treatment and is an important challenge for clinicians. The risk of contracting disease, such as bacteraemia, is increased by antibiotic-resistant enterococci colonisation (Lebreton et al., 2014; Tedim et al., 2015). One of the most common problems in hospitals is an increasing presence of vancomycin-resistant *Enterococcus faecium* (VREF) (Özsoy and İlki, 2017). This pathogen is proving problematic for many countries worldwide, especially developing countries that are generally associated with poor hospital hygiene practice. Furthermore, the ability of these bacteria to survive for prolonged periods of time may contribute to cross-contamination between patients (Sood et al., 2008; Lee et al., 2013), particularly in neonatal intensive care units (NICUs), where neonates are at high risk of nosocomial infection.

However, *E. faecium* clonal complex 17 (CC17) has received more attention as a successful nosocomial pathogen and hospital adapted strain. This clone appears to be a one of the universally epidemic clones of ampicillin-resistant *E. faecium* (AREF). It is associated with hospital outbreaks and clinical infections and is specified as a high risk enterococcal clone. In addition, it can acquire vancomycin resistance genes and other virulence factors (Werner et al., 2008; Hollenbeck and Rice, 2012; Garrido et al., 2014). A study by Willems (2005) conducted on 411 vancomycin-resistant enterococci (VRE) and vancomycin-susceptible *E. faecium* (VSE) strains, isolated from different continents, identified a genetic lineage of *E. faecium* (CC17). These strains were recovered from multiple sources including human, non-human and

hospital/clinical samples. This clone is characterised with acquisition of surface protein (Esp), which is located in a pathogenicity Island, ampicillin resistance and an association with outbreaks in hospitals. Figure 5-1, which was adapted from Top et al. (2008), confirms that the infections caused by ampicillin-resistant *E. faecium* (AREF) belonging to clonal complex CC17. The figure shows eBURST clustering of 331 MLST profiles representing 855 *E. faecium* isolates from the MLST database. It indicates the sources of specific clusters of STs, including CC17 comprising hospital outbreaks and clinical isolates and ST-80 which is the focus of this chapter (see 5.3.3 below).

Figure 5-1 eBURST clustering of 331 MLST profiles including ST-80, representing 855 E. faecium isolates from the database (www.mlst.net).

Dashed lines indicate the sources of specific clusters of STs, including CC17 comprising hospital outbreaks and clinical isolates. Figure adapted from Top et al. (2008).

5.1.3. Virulence Factors in *E. faecium*

Several virulence factors exist which contribute to the ability of enterococci to persist in the host and cause disease. The most distinguished of the enterococcal virulence factors are the surface adhesions proteins such as enterococcal surface protein (Esp). This protein has been shown to contribute to adherence and biofilm formation in *E. faecium*. In addition, it was found to be associated with nosocomial outbreaks and specifically enriched among hospital associated isolates. (Leavis et al., 2004; Soares et al., 2014; Biswas et al., 2016). Other virulence factors are the Ebp pili, which are important for adherence and play a significant role in biofilm formation. Ebp pili are encoded by the *ebp* operon which consists of *ebpA, ebpB* and *ebpC* (Sillanpaa et al., 2010; Sillanpaa et al., 2013). Adhesion-associated protein EfaA has been also identified in *E. faecium*, which believed to be involved in cell wall adherence (Soheili et al., 2014). Additional virulence genes harboured by *E. faecium* strains are associated with human infections such as *gelE, asa1, cylA*, and *hyl*, (Ferguson et al., 2016).

However, enterococci usually have either intrinsic resistance or acquired resistance to multiple antimicrobial agents and most of the antibiotics used in humans (Ünal et al., 2017). Indeed, there is concern in hospital settings due to resistance to several antimicrobial agents commonly used. Enterococci are resistant to numerous classes of antibiotics, including β-lactams and aminoglycosides (Faron et al., 2016). They are also able to acquire resistance to vancomycin, which is observed more frequently in *E. faecium* among enterococcal species.

Vancomycin resistant *E. faecium* VREfm is an important health concern and is a globally disseminated pathogen. Infections caused by vancomycin-resistant enterococci (VRE) are associated with higher mortality and there could be limited treatment options (DiazGranados and Jernigan, 2005; Cheah et al., 2013). However, since the 1980s ampicillin and vancomycin are the most significant resistance traits known to have been acquired by *E. faecium* (Cetinkaya et al., 2000; Arias and Murray, 2012). Ampicillin-resistant *E. faecium* isolates have been reported by Lester and colleagues in 2008 and that these isolates are increasing in numbers in many European hospitals. (Lester et al., 2008). In one study, it was reported that *E. faecium* increased at least from 1.4% in 2001 to 4.3% in 2014 in European hospitals, while in the USA the rates also increased from 3.0% in 2001 to 5.4% in 2010 (Mendes et al., 2016).

Premature, low birth weight and immunodeficient infants are usually admitted for prolonged periods in the NICU. They are exposed to multidrug-resistant organisms that have readily adapted to the healthcare setting and hospital environment. The majority of these organisms are high risk clones belonging to the nosocomial ESKAPE pathogens group (Santajit and Indrawattana, 2016; Navidinia, 2016). Furthermore, these preterm infants are also colonised by pathogenic bacteria recovered from the mother and the environment. Sepsis is one of the main causes of death in the NICU; risk factors for late-onset sepsis (LOS) include immaturity, mechanical ventilation, and intravascular catheters (Dong and Speer, 2015).

Additionally, nasogastric feeding tubes are reported to act as a locus for bacterial attachment and proliferation of several opportunistic pathogens, and this is

suggested to be a significant risk factor in the neonatal infections (Hurrell et al., 2009a; b). On the other hand, powdered infant formula (PIF) and mother's milk or donor human milk may be associated with contamination. For instance, *Cronobacter* species have been recovered from powdered infant formula (PIF), which is of serious concern for neonatal health (Craven et al., 2010; Fei et al., 2017). Bacteria may contaminate donor human milk due to different means such as through storage, during collection, or during processing (Froh et al., 2018).

Biofilms represent another virulence factor which may contribute to bacterial pathogenicity. For instance, adhesion is a necessary step for biofilm formation, which allows bacteria to bind to urinary and intravascular catheters and other devices. Furthermore, biofilms contribute to bacterial protection from killing by antibiotics and phagocytosis. In enterococcal strains, for example, biofilm formation is associated with antimicrobial resistance and it is dependent on complex virulence factors (Lee, 2017). The ability of the bacteria to form biofilms on medical devices including catheters and enteral feeding tubes may result in infection of preterm infants in the NICU. Preterm infants are therefore of particular concern, especially those born with low birth weight and underdeveloped immune systems fed via a nasogastric tube.

5.1.4. Aim and objectives

Persistence of bacterial biofilms inside nasogastric feeding tubes has been associated with the presence of infectious organisms. Preterm infants are at particularly high risk of acquiring infection because their immune systems are underdeveloped. In addition, cross-transmission among neonates in the NICU may occur due to prolonged hospitalisation. For this reason, it was decided to characterise these strains, which have been collected from paired neonatal feeding tubes and faeces. The acquisition of the collection of strains assessed in this study represented a unique opportunity to investigate pathogens that colonise neonates over time and provide answers to some of the key issues that are central to this thesis, to help inform and improve pathogen control measures in NICUs in the future. This study is focused on longitudinal study for twin babies aimed to compare potentially

pathogenic isolates within and between the feeding tubes and faeces of two twin babies over time. This will be achieved according to the following objectives:

- Whole-genome sequencing of *E. faecium* isolates and SNP analysis based on the core genome, in order to determine the relatedness of strains isolated from feeding tubes and faeces from both babies over time. Additionally, to determine whether some of the strains might have transferred between the two babies
- Genomic analysis of *E. faecium* isolates to determine the presence or absence of virulence and antibiotic resistance genes.
- Characterisation of a range of physiological traits, such as capsule and biofilm formation, serum resistance and acid tolerance to evaluate the possible risk to neonates acquiring these organisms through feeding tubes.
- *In vitro* virulence characterisation of *E. faecium* isolates using epithelial cell line Caco-2, to determine bacterial attachment and invasion abilities.

5.2. Materials and Methods

The methodology of this chapter is described in detail in Chapter 2 (Section 2). The samples had been collected by Pauline Ogrodzki from two premature babies (twins) from the neonatal intensive care unit of Queens Medical Centre (Table 5-1). The patients were born premature at 25 weeks and 5 days gestation. The first patient was a low-birth weight (660 g) preterm male neonate and the second preterm male was also low-birth weight (880 g). The samples were collected from feeding tubes and faecal samples from both neonates at different times. The first sample was collected at 4 weeks of age and the last sample was collected at 13 weeks. The neonates had been fed breast milk, fortified and pre-med formula during this period of time. However, because of the ethical considerations, storage of the original faecal material collected from the two premature babies was not permitted. Therefore, feeding tube and faeces samples were incubated in Brain Heart Infusion (BHI) growth medium at 37 °C for 24h. The samples were then mixed with 20% glycerol and stored in 1.5 ml aliquots at -80°C until required for analysis. Differential media were used in the present study for identification of these isolates, in order to distinguish between

isolates as an initial presumptive identification. Brain heart infusion agar (BHIA) was used for general bacteria and Violet Red Bile Glucose Agar (VRBGA) for Enterobacteriaceae. In addition, De Man, Rogosa, Sharpe Agar (MRSA) and Bifidus Selective Media Agar (BSMA) were used for Lactic acid bacteria and Bifidobacteria.

The provisional identification was performed according to their morphological appearance, Gram stain, catalase and oxidase reactions. The isolated organisms were identified using 16S rRNA sequencing. However, among these isolates, a total of 24 *E. faecium* isolates were used in this study, as shown in Table 5.1, selected as representative strains and subjected to whole-genome sequencing by Pauline Ogrodzki using the Illumina MiSeq sequencer. All these isolates were analysed to identify the presence and absence of different virulence genes potentially associated with colonising the host. Furthermore, a range of phenotyping methods were applied such as those for biofilm formation, capsule production, acid tolerance, and serum resistance. Moreover, evaluating the potential virulence of these strains using *in vitro* attachment and invasion assays with intestinal cells lines (Caco-2).

Table 5-1 *E. faecium* strains used in this study.

Strain	Neonate	Collection date	Sample source	Organism
2367	1	23/07/15	Faecal	*E. faecium*
2368	1	10/08/15	Faecal	*E. faecium*
2372	1	24/08/15	Faecal	*E. faecium*
2375	1	15/09/15	Faecal	*E. faecium*
2376	1	24/09/15	Faecal	*E. faecium*
2379	1	20/10/15	Faecal	*E. faecium*
2382	1	22/10/15	Faecal	*E. faecium*
2393	1	12/08/15	NG tube	*E. faecium*
2395	1	19/08/15	NG tube	*E. faecium*
2397	1	08/09/15	NG tube	*E. faecium*
2399	1	27/09/15	NG tube	*E. faecium*
2401	1	01/10/15	NG tube	*E. faecium*
2407	2	31/07/15	Faecal	*E. faecium*
2409	2	16/08/15	Faecal	*E. faecium*
2410	2	20/08/15	Faecal	*E. faecium*
2413	2	10/09/15	Faecal	*E. faecium*
2417	2	30/09/15	Faecal	*E. faecium*
2418	2	16/10/15	Faecal	*E. faecium*
2425	2	20/10/15	Faecal	*E. faecium*
2430	2	16/07/15	NG tube	*E. faecium*
2433	2	17/08/15	NG tube	*E. faecium*
2436	2	08/09/15	NG tube	*E. faecium*
2439	2	12/10/15	NG tube	*E. faecium*
2462	2	11/10/15	NG tube	*E. faecium*

5.3. Results

5.3.1. Isolation and identification of bacterial strains

In general, samples were streaked onto differential media to give an initial presumptive identification by distinguishing visually by the appearance of the colony. The majority of these isolates were revealed as Gram-positive cocci and few isolates were identified as Gram-negative. These isolates were subjected to 16S rRNA sequencing and were identified as *E. faecium*, *S. aureus, E. faecalis, S. haemolyticus, S. epidermidis, Streptococcus, K. pneumoniae* and *K. oxytoca* as shown in Table 5-2. The most common species among the isolated strains was *E. faecium*. This species

represented 30% of isolates, which were recovered from feeding tubes (n=10) and from faecal samples (n=14). *S. aureus* was the next most common species at 20% (9 isolates were recovered from feeding tubes and 7 isolates from faecal samples). *E. faecalis* was also identified among the isolates at 15% prevalence; 7 strains were recovered from feeding tubes and 5 strains from faecal samples. In addition, 11.3% *S. haemolyticus* (n=9), 10% *S. epidermidis* (n=8), 7.5% *Streptococcus* (n=6), 3.8% *K. pneumoniae* (n=2) and 2.5% *K. oxytoca* (n=2) were also identified (Figure 5-2).

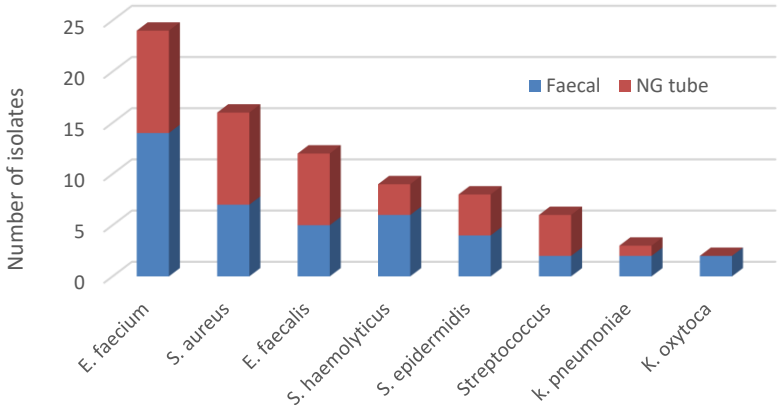


Figure 5-2 Species identity of clinical strains collected from neonatal gastric feeding tubes and faecal samples, expressed as the total number strains identified at each site. The most commonly isolated species was *E. faecium*.

Table 5-2 Biochemical tests and 16S rRNA of *E. faecium* strains

Strain	Neonate	Collection date	Sample source	Gram stain	Oxidase	Catalase	16S rRNA	Feeding regime
2367	1	23/07/15	Faecal	G +ve	- ve	-ve	*E. faecium*	
2368	1	10/08/15	Faecal	G +ve	- ve	-ve	*E. faecium*	
2372	1	24/08/15	Faecal	G +ve	- ve	-ve	*E. faecium*	
2375	1	15/09/15	Faecal	G +ve	- ve	-ve	*E. faecium*	
2376	1	24/09/15	Faecal	G +ve	- ve	-ve	*E. faecium*	
2379	1	20/10/15	Faecal	G +ve	- ve	-ve	*E. faecium*	
2382	1	22/10/15	Faecal	G +ve	- ve	-ve	*E. faecium*	pre-made formula
2393	1	10/08/15	NG tube	G +ve	- ve	-ve	*E. faecium*	+
2395	1	19/08/15	NG tube	G +ve	- ve	-ve	*E. faecium*	breast milk
2397	1	08/09/15	NG tube	G +ve	- ve	-ve	*E. faecium*	+
2399	1	27/09/15	NG tube	G +ve	- ve	-ve	*E. faecium*	fortifier
2401	1	01/10/15	NG tube	G +ve	- ve	-ve	*E. faecium*	
2407	2	31/07/15	Faecal	G +ve	- ve	-ve	*E. faecium*	
2409	2	16/08/15	Faecal	G +ve	- ve	-ve	*E. faecium*	
2410	2	20/08/15	Faecal	G +ve	- ve	-ve	*E. faecium*	
2413	2	10/09/15	Faecal	G +ve	- ve	-ve	*E. faecium*	
2417	2	30/09/15	Faecal	G +ve	- ve	-ve	*E. faecium*	
2418	2	16/10/15	Faecal	G +ve	- ve	-ve	*E. faecium*	
2425	2	20/10/15	Faecal	G +ve	- ve	-ve	*E. faecium*	
2430	2	16/07/15	NG tube	G +ve	- ve	-ve	*E. faecium*	
2433	2	17/08/15	NG tube	G +ve	- ve	-ve	*E. faecium*	
2436	2	08/09/15	NG tube	G +ve	- ve	-ve	*E. faecium*	
2439	2	11/10/15	NG tube	G +ve	- ve	-ve	*E. faecium*	
2462	2	12/10/15	NG tube	G +ve	- ve	-ve	*E. faecium*	

Genomic DNA was extracted from these isolates using the GenElute™ Bacterial Genomic DNA Kit (Sigma Aldrich). The Illumina Miseq reagent kit was used for Whole-genome sequencing. A core-genome alignment was constructed and the CSI Phylogeny program was used to identify Single nucleotide polymorphisms (SNPs) to confirm the speciation of the *E. faecium* strains (Figure 5-3). As mentioned in an earlier chapter, Pauline Ogrodzki (a member of our research group at NTU), carried out the whole-genome sequencing and SNP analysis.

5.3.1.1. Multi locus sequence typing (MLST)

Traditionally, MLST was achieved by PCR amplification of seven housekeeping genes based on the MLST scheme database and followed by Sanger sequencing. In the present study, the genetic diversity of different sequence types (ST) of *E. faecium* strains was analysed to compare the isolates obtained from different sites. These strains were isolated from feeding tubes and faecal samples during a 3-month period collected from two premature babies in the NICU. The sequence types were identified using whole-genome sequence (WGS) data and the MLST web-based

service of the Center for Genomic Epidemiology. The database showed fragments of seven housekeeping genes for twenty-four *E. faecium* strains were unique and they all belonged to the same ST-80, as shown in Table 5-3. However, all strains were typed as ST80 belonging to clonal complex (CC17). These results indicate that these isolates from different sites are clonally identical and transfer between two premature neonates in the NICU may have occurred.

Table 5-3 MLST profile of 24 representative strains of *E. faecium* isolated from feeding tubes and faecal samples from two premature babies in an NICU. The results indicate that all isolates were ST-80 and belonged to clonal complex (CC17).

Isolate	Neonate	Sample Source	Isolation date	adk	atpa	addi	gdh	gyd	psts	purk	Sequence Type	Clonal complex
2367	1	Faecal	23/07/15	1	9	1	1	12	1	1	ST-80	CC17
2368			10/8/2015	1	9	1	1	12	1	1	ST-80	CC17
2372			24/08/15	1	9	1	1	12	1	1	ST-80	CC17
2375			15/09/15	1	9	1	1	12	1	1	ST-80	CC17
2376			24/09/15	1	9	1	1	12	1	1	ST-80	CC17
2379			20/10/15	1	9	1	1	12	1	1	ST-80	CC17
2382			22/10/15	1	9	1	1	12	1	1	ST-80	CC17
2393		NG tube	12/08/15	1	9	1	1	12	1	1	ST-80	CC17
2395			20/08/15	1	9	1	1	12	1	1	ST-80	CC17
2397			08/09/15	1	9	1	1	12	1	1	ST-80	CC17
2399			01/10/15	1	9	1	1	12	1	1	ST-80	CC17
2401			1/10/15	1	9	1	1	12	1	1	ST-80	CC17
2407	2	Faecal	31/07/15	1	9	1	1	12	1	1	ST-80	CC17
2409			16/08/15	1	9	1	1	12	1	1	ST-80	CC17
2410			20/08/15	1	9	1	1	12	1	1	ST-80	CC17
2413			10/9/2015	1	9	1	1	12	1	1	ST-80	CC17
2417			30/09/15	1	9	1	1	12	1	1	ST-80	CC17
2418			16/10/15	1	9	1	1	12	1	1	ST-80	CC17
2425			20/10/15	1	9	1	1	12	1	1	ST-80	CC17
2430		NG tube	19/07/15	1	9	1	1	12	1	1	ST-80	CC17
2433			20/08/15	1	9	1	1	12	1	1	ST-80	CC17
2436			10/09/15	1	9	1	1	12	1	1	ST-80	CC17
2439			12/10/15	1	9	1	1	12	1	1	ST-80	CC17
2462			11/10/15	1	9	1	1	12	1	1	ST-80	CC17

The MLST scheme is based on seven housekeeping genes - adenylate kinase (*adk)*, ATP synthase alpha subunit (*atpA*), D-alanine:D-alanine ligase (*ddl*), glyceraldehyde-3-phosphate dehydrogenase (*gyd*), glucose-6 phosphate dehydrogenase (*gdh*), phosphor ribosyl aminoimidazol carboxylase ATPase subunit (*purK*),and phosphate ATP-binding cassette transporter *(pstS).*

5.3.1.2. Core genome phylogenetic analysis of *E. faecium*

Whole-genome sequencing of *E. faecium* isolates was performed using the Illumina Miseq as mentioned earlier and a maximum likelihood phylogeny of the 24 *E. faecium* strains was constructed from a core genome alignment. The CSI Phylogeny program was used to identify SNPs. In the present study, twenty-four *E. faecium* isolates were

analysed based on core genome (Figure 5-3). All of these isolates belong to ST80 based on sequences of the conserved regions of the seven housekeeping genes of *E. faecium* MLST scheme.

Whole genome sequence data revealed a number of SNP differences ranging from 12 to 287 SNPs between the isolates from the two premature twin babies in the NICU. For instance, strain 2393 was distant to the strain 2397 by 75 SNPs, both strains were isolated from baby 1 from feeding tube samples taken four weeks apart. Whereas, the two isolates 2433 and 2395 differed from each other by a very small of SNPs (12), and these 2 strains were isolated from feeding tubes from neonates 1 and 2 at different time points 3 days apart. This might be explained by the transfer of the strain from baby 2 to baby 1. Similarly, strain 2425 and 2367 from faecal samples from different neonates, with 15 SNPs difference across a 12- weeks time period. Strains 2436 and 2417 which differed by only 21 SNPs, were isolated from infant 2 at different neonatal sites, feeding tube and faecal sample during a 3-week period. The interesting observation that the first strain 2430 isolated from baby 2 feeding tube on 16-07-2015 differed from the last isolate 2425 by 23 SNPs collected on 20-10-2015 from baby 2 faecal sample. This may indicate that the same strain could persist in the baby's intestinal tract over this time. Another important observation is that the same strain 2430 differed from strain 2439 by 23 SNPs, which was isolated after at least 11 weeks. The other two isolates 2413 and 2425 were obtained from neonate 2 faecal sample at 5 weeks. These strains had a maximum of 17 SNPs difference. The outlying single isolate 2409 clustered alone with a high SNPs difference of 287 SNPs compared with strain 2376. Suggesting that they were acquired independently of each other.

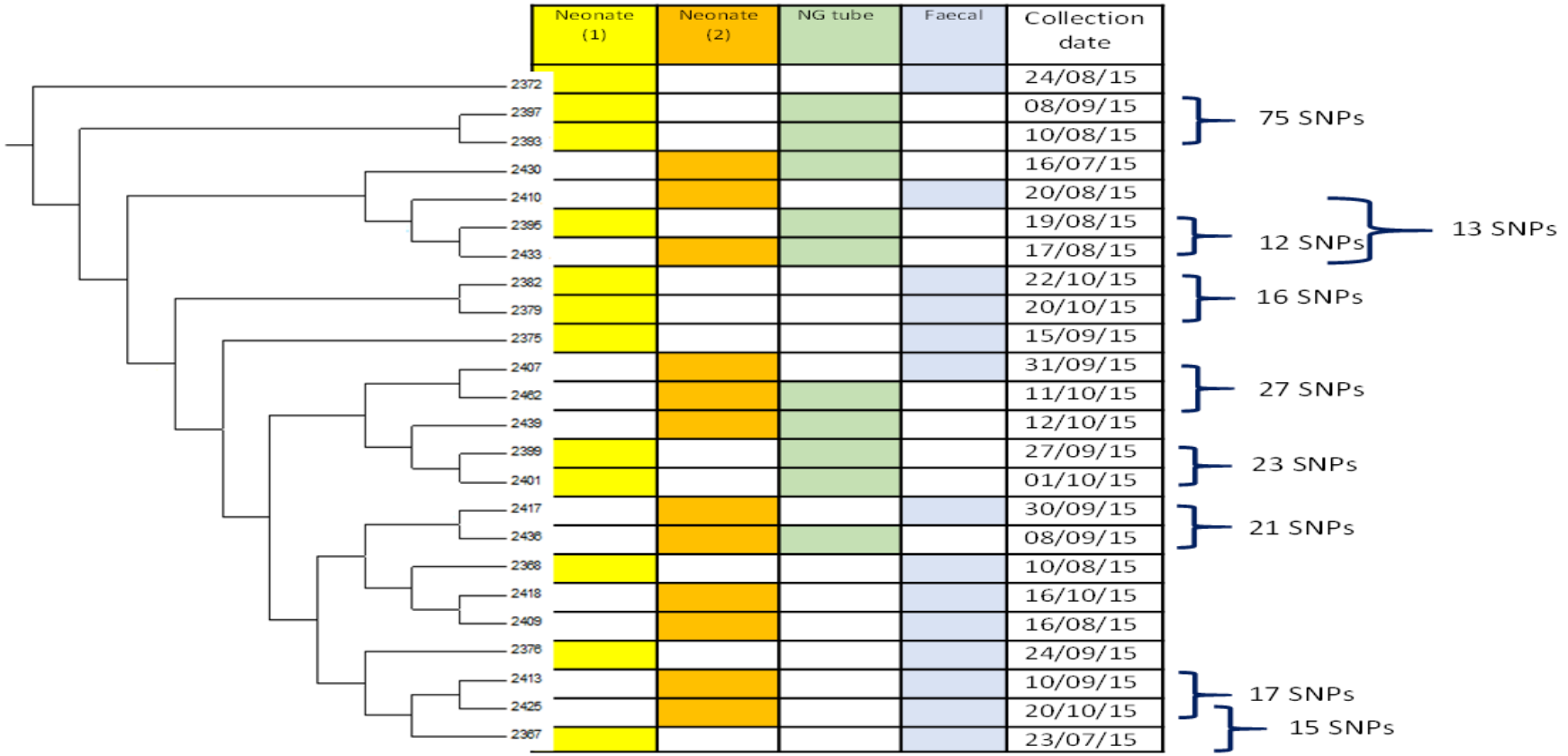


Figure 5-3 Single nucleotide polymorphism analysis tree of *E. faecium* isolates

E. faecium isolates collected from feeding tubes and faecal samples from twins in the NICU of QMC during different time periods are represented in different colours. Yellow indicates baby 1, orange colour indicates baby 2, while green indicates bacteria detected in feeding tubes and blue represents bacteria detected in faeces of the infant.

5.3.1.3. 5.3.5 Screening of *E. faecium* for virulence factors

Genome screening was performed to examine the presence and absence of selected genes that may contribute to virulence or physiological features. Table 5-4 shows a list of the proposed virulence genes classified into the major virulence factors of *E. faecium* strains, including a gene encoding enterococcal surface protein (Esp), which is located on a pathogenicity island and is involved in biofilm formation and contributes to pathogenesis (Top et al., 2013; Zou and Shankar, 2016;). Gene *gltK*, encoding a putative glutamate transport system permease protein, was identified in high prevalence and may contribute to bile resistance in *E. faecium* (Zhang et al., 2013b). It well known that bacteria that colonise the human intestinal tract must be able to tolerate bile in order to survive. However, other virulence genes were also noted in this strain such as the *acm* gene, which confers collagen-binding adhesin properties of *E. faecium* and contributes to the pathogenesis in experimental endocarditis. In addition, EcbA, *E. faecium* collagen-binding protein A, encodes for fibrinogen and recognises adhesive matrix molecules. Furthermore, the *efaA* gene was hypothesised and believed to be involved in cell wall adherence (Sava et al., 2010; Soheili et al., 2014; Yang et al., 2016). Additionally, other virulence genes were observed among *E. faecium* strains, as shown in Table 5-4. Further details regarding these genes are provided in the Discussion section (5.4).

5.3.1.4. 5.3.6 Plasmids and resistance genes

All isolates were screened for identifying plasmids among 24 *E. faecium* isolated from feeding tubes and faecal samples from two premature babies in the NICU. These isolates were analysed using the PlasmidFinder database and were BLAST'd against the assembled genomes. All these strains were found to carry multiple plasmids. Six different rep-family plasmids were detected among 24 *E. faecium* isolates (*repUS15, rep11, rep17, rep2, rep1* and *rep21*). The predominant rep-family, *repUS15* and *rep2* were identified in all *E. faecium* isolated in this study. Other rep-families, *rep11* and *rep17*, were also observed among all *E. faecium* isolates except strain 2372 isolated from infant 1, which do not carry *rep17*. *rep11* was not detected only in strain 2409 isolated from infant 2. In contrast, *rep1* and *rep21* are rare plasmids found among *E. faecium*

isolates. *rep21* was detected only in two isolates 2425 and 2436 were isolated from infant 2. *rep1* was found only in three other strains, 2395 isolated from infant 1 and strains 2410 and 2433 isolated from infant 2 (Table 5-5).

However, all of these *E. faecium* isolates were also investigated for the presence of the antimicrobial resistance genes. The antibiotic resistance genes were identified using ResFinder 2.1 software by the Center for Genomic Epidemiology website (https://cge.cbs.dtu.dk/services/ResFinder/). Two genes encoding resistance to aminoglycosides were identified among all *E. faecium* isolated. For instance, aminoglycoside resistance gene, *aph(3')-III ant(6)-Ia* was shared among all of *E. faecium* isolates, the exception was strain 2417 which lacked this particular resistance gene. In contrast, only two strains 2410 and 2433 isolated from infant 2 were found to harbour the second aminoglycoside gene *aac(6')-aph(2'').* The other four encountered genes, coding for macrolide resistance - that is, *msr*(C) and *erm*(B) - all isolates possessed these genes except for strain 2417 which lacked *erm*(B). While *mph*(C) and *vat*(B) were not detected among all isolates evaluated, this excluded strain 2433, which was positive for *mph*(C) and only strain 2436 was positive for *vat*(B). Both these strains were isolated from the feeding tube from infant 2 after a 3-week period. However, tetracycline resistance gene *tet*(S) was present in all isolates and fluoroquinolone *nor*(A) was harboured by only two strains 2462, which was isolated from the feeding tube of infant 2 and strain 2375 isolated from infant 1 faecal sample, as shown in Table 5-5.

5.3.2. Antimicrobial Susceptibility

Antibiotic susceptibility profiles were determined for the 24 *E. faecium* strains. This involved screening the sensitivity to 9 different antibiotic groups: penicillins, aminoglycosides, carbapenems, cephalosporins, chloramphenicol, tetracycline, macrolide, glycopeptides and trimethoprim. The antibiogram (Table 5-6) was generated using the Kirby-Bauer disk diffusion method. All 24 *E. faecium* isolates were susceptible to vancomycin, gentamicin and doxycycline, but were found to be highly resistant to most antibiotics tested in this study. This study determines that all of the *E. faecium* isolates were multidrug resistant and exhibited similar levels of resistance

relative to each other. Among the 24 *E. faecium* isolates, 9 different antibiotic resistance genes were detected including aminoglycoside, macrolide tetracycline resistance genes.

Table 5-4 shows the results of the genomic comparison of 24 *E. faecium* strains recovered from feeding tubes and faecal samples from two premature babies in NICU.

				Virulence genes															
Isolate	**Neonate**	**Sample Source**	**Isolation date**	*acm*	*ebpA*	*ebpB*	*ebpC*	*srtC*	*ecbA*	*efaA*	*esp*	*sqrA*	*manY_2*	*pyrF*	*purD*	*pyrF*	*pyrK_2*	*Cyl1,2*	*cylABM*	*gltK*
2372	1	Faecal	24/08/15	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+
2397	1	NG tube	08/09/15	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+
2393	1	NG tube	12/08/15	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+
2430	2	NG tube	16/07/15	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+
2410	2	Faecal	20/08/15	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+
2395	1	NG tube	19/08/15	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+
2433	2	NG tube	17/08/15	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+
2382	1	Faecal	22/10/15	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+
2379	1	Faecal	20/10/15	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+
2375	1	Faecal	15/09/15	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+
2407	2	Faecal	31/09/15	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+
2462	2	NG tube	11/10/15	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+
2439	2	NG tube	12/10/15	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+
2399	1	NG tube	27/09/15	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+
2401	1	NG tube	01/10/15	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+
2417	2	Faecal	30/09/15	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+
2436	2	NG tube	08/09/15	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+
2368	1	Faecal	10/8/2015	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+
2418	2	Faecal	16/10/15	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+
2409	2	Faecal	16/08/15	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+
2376	1	Faecal	24/09/15	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+
2413	2	Faecal	10/9/2015	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+
2425	2	Faecal	20/10/15	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+
2367	1	Faecal	23/07/15	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+

The results indicate that all isolates harboured a number of virulence-associated genes. (+) = present, (-) = absent.

Table 5-5 shows the results of plasmids and antibiotic resistance gene profiles of 24 *E. faecium* strains recovered feeding tubes and faecal samples from twines in NICU at QMC.

Isolate	Neonate	Sample Source	Isolation date	Plasmid						Beta-lactam	Aminoglycoside		MLS - Macrolide				Tetracy-cline	Fluoroqu-inolone
				repUS15	Rep11	Rep17	Rep2	Rep1	Rep21	*blaZ*	*aac(6')-aph(2'')*	*aph(3')-III ant(6)-Ia*	*msr(C)*	*erm(B)*	*mph(C)*	*vat(B)*	*tet(S)*	*norA*
2372	1	Faecal	24/08/15	+	+	-	+	-	-	-	-	+	+	+	-	-	+	-
2397	1	NG tube	08/09/15	+	+	+	+	-	-	-	-	+	+	+	-	-	+	-
2393	1	NG tube	12/08/15	+	+	+	+	-	-	-	-	+	+	+	-	-	+	-
2430	2	NG tube	16/07/15	+	+	+	+	-	-	-	-	+	+	+	-	-	+	-
2410	2	Faecal	20/08/15	+	+	+	+	+	-	-	+	+	+	+	-	-	+	-
2395	1	NG tube	19/08/15	+	+	+	+	+	-	-	-	+	+	+	-	-	+	-
2433	2	NG tube	17/08/15	+	+	+	+	+	-	+	+	+	+	+	+	-	+	-
2382	1	Faecal	22/10/15	+	+	+	+	-	-	-	-	+	+	+	-	-	+	-
2379	1	Faecal	20/10/15	+	+	+	+	-	-	-	-	+	+	+	-	-	+	-
2375	1	Faecal	15/09/15	+	+	+	+	-	-	-	-	+	+	+	-	-	+	+
2407	2	Faecal	31/09/15	+	+	+	+	-	-	-	-	+	+	+	-	-	+	-
2462	2	NG tube	11/10/15	+	+	+	+	-	-	+	-	+	+	+	-	-	+	+
2439	2	NG tube	12/10/15	+	+	+	+	-	-	-	-	+	+	+	-	-	+	-
2399	1	NG tube	27/09/15	+	+	+	+	-	-	-	-	+	+	+	-	-	+	-
2401	1	NG tube	01/10/15	+	+	+	+	-	-	-	-	+	+	+	-	-	+	-
2417	2	Faecal	30/09/15	+	+	+	+	-	-	-	-	-	+	-	-	-	+	-
2436	2	NG tube	08/09/15	+	+	+	+	-	+	-	-	+	+	+	-	+	+	-
2368	1	Faecal	10/8/2015	+	+	+	+	-	-	-	-	+	+	+		-	+	-
2418	2	Faecal	16/10/15	+	+	+	+	-	-	-	-	+	+	+	-	-	+	-
2409	2	Faecal	16/08/15	+	-	+	+	-	-	-	-	+	+	+	-	-	+	-
2376	1	Faecal	24/09/15	+	+	+	+	-	-	-	-	+	+	+	-	-	+	-
2413	2	Faecal	10/9/2015	+	+	+	+	-	-	-	-	+	+	+	-	-	+	-
2425	2	Faecal	20/10/15	+	+	+	+	-	+	-	-	+	+	+	-	-	+	-
2367	1	Faecal	23/07/15	+	+	+	+	-	-	+	-	+	+	+	-	-	+	-

(+) = present, (-) = absent.

Table 5-6 Sensitivity for E. faecium strains to antibiotic agents.

Isolate	Neonate	Sample Source	Carbapenems		Cephalosporins				Aminoglycosides		Tetra	Fluoroqu	Glycoope	Penicillins	Macrolide	ESBL	
			IPM 10 µg	MEM 10 µg	ZOX 30µg	CAZ 30µg	CRO 30µg	CFP 75µg	GM 10 µg	TOB 10 µg	DO 30µg	CIP 5µg	VA 5µg	AMP 10 µg	CLR 15µg	CTX+CV 30/10µg	CAZ+CV 10/1µg
2367	1	Faecal	R	R	R	R	R	R	S	R	S	R	S	R	R	R	R
2368			R	R	R	R	R	R	S	R	S	R	S	R	R	R	R
2372			R	R	R	R	R	R	S	R	S	R	S	R	R	R	R
2375			R	R	R	R	R	R	S	R	S	R	S	R	R	R	R
2376			R	R	R	R	R	R	S	R	S	R	S	R	R	R	R
2379			R	R	R	R	R	R	S	R	S	R	S	R	R	R	R
2382			R	R	R	R	R	R	S	R	S	R	S	R	R	R	R
2393		NG tube	R	R	R	R	R	R	S	R	S	R	S	R	R	R	R
2395			R	R	R	R	R	R	S	R	S	R	S	R	R	R	R
2397			R	R	R	R	R	R	S	R	S	R	S	R	R	R	R
2399			R	R	R	R	R	R	S	R	S	R	S	R	R	R	R
2401			R	R	R	R	R	R	S	R	S	R	S	R	R	R	R
2407	2	Faecal	R	R	R	R	R	R	S	R	S	R	S	R	R	R	R
2409			R	R	R	R	R	R	S	R	S	R	S	R	R	R	R
2410			R	R	R	R	R	R	S	R	S	R	S	R	R	R	R
2413			R	R	R	R	R	R	S	R	S	R	S	R	R	R	R
2417			R	R	R	R	R	R	S	R	S	R	S	R	R	R	R
2418			R	R	R	R	R	R	S	R	S	R	S	R	R	R	R
2425			R	R	R	R	R	R	S	R	S	R	S	R	R	R	R
2430		NG tube	R	R	R	R	R	R	S	R	S	R	S	R	R	R	R
2433			R	R	R	R	R	R	S	R	S	R	S	R	R	R	R
2436			R	R	R	R	R	R	S	R	S	R	S	R	R	R	R
2439			R	R	R	R	R	R	S	R	S	R	S	R	R	R	R
2462			R	R	R	R	R	R	S	R	S	R	S	R	R	R	R

Table 5-6 R= resistant, S= susceptible, Imipenem (IMI), Meropenem (MEM), ceftizoxime (ZOX), Ceftazidime (CAZ), Ceftriaxone (CRO), Cefoperazone (CFP), clarithromycin (CLR), doxycycline (DO), Ampicillin (AMP), Ciprofloxacin (CIP), Vancomycin (VA), Tobramycin (TOB) and Gentamicin (CN), Ceftazidime + Clavulanic acid (CAZ+CV), Cefotaxime + Clavulanic acid (CTX+CV), Fluroqu= Fluoroquinolone, Tetra= tetracycline, Glycope= glycopeptide.

5.3.3. Biofilm formation

Biofilm formation is recognised as an important virulence factor and has been related to colonisation of medical equipment, such as catheters and feeding tubes and is linked to various human diseases. In this study, the ability of *E. faecium* isolates to produce biofilms was studied on plastic surfaces at different temperatures (Figure 5-4).

All these strains demonstrated the ability to form high amounts of biofilms at 37 °C in infant formula. On the other hand, a low amount of biofilm formation at 25 °C was observed in the same formula. Paired t-test showed that *E. faecium* strains formed significantly more biofilm in PIF at 37 °C than at 25 °C ($p < 0.0001$), as shown in Figure 5-5. The highest amount of biofilm formation was observed in strain 2409 at 37 °C, which was isolated from the faecal sample from neonate 2. However, no significant differences ($p < 0.0001$) were noted between the isolates; e.g. 2368, 2401, 2407, 2410, 2417 and 2430 when compared to strain 2409. These strains were considerably higher producers of biofilm when compared with other isolates from both neonates. Conversely, the lowest amount of biofilm formation was observed in strains 2393, 2413, 2425 and 2397 when compared to the highest biofilm producer, strain 2409. They showed a statistically significant difference when analysed by means of one-way ANOVA followed by multiple comparisons with a Dennett's test. Of note, none of the *E. faecium* isolates produced statistically significant biofilm at 25 °C when compared to the negative control, except for strain 2401 (Figure 5-4).

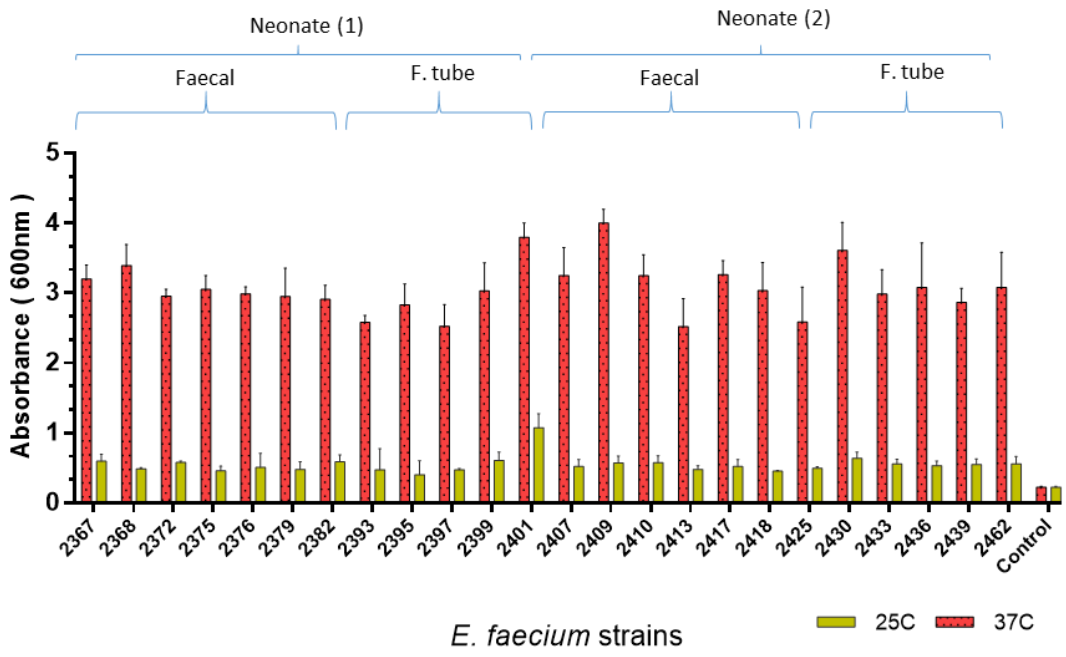


Figure 5-4 Biofilm formation at 25 °C and 37 °C in infant formula using the crystal violet adsorption assay.

All strains showed an ability to form more biofilm at 37 °C than at 25 °C in infant formula. The highest amount of biofilm formation was observed in strain 2409 at 37 °C, while at 25 °C the highest amount was observed in strain 2401 when compared to the negative control.

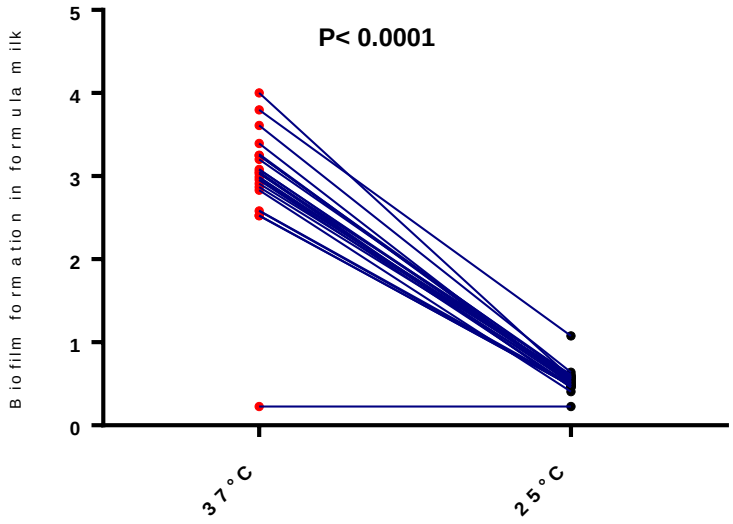


Figure 5-5 Paired t-test shows *E. faecium* strains form significantly more biofilm in PIF at 37°C than at 25°C ($p < 0.0001$).

5.3.4. Acid resistance

The main route of bacteria colonising the human intestine is via oral ingestion. Bacteria need to survive and grow in the high acidic conditions of the stomach, in order to cause infection. Acid tolerance is therefore one of the important virulence factors, and in this study, the ability of 24 *E. faecium* isolates to survive and grow under highly acidic conditions were investigated. These strains were exposed to pre-adjusted infant formula at pH 3.5 for two hours and the results are presented in Figure 5-6. All *E. faecium* strains were clearly resistant to exposure to low acidity for 2 hours. The viability of all these isolates decreased to 0.3-0.5 log cycles during the first 30 mins of acid exposure. From 30 mins to 120 minutes, the viable count cells for all strains tested did not show significant reduction, revealing a 0.2-0.3 log cycle decline during this period. No significant variation was observed between strains isolated from feeding tubes and faecal samples from the two premature babies. It is therefore likely that the same clone of bacteria has colonised the feeding tubes during the three-month period and has transferred between the twins.

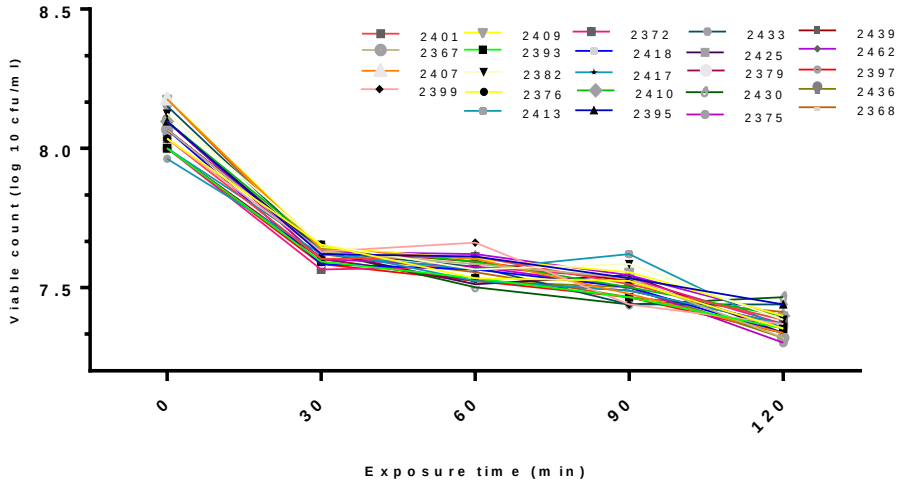


Figure 5-6 Survival curve of 24 *E. faecium* strains in infant formula at pH 3.5.

E. faecium strains (n=24) were assessed for their tolerance to pH 3.5 for two hours. The viable counts were determined by the Miles & Misra method. All strains showed an ability to resist pH 3.5 for the total 2 hours. No significant variation was observed between strains isolated from feeding tubes and faecal samples from two premature babies.

5.3.5.Heat tolerance

Heat resistance is an essential bacterial virulence factor for survival of microorganisms in different environmental stresses. It could pose a risk to the human health when bacteria become resistant to heat treatment, especially in PIF. In the present study, 24 *E. faecium* isolates were investigated to determine their ability to tolerate heat at 55 °C. D-values were determined at the time required to drop 1 log cycle in the viable cell count at 55 °C. D-values were quantified by linear regressions (−1/slope of the regression line) from the linear of the bacterial survival curves, and categorised as following: strain to heat tolerance (D55 = ≤5), moderate strain to heat tolerance (D55= >5 & ≤10) and resistant strain to heat tolerance (D55 = >10). All *E. faecium* isolates (n=24) showed a high level of heat resistance as shown in Figure 5-7, the D-values ranged from 15.7 to 24.5 min (Table 5-7). However, some levels of variation in thermo-tolerance were observed between the strains. For example, strain 2401 had showed high heat resistance, with D55 of 24.5 minutes at 55 °C. While strain 2413 showed lower heat-resistance compared with other isolates, D55 of 15.7minutes at 55 °C (Table 5-7).

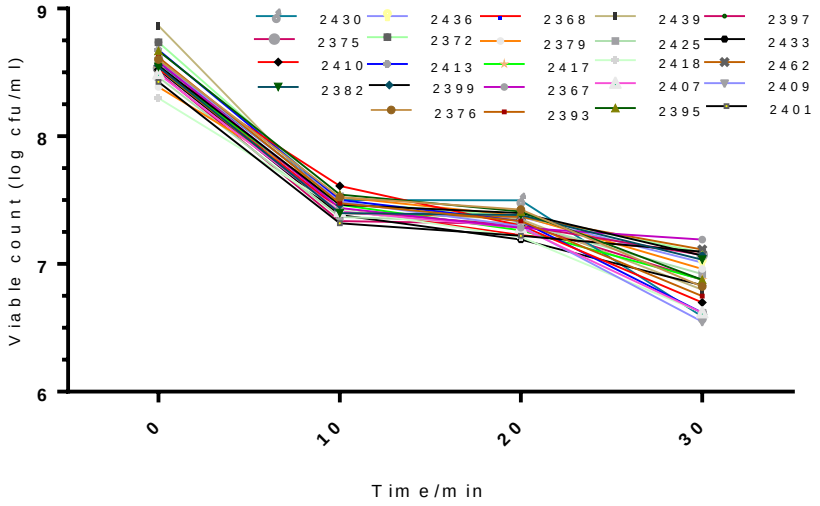


Figure 5-7 Survival of *E. faecium* isolates (n=24) in liquid infant formula at 55°C.

E. faecium strains (n=24) were assessed for their tolerance to heat resistance during their growth in liquid infant formula at 55 °C. The viable counts were determined by the Miles & Misra method. All strains showed an ability to a high level of heat resistance.

Table 5-7 D-values for *E. faecium* isolates (n=24) in liquid infant formula at 55°C.

Isolate	Neonate	Sample Source	Isolation date	Sequence Typing	D55 (min)	Category
2372	1	Faecal	24/08/15	ST-80	19.8	Resistant
2397	1	NG tube	08/09/15	ST-80	20.4	Resistant
2393	1	NG tube	12/08/15	ST-80	17.3	Resistant
2430	2	NG tube	16/07/15	ST-80	16.7	Resistant
2410	2	Faecal	20/08/15	ST-80	17.3	Resistant
2395	1	NG tube	19/08/15	ST-80	18.1	Resistant
2433	2	NG tube	17/08/15	ST-80	19.1	Resistant
2382	1	Faecal	22/10/15	ST-80	22.1	Resistant
2379	1	Faecal	20/10/15	ST-80	22.6	Resistant
2375	1	Faecal	15/09/15	ST-80	22.2	Resistant
2407	2	Faecal	31/09/15	ST-80	17.4	Resistant
2462	2	NG tube	11/10/15	ST-80	23.3	Resistant
2439	2	NG tube	12/10/15	ST-80	15.8	Resistant
2399	1	NG tube	27/09/15	ST-80	22.7	Resistant
2401	1	NG tube	01/10/15	ST-80	24.5	Resistant
2417	2	Faecal	30/09/15	ST-80	19.3	Resistant
2436	2	NG tube	08/09/15	ST-80	21.9	Resistant
2368	1	Faecal	10/8/2015	ST-80	22.3	Resistant
2418	2	Faecal	16/10/15	ST-80	19.4	Resistant
2409	2	Faecal	16/08/15	ST-80	15.8	Resistant
2376	1	Faecal	24/09/15	ST-80	18.4	Resistant
2413	2	Faecal	10/9/2015	ST-80	15.7	Resistant
2425	2	Faecal	20/10/15	ST-80	21.3	Resistant
2367	1	Faecal	23/07/15	ST-80	23.9	Resistant

5.3.6. Human serum

Once pathogenic bacteria succeed in passing through the membrane epithelial barrier, cells must be able to evade host defence mechanisms, such as serum bactericidal activities and macrophages. In this study, bactericidal assays were conducted on 24 *E. faecium* isolates exposed to human serum for three hours. As a positive control, *S.* Enteritidis was used, whilst *E. coli* K12 was used as a negative control. The results of this experiment are shown in Figure 5-8. All these strains demonstrated a high level of resistance to human serum when compared with negative and positive controls. It was revealed that an increase in cell numbers occurred after 3-hours incubation. There was a slight variation between *E. faecium* strains in their response to the human serum. Differentiation between the viable counts of bacteria at time zero could be a possible

reason of the variations among these strains. These strains showed an increase in their numbers after the first hour of incubation with human serum. While strain 2367 isolated from the faecal sample from neonate 1, showed a decrease of recovered viable cell numbers at the first hour of incubation. However, all of the strains isolated from the twin infants showed an ability to tolerate human serum after the final time point. This explain why such bacteria could survive and travel through the bloodstream and infect different host organs such as brain.

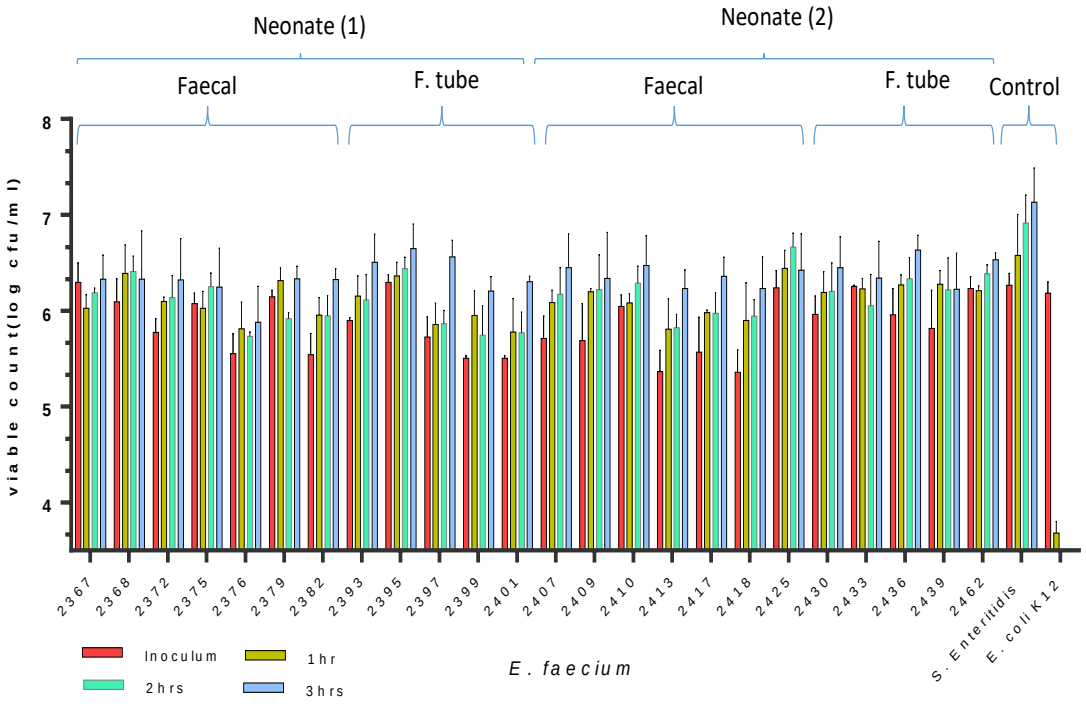


Figure 5-8 Sensitivity of 24 *E. faecium* to human serum over 3 hours of incubation at 37 °C.

All 24 *E. faecium* strains isolated from twin infants at different neonatal sites, showed an ability to tolerate human serum after the final time point.

5.3.7. Blood haemolysis

Haemolysing blood cells is an important virulence factor and is recognised as an important mechanism for many pathogenic bacteria to lyse red blood cells. The production of haemolysin was determined by streaking bacterial cultures on TSA agar supplemented with 5% horse blood. Haemolytic activity of the twenty-four *E. faecium* strains isolated from feeding tubes and faecal samples from two premature babies in an NICU was investigated. All isolates were able to induce α haemolysis on

horse blood (Table 5-8). The haemolysis activity expressed by these strains to lyse red blood cells might be one of the important key virulence in persistence of these isolates in bloodstream and causing sepsis.

5.3.8. Protease production

Protease activity by pathogens are well known as a virulence factor that plays an important role in pathogenesis, by contributing to immune evasion of many pathogens. In this study, the protease activity assay was performed to determine the expression of protease on skimmed milk at 37 °C for 72 hours. The results indicated that all *E. faecium* strains (n = 24) had revealed a positive result for production of protease activity (Table 5-8). Protease activity is defined as a clear circle on the skimmed milk agar. *B. cereus* was used as a positive control and *E. coli* NTU 407 was used as a negative control.

5.3.9. Lipase

Lipase activity was assessed by using tributyrin agar media, according to the methodology described in detail in Chapter 2 (Section 2). A clear halo around the colonies indicates a positive reaction by the tested strain. Lipase activity was observed amongst all strains (n = 24) collected from two premature babies in this study, as shown in the Table 5-8.

5.3.10. Capsule production

Table 5-8 shows the results of capsule production in two different infant formula agar media, soya-based infant formula (IF1) and whey -based infant formula (IF2). All of twenty-four *E. faecium* strains were negative for capsule production on both media.

Table 5-8 Results summary of biofilm formation, capsule production on milk agar, acid resistance, haemolysins, protease and lipase, among *E. faecium* strains (n=24).

Isolate	Neonate	Sample Source	Isolation date	Sequence Typing	Capsule (Mucoid)		Biofilm formation in PIF		Haemolysis	Lipase	Protease	Feeding regime
					IF1	IF2	25°C	37°C	HB			
2372	1	Faecal	24/08/15	ST-80	-	-	Low	High	Alpha (α)	+	+	Breast Milk + Pre-made Formula + Fortifier
2397	1	NG tube	08/09/15	ST-80	-	-	Low	High	Alpha (α)	+	+	
2393	1	NG tube	12/08/15	ST-80	-	-	Low	High	Alpha (α)	+	+	
2430	2	NG tube	16/07/15	ST-80	-	-	Low	High	Alpha (α)	+	+	
2410	2	Faecal	20/08/15	ST-80	-	-	Low	High	Alpha (α)	+	+	
2395	1	NG tube	19/08/15	ST-80	-	-	Low	High	Alpha (α)	+	+	
2433	2	NG tube	17/08/15	ST-80	-	-	Low	High	Alpha (α)	+	+	
2382	1	Faecal	22/10/15	ST-80	-	-	Low	High	Alpha (α)	+	+	
2379	1	Faecal	20/10/15	ST-80	-	-	Low	High	Alpha (α)	+	+	
2375	1	Faecal	15/09/15	ST-80	-	-	Low	High	Alpha (α)	+	+	
2407	2	Faecal	31/09/15	ST-80	-	-	Low	High	Alpha (α)	+	+	
2462	2	NG tube	11/10/15	ST-80	-	-	Low	High	Alpha (α)	+	+	
2439	2	NG tube	12/10/15	ST-80	-	-	Low	High	Alpha (α)	+	+	
2399	1	NG tube	27/09/15	ST-80	-	-	Low	High	Alpha (α)	+	+	
2401	1	NG tube	01/10/15	ST-80	-	-	Low	High	Alpha (α)	+	+	
2417	2	Faecal	30/09/15	ST-80	-	-	Low	High	Alpha (α)	+	+	
2436	2	NG tube	08/09/15	ST-80	-	-	Low	High	Alpha (α)	+	+	
2368	1	Faecal	10/8/2015	ST-80	-	-	Low	High	Alpha (α)	+	+	
2418	2	Faecal	16/10/15	ST-80	-	-	Low	High	Alpha (α)	+	+	
2409	2	Faecal	16/08/15	ST-80	-	-	Low	High	Alpha (α)	+	+	
2376	1	Faecal	24/09/15	ST-80	-	-	Low	High	Alpha (α)	+	+	
2413	2	Faecal	10/9/2015	ST-80	-	-	Low	High	Alpha (α)	+	+	
2425	2	Faecal	20/10/15	ST-80	-	-	Low	High	Alpha (α)	+	+	
2367	1	Faecal	23/07/15	ST-80	-	-	Low	High	Alpha (α)	+	+	

5.3.11. Assessment of the pathogenicity of *E. faecium* using tissue culture

5.3.11.1. Attachment to Caco-2 Intestinal Epithelial Cells

There are a number of virulence factors which enable bacteria to cause infections such as attachment and invasion. In this chapter, the potential virulence of 24 *E. faecium* isolates was investigated to assess the ability of these strains to attach and invade epithelial Caco-2 cells. *E. coli* K12 and *S.* Enteritidis were used as negative and positive controls (Figure 5-9). All strains from different sources were able to adhere to the Caco-2 cell line. These isolates were significantly higher than that of the negative

control strain *E. coli* 1230. However, there were some different levels of adherence to Caco-2 cells among strains. Strain 2309, isolated from neonate 2 faecal sample, was the strain with the highest adhesion potential among all tested strains, although there was no significant difference in attachment levels among strains when compared to the most adhesive strain 2309. Some observable differences were noted between strains 2372, 2401, 2417 and 2462 when compared with the positive control. An unpaired t-test shows no significant difference in the ability of the bacteria to adhere to Caco-2 between all strains isolated from baby 1 and with baby 2 ($p = 0.6048$). Also, no significant difference was observed between all strains isolated from feeding tubes from both babies compared with the faeces sample from both babies ($p = 0.2073$).

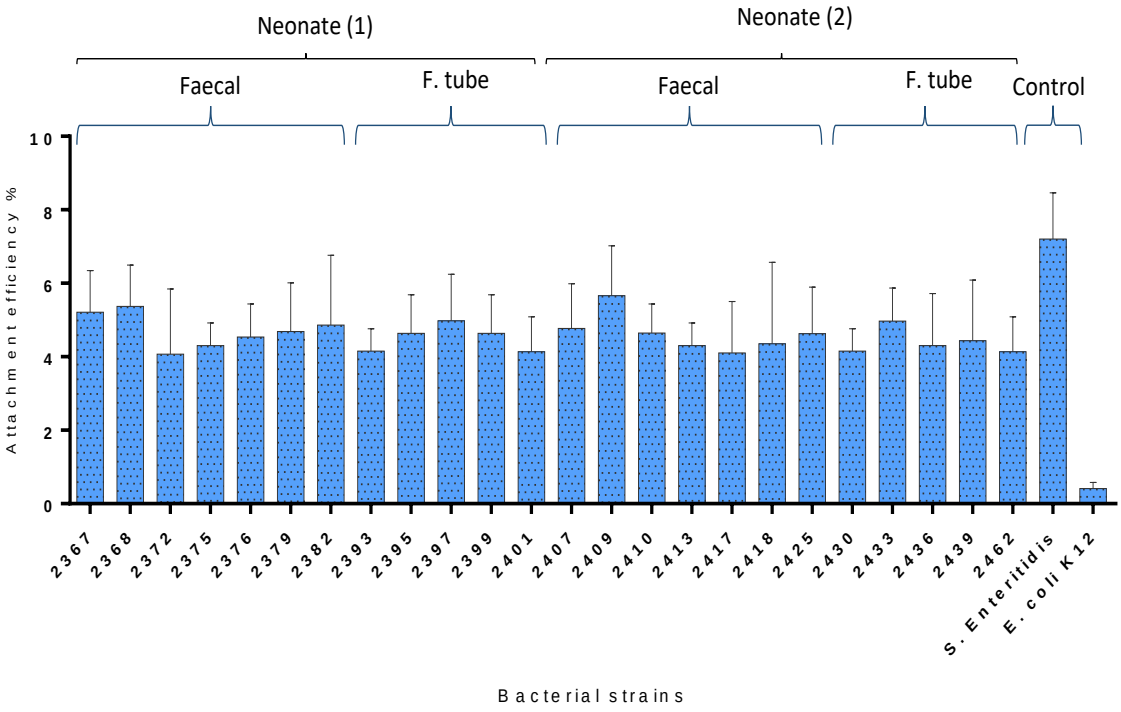


Figure 5-9 Attachment of *E. faecium* strains to the human Caco-2 cell line.

The figure shows the differences in attachment levels to the human epithelial cell line Caco-2 among *E. faecium* strains. All strains were able to attach to the epithelial cell line when compared to *E. coli* 1230, which was used as the negative control, and *S.* Enteritidis 358, which was used as the positive control.

5.3.11.2. Bacterial invasion of Caco-2 human epithelial cells

The gentamicin protection assay was employed in order to determine the ability of *E. faecium* strains to invade the Caco-2 cells. All strains were able to invade the Caco-2 cells at a low level; however, there was a variation amongst *E. faecium* isolates in their ability

to invade this cell line (Figure 5-10). The highest invasion efficiency was achieved by strains 2399, 2372, 2433,2393, 2367 and 2462, conversely, strain 2417 and 2418 showed the lowest invasion levels among the isolates tested. Although there was slight variation among *E. faecium* isolates in their ability to invade Caco-2 cells, there was no significant difference in invasion levels when analysed by means of one-way ANOVA ($P < 0.001$), followed by multiple comparisons with a Dennett's test. Unpaired t-test also shows no significant difference in the ability to invade to Caco-2 between all strains isolated from baby 1 and compered with baby 2 ($p = 0.0533$). Also no significant difference was observed between all strains isolated from feeding tubes from both babies compared with faecal samples from both babies ($p = 0.2073$).

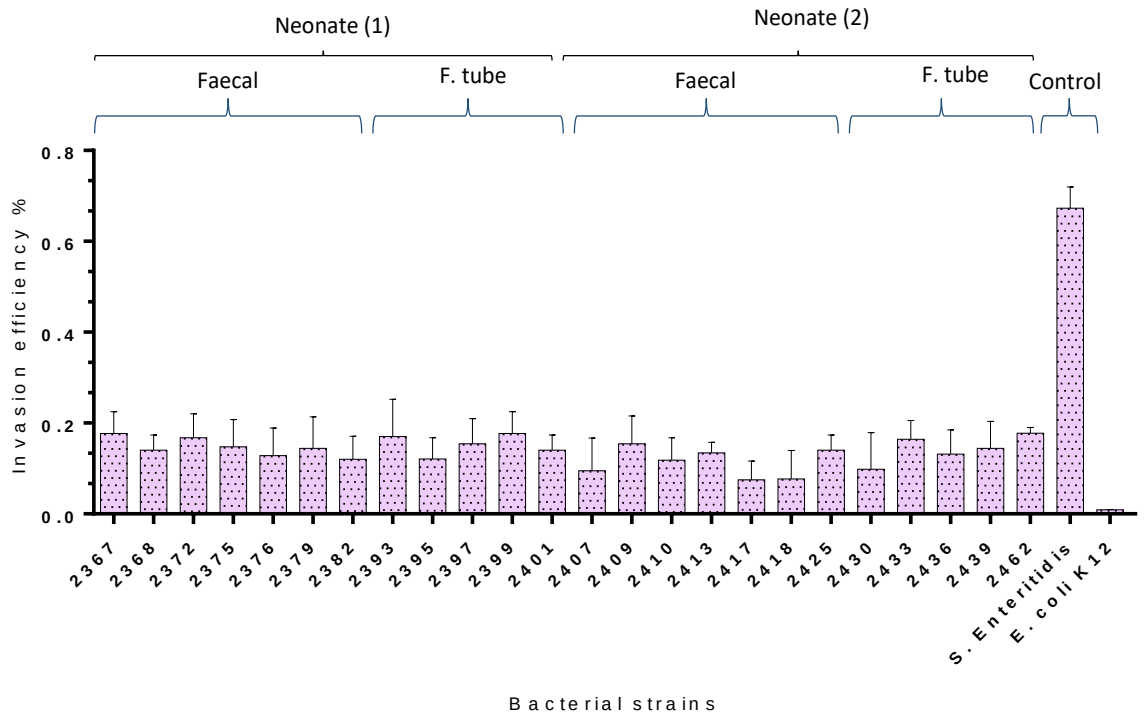


Figure 5-10 Invasion efficiency of *E. faecium* strains to the mammalian Caco-2 cell line.

The bar chart shows the differences among *E. faecium* strains with regards to their ability to invade the human epithelial cell line Caco-2, by performing the gentamicin protection assay. All of strains were able to invade the Caco-2 cells by low level. *S.* Enteritidis 358 and *E. coli* 1230 were used as positive and negative control strains, respectively.

5.4. Discussion

Enterococci are one of the leading causes of nosocomial infection in the hospital setting, particularly in Neonatal Intensive Care Units (NICU). Most enterococcal infections are known to be caused by *E. faecalis* and *E. faecium* among various species of *Enterococcus* (Sood et al., 2008; Mutters et al., 2013; Abamecha et al., 2015; Adesida et al., 2017). Multidrug-resistant enterococci are a major concern in hospitals. They are most commonly recovered in NICUs (Moemen et al., 2015; Wassilew et al., 2018). The ability of enterococci to survive for prolonged periods of time may contribute to cross-contamination, through healthcare workers' hands, surfaces or medical devices. The use of medical devices is one of the main clinical risk factors in neonatal infection including nasogastric enteral feeding tubes (NEFTs). These tubes may also contribute to the colonisation by pathogenic microorganisms which are often required for feeding new-borns (Gómez et al., 2016). Furthermore, preterm infants in Intensive Care Units (ICUs) are high-risk patients, due to their increased susceptibility to infections during hospitalisation (Christina et al., 2015; Ramasethu, 2017). Their exposure to pathogens from the surrounding environment may result in a potential infection. In addition, cross-transmission among neonates in the NICU may potentially occur due to prolonged hospitalisation. The colonisation of the enteral feeding tubes by nosocomial bacteria and their impact on earlier gut colonisation of the premature newborn babies was investigated in a recent study (Gomez et al., 2016). Their study indicated that *E. coli, K. pneumoniae, S. aureus, S. epidermidis, Serratia marcescens, E. faecium* and *E. faecalis* were the predominant isolates in faeces and milk samples amongst 4,000 bacterial strains recovered (Gomez et al., 2016). The findings of present study have striking similarities with Gomez, 2016.

Therefore, the present longitudinal study was aimed to compare potentially pathogenic isolates within and between the feeding tubes and faeces of two twin babies in the NICU over time. Subsequently, feeding tubes and faecal samples were collected from two premature babies (twins) in the NICU of Queen Medical Centre (QMC). The first patient was a low-birth weight (660 g) preterm male neonate and the second preterm male was also low-birth weight (880 g). Both neonates received antibiotics, and were initially fed with breast milk, fortifier and followed by pre-made formula.

Accurate identification of bacterial isolates is significant, particularly in the clinical field. For instance, correct identification of species and sub-species groups may be essential to selecting the appropriate choice of antibiotic therapy. The isolates collected from two premature babies were identified using 16S rRNA sequencing. Indeed, it is difficult to distinguish among closely related species by using 16S rRNA gene as the limited resolution of this method. A recent study by Ogrodzki et al. (2017) indicated that the strains isolated from 2 different feeding tubes of the same neonate were identified as *E. faecalis* by using 16S rRNA gene. However, the genomic profiling of *E. faecalis* confirmed these strains formed two distinct clusters according to their sequence type. For this reason, representative strains in this study were later confirmed by Whole-genome sequencing (WGS) to help provide a complete picture. These isolates were revealed to be Gram-positive cocci while few isolates were identified as Gram-negative. In the current study, it was revealed that *E. faecium* was the predominant species isolated from feeding tubes and faecal samples that persisted unaffected by antibiotics and other factors in the NICU (Figure 5-2). *E. faecium*, *S. aureus* and *E. faecalis* showed higher prevalence compared to Gram-negative *K. pneumoniae* and *K. oxytoca* during the three-month period.

Despite different bacterial cultures being used, my sample collection was characterised by a lack of detectable *Bifidobacterium* spp., in addition to low levels of Gram-negative isolates. It may be possible that those premature neonates received antibiotics during hospitalisation. This may prevent or affect these bacteria from colonising and also reduce the level of Gram-negative strains. A previous studies showed that the reduced *Bifidobacterium* colonised preterm infants who treated with antibiotics (Hussey et al., 2011; Barrett et al., 2013). The early exposure to antibiotics can be considered as a possible explanation for the low percentage of bacterial species in the feeding tubes and faecal samples. This observation is in agreement with Ubeda et al. (2010), who revealed that bacteria associated with hospital-acquired infections can become one of the most dominant in the gut microbiota as a result of antibiotic therapy.

However, among the most common species isolated from feeding tubes and faecal samples from the two premature babies in the NICU, *E. faecium* showed the highest

percentage prevalence. For this reason, twenty-four *E. faecium* isolates were selected as representative strains in this study and were subjected to whole-genome sequencing. Traditionally *E. faecium* have been observed as low-grade pathogens, however, in the last two decades enterococci have emerged as an increasingly important cause of nosocomial infections. In addition, they have been identified to be responsible for the majority of healthcare-associated enterococcal infections. This is attributed to their ability to acquire resistance to various antibiotics, to receive foreign genetic material or undergo mutation due the transfer of transposons and plasmids. (Simjee et al., 2002; Sood et al., 2008; Palmer et al., 2011; Arias and Murray, 2012).

However, previous epidemiological studies of *E. faecium* using multilocus sequence typing (MLST), revealed that the clonal complex lineage CC17 are considered a high-risk clonal group. This clonal associated with hospital outbreaks, recovered frequently from hospitalised patients has become endemic in most countries. At the same time, this clone is characterised by susceptibility to ampicillin and carriage of the *esp* gene, indicating presence of a putative pathogenicity island (Deplano et al., 2007; Top et al., 2008; Lester et al., 2008). The present study exploited this opportunity, and MLST analysis of *E. faecium* isolates was performed to determine whether these isolates belong to CC17 or not.

It was found that all strains were typed as ST80, belonging to clonal complex (CC17). Furthermore, they were resistant to ampicillin and were found to carry the virulence-associated gene *esp*. My results are supported by previous studies which stated that the CC17 lineage is largely characterised by resistance to ampicillin and possess the virulence gene *esp*. (Deplano et al., 2007; Top et al., 2008; Kwon et al., 2012).

Despite the high percentage of *E. faecium* isolates recovered during the NICU stay, no genetic diversity was observed, and the ST80 clone persisted throughout most of the studied period. These results led to the conclusion that these isolates from different sites are same clone and are able to persist and be transferred between the two premature neonates in the NICU (Table 5-3). Based on MLST and phylogenetic analysis of whole genome sequences, the isolates recovered from feeding tubes and faeces samples of the two neonates are likely derived from the same local source. Nevertheless,

the ampicillin resistant *E. faecium* isolates are an increasing problem in NICUs. The treatment of the two premature babies with vancomycin and gentamicin may have created selective antibiotic pressures, contributing to the presence of vancomycin-resistant *E. faecium* (VREF) through mutation. Furthermore, Enterococci have the ability to acquire antimicrobial resistance via horizontal transfer of mobile genetic elements (Palmer et al., 2011). Previous studies have shown that within enterococci, vancomycin resistance possibly spreads through genetic mobile elements such as the *vanA* type frequently found on mobilisable or conjugative plasmids (Migura et al., 2007; Sletvold et al., 2010; Hegstad et al., 2010).

Significant rates of antimicrobial resistance was observed for *E. faecium* isolates, not only for ampicillin resistance, but resistance to multiple antimicrobial drugs, such as imipenem, meropenem, ceftizoxime, ceftazidime, ceftriaxone, cefoperazone, clarithromycin, ciprofloxacin, tobramycin and also to ESBLs, ceftazidime + clavulanic acid and cefotaxime + clavulanic acid. Some drug susceptibility was reported to vancomycin, gentamicin and doxycycline. Overall, feeding tubes and faecal isolates from both neonates had displayed the similar patterns of antibiotic resistance. However, several previous studies have identified multidrug-resistant *E. faecium* in NICUs, especially vancomycin resistant strains (Hufnagel et al., 2007; Cilo et al., 2014; Shantala et al., 2014).

The persistence of antibiotic-resistant *E. faecium* acquired by premature infants during hospitalisation in the NICU may lead to an increased risk among these neonates. The gut microbiota composition during the first 3 months may have important health consequences and may be linked to numerous problems later in life, particularly those associated with development of the immune system. (Cahenzli et al., 2013; Tanaka and Nakayama, 2017). For example the genus *Bifidobacterium* is considered to be good for health, however my collection in this study showed a lack of this genus, which might be a result of antibiotics used during hospitalisation as I mentioned previously. Indeed, the persistence of multidrug-resistant strains among neonates may pose a risk to other neonates acquiring these organisms in the NICU, particularly preterm infants with very low birthweight.

Enterococci are clinically significant pathogens and are considered to be a major concern and are able to acquire resistance to many antibiotics (Billington et al., 2014; Daniel et al., 2015). All *E. faecium* isolates were investigated for the presence of the antimicrobial resistance genes in this study. Aminoglycoside resistance gene *aph(3')-III ant(6)-Ia* were detected among all isolates except strain 2417. These isolates were found to be highly resistant to tobramycin and it is primarily due to the acquisition of this resistance gene. I presume the resistance of strain 2417 to tobramycin and lack of aminoglycoside resistance gene may be due to the action of other resistance mechanisms. Furthermore, all isolates were observed to be phenotypically susceptible towards gentamicin, and lacking the aminoglycoside gene *aac(6')-aph(2'')*, except for two strains 2410 and 2433, which carried this gene but without resistance to gentamicin. Indeed, this gene is well known to play a significant role in mediating high-level gentamicin-resistance among strains (Behnood et al., 2013). These results suggest that the susceptibility observed among these strains may be caused by inactive gene products or by low levels of genes expression (Table 5-5). Another important observation in this study was that all isolates were found to carry genes coding for macrolide resistance *msr*(C), *erm*(B) and tetracycline resistance gene *tet*(S). These genes were identified in this study are similar to those reported by previous studies were isolated from diverse samples: clinical isolates, animals and food (Cauwerts et al., 2007; Thumu and Halami, 2014; Beukers et al., 2015). Macrolide resistance conferred by the *erm* (B) gene has spread in different parts of the world (Qin et al., 2012; Lam et al., 2013; Szakacs et al., 2014; El Ashkar et al., 2017).

Moreover, other antimicrobial resistance genes were harboured among few of the *E. faecium* strains. For example, only strain 2433 was positive for the macrolide resistance gene *mph*(C) and strain 2436 was positive for *vat*(B). Fluoroquinolone resistance gene *nor*(A) was harboured by only two strains (2375 and 2462) and the Beta-lactam blaZ was harboured by 3 strains, 2367, 2433 and 2462 (Table 5-5). This is not surprising as *E. faecium* can more easily acquire antibiotic resistance (Hollenbeck and Rice, 2012; Cattoir and Giard, 2014) and are believed to be highly transferable plasmids or transposons. An important observation in this study was that these isolates were found to carry multiple plasmids. Six different rep-family plasmids were detected among 24 *E.*

faecium isolates (*rep15, rep11, rep17, rep1, rep2* and *rep21*) as shown in Table 5-5. This finding is consistent with the fact that clinical enterococcal isolates often harbour several types of plasmids (Migura et al., 2007; Rosvoll et al., 2010; Zhu et al., 2010; Rosvoll et al., 2012). Additionally, this is in agreement with previous studies, which have described the *rep17, rep1* and *rep2* plasmids in *E. faecium* clinical isolates (Mikalsen et al., 2015; Freitas et al., 2016). It is possible that plasmids could be a potential source of transferable antimicrobial resistance. The rise of drug resistance in *E. faecium* strains is a serious problem for treatment and control, especially among neonates in NICUs. It would appear that neonates born with low birth weights and underdeveloped immune systems in NICUs are at high risk, particularly those fed via nasogastric tubes. It was interesting to note that *E. faecium* was revealed to be the most predominant species isolated as well as the species persisting during the studied period. This is also why it was decided to characterise and type the isolates obtained from feeding tubes and faecal samples from the two premature babies.

Pathogen typing is essential to identify and trace the epidemic and phylogenetic relationships among the isolates that have common origins. Also, it is important in clinical settings for patient diagnosis, treatment and helps in tracing contaminants to their original source. (Squires et al. 2015). Although PFGE is the most commonly used method for typing and outbreak tracking, there are limitations of PFGE to resolve bands; they could be obscured by bands of similar or nearly identical size (Davis et al., 2003). However, the SNP-based typing assay has proven to be more discriminatory and has been employed to infer the evolution of outbreak isolates which show too little sequence diversity (Turabelidze et al., 2013). In the present study, whole-genome sequencing of isolates was performed, and a maximum likelihood phylogeny of the 24 *E. faecium* isolates was constructed from a core genome alignment. The SNP analysis was undertaken in order to observe the relatedness of *E. faecium* strains recovered from the feeding tubes and faecal samples among two premature babies over time. Isolate 2425 was used as the reference for SNP comparisons within this collection, which was isolated on 20/10/2015 from neonate 2.

However, strain 2372 clustered alone with high SNP differences of 211 SNPs when compared with reference strain 2425. Both isolates were recovered from faecal samples from baby 1 at different time points, suggesting that they were acquired independently of each other. The interesting observation is that of the numbers of SNP was very small (12 SNPs) between strains 2433 isolated from feeding tube neonate 2 and strain 2395 isolated from feeding tube neonate 1 after 2 days (Figure 5-3). This observation suggests that possible cross contamination and transfer by this organism from neonate 2 to neonate 1. Also, SNP-based analysis of the strain 2433 isolated from feeding tube displayed related close phylogenetic relation to strain 2410 isolated 3 days later from the faecal sample of the same neonate 2. Only 13 SNPs were identified between them, which confirms the source of this strain to be ingested by the neonate through the feeding tube.

In addition, strain 2433 and 2395, also isolated from feeding tubes from both neonates, was shown by the small number of SNP differences (12 SNPs). This finding can be explained by the transfer of the strain from baby 2 to baby 1. Another important observation in this study was that strain 2413 and 2425 were obtained from the same infant 2 from faecal samples during a 5-week period (Figure 5-3). These isolates differed from each other by 17 SNPs difference between them, indicating the same strain could persist in the baby's intestinal tract over this period of time. Furthermore, strain 2367 isolated from neonate 1 was highly identical to strain 2425 (only 15 SNPs difference), which were recovered after nearly 3 months from baby 2. This observation led to the conclusion that the same clone has persisted among two neonates during the majority of the study period. The SNP typing of the isolates were highly similar, as well as the MLST profile also indicated all of these isolates were typed as ST80 belonging to clonal complex (CC17). The last isolate 2382 from neonate 1 is virtually identical to the original isolate 2430 from neonate 2, which was isolated after 3 months and had only 24 SNP differences. This confirms the circulation of the *E. faecium* ST80 clone between the twin babies within the NICU. The SNP typing suggested that strains had as little as 12 to 27 SNPs difference from each other, thus revealing a remarkable degree of homology between these strains. The SNP differences observed between strains might be the

result of microevolution and adaptation to two different niches (i.e. the gut flora and the feeding tube), as they are two different environments.

The small number of SNP differences suggest a common source of origin. It might be that the potential source of contamination could be the mother's milk or it could either be PIF itself, in addition, the tools used to prepare formula or the carer's hands. However, a current limitation of my study is the lack of environmental/carer samples or samples of mother's milk and PIF, which have not been screened to compare with feeding tubes and faecal isolates. For this reason, it is not possible to determine the source of the *E. faecalis* strains that colonised the infants and their feeding tubes. The ability of this clone to colonise and persist in feeding tubes and faecal samples among two neonates for three months has been demonstrated in this study, and this could be explained by their possession of essential bacterial virulence factors such as the ability to form biofilm.

Biofilm formation is one of important factors reported as a virulence-associated trait, by helping to maintain cell survival on biotic and abiotic surfaces (Tomaras et al., 2003). For example, a study by Ogrodzki et al. (2017) reported that bacterial biofilms distributed inside the nasogastric feeding tube was visualised using optical coherence tomography. Indeed, the ability of bacteria to form biofilms inside feeding tubes are a major concern. It is believed that bacterial biofilms would be capable to develop on medical device surfaces and are an important risk factor for microbial spreading within the host and increased chance of disease (Perciva et al., 2015). In this study, the ability of *E. faecium* isolates to produce biofilms was studied on plastic surfaces using two temperatures, 25 °C and 37 °C. All strains demonstrated the ability to form high amounts of biofilm in infant formula at 37 °C when compared with at 25°C.

There was no significant difference ($p > 0.05$) among all isolates in the capacity of biofilm formation at 25 °C, except for strain 2401, which showed the highest amount of biofilm formation. The reason for the difference in capacity of biofilm formation might be influenced by gene expression. Although there are some differences among all strains with their capacity to form biofilm at 37 °C, all strains demonstrated the ability to form high amounts of biofilms in infant formula at this temperature. Indeed, numerous genes have been reported by several earlier studies to be associated with biofilm production,

particularly the presence of *esp* gene. This gene encodes the enterococcal surface protein (Esp), which is located on a pathogenicity island (Leavis et al., 2004; Van Schaik et al., 2010). This gene is also predominantly present in hospital acquired isolates, which has a role in biofilm formation and contributes to pathogenesis (Heikens et al., 2007; Top et al., 2013). In this study, the results indicate that the analysed genomes of the 24 *E. faecium* isolates were found to harbour the *esp* gene. Additionally, these strains revealed high amounts of biofilm production at 37 °C compared to at 25 °C as mentioned earlier.

This finding was in agreement with previous study by Van Wamel et al. (2007). Who reported that *esp*-positive *E. faecium* strains showed significantly increased levels to binding of polystyrene and biofilm development when grown at 37 °C compared to 21 °C. This result may suggest a correlation between the *esp* gene and biofilm formation. However, there is conflict regarding the role of the *esp* gene in biofilm development. Some studies have suggested that the *esp* gene plays an important role in biofilm formation. (Heikens et al., 2007). While other studies have reported that it is not necessarily required to produce biofilm (Kafil and Mobarez, 2015; Garg et al., 2017).

So far, several virulence genes have been reported to be involved in biofilm formation by *E. faecium*. For instance, the *sgrA* gene, which binds to extracellular matrix proteins and is implicated in biofilm development (Hendrick et al., 2009). Locus *ebpABC*, which are putative major pilus subunits are also important for the cell's ability to form biofilm (Sillanpaa et al., 2010). However, analysing the genomes in the current work revealed that the *sgrA* gene and *ebpABC* locus have been detected in the genomes of the tested *E. faecium*. Therefore, biofilm production in *E. faecium* strains belonging to clonal complex (CC17) could enhance colonisation and persistence in enteral feeding tubes, and also increase the probability of causing nosocomial infections and outbreaks.

Translocation of *E. faecium* from the gut into the bloodstream can cause serious bloodstream infections (Arias and Murray, 2012), and additionally, they are able to resist the host immune response (Wilson et al., 2002). In this study, a bactericidal assay was conducted on the 24 *E. faecium* isolates by exposing them to human serum for three hours. All strains demonstrated a high level of resistance to human serum when

compared to the negative and positive controls. There was a slight variation between *E. faecium* strains in their response to the human serum. Differences between the viable counts of bacteria at time zero could be a possible reason for the variation observed between these strains. However, all strains isolated from the feeding tubes and faeces of the twin infants showed an ability to tolerate human serum after the final time point. This would indicate that these bacteria could survive and travel in the bloodstream, which may lead to infection of different host organs, such as the brain. A recent study by Zhang et al. (2017b) identified genes required for the growth of *E. faecium* E745 in human serum. These genes, *pyr K_2, pyrF, purD,* and *purH*, are involved in nucleotide biosynthesis and *manY_2* encoding a phosphotransferase system subunit. The study showed that these genes are crucial for the growth of *E. faecium* E745 in human serum and contributes to the pathogenesis of this strain. The *pyrK_2, pyrF, purD and purH* genes have been detected in all *E. faecium* genomes in this study, while the *manY_2* gene was not detected in the genomes analysed. The results of the current analysis might explain that the genes detected are associated with the ability of these strains to survive in human serum. These findings increase the clinical concern to neonates with compromised immune systems in the NICU, which are highly susceptible to infections.

Other virulence factors such as haemolysin which were detected among the tested *E. faecium* strains. The present study showed that all *E. faecium* strains were able to induce α haemolysis (partial haemolysis) on horse blood. This result is inconsistent with a previous study by Kivanc et al. (2011), which reported that all *E. faecium* isolates derived from breast milk samples were found to be non-haemolytic strains, although such factors are recognised to be important in contributing to bacterial pathogenesis and disease. Previous studies indicated that cytolysin produced by *Enterococcus* spp. are capable of lysing human, horse, and rabbit erythrocytes. Expression of cytolysin is controlled by an operon having genes encoded *cyl1, cyl2, cylA, cylM*, and *cylB*. These genes have been attributed to haemolytic activity previously (Cox et al., 2005; Solheim et al., 2009; Van Tyne et al., 2013). However, none of my isolates harboured these genes, suggesting that they have acquired other genes that possibly have a role in haemolysin activity.

This study was extended to investigate other virulence factors that are assumed to play an important role in microbial pathogenicity including biological activity of lipase and protease.

Such virulence factors have been reported to play a crucial role in bacterial infections (Furumura et al., 2006; Sava et al., 2010). For example, a previous study by Asha et al. (2005) reported that lipase activity was detected in clinical isolates of *E. faecium* at a high frequency when compared to strains of non-clinical origin. All *E. faecium* strains in the present study had detectable lipolytic and proteolytic activity. These activities may contribute to these isolates becoming more harmful to neonates, particularly, preterm infants in NICUs. A remarkable degree of homology was revealed between feeding tube and faecal sample strains isolated from the twin infants. Moreover, these strains harboured the same genes and displayed a low number of SNPs difference from each other. This observation may support the suggestion that these isolates are likely to be the same clone and have a common source of origin.

Acid tolerance is also one of the important characteristics of bacterial cells. The ability of bacteria to survive and grow in the highly acidic conditions of the stomach is a significant feature which may present the opportunity of bacterial colonisation and potentially cause infections in the patient. Thus, in present study, the ability of 24 *E. faecium* to survive and grow under acidic conditions was investigated. All *E. faecium* strains were clearly resistant to exposure to low acidity for 2 hours. These results are consistent with the study reported by Ispirli et al. (2015), where *E. faecium* isolated from the human gut are characteristically resistant to low pH and bile salts. Similarly, Mubarak and Soraya (2018) also found that *E. faecalis* (ATCC-29212) has an excellent growth capability to adapt to a very acidic environment. Zhang et al. (2013b) reported that the *gltK* gene is responsible for bile resistance in *E. faecium* E1162. Data from the present study indicated that all *E. faecium* strains that harboured the *gltK* gene and are able to grow and resist conditions of low acidity. This suggests that this gene may contribute to resistance to acidic conditions and can be considered as a feature which may result in infections of neonates. The interesting observation was that there was no significant variation of the acidic resistance of the isolates analysed in the

present study. Therefore, it is likely that the same clone of bacteria has colonised the feeding tubes and the gut of the neonates and were excreted in the faeces, during the three-month period.

In the present study, the heat tolerance analysis was carried out on the 24 *E. faecium* isolates, in order to determine their ability to tolerate heat at 55 °C for 30 minutes in liquid infant formula. All *E. faecium* isolates showed a high level of heat resistance. *E. faecium* has been recognised to be heat-resistant, for example, a study by McAuley et al. (2012) reported that *E. faecium* is one of the most heat resistant species to be isolated from pasteurised milk collected from dairy factories. This was validated in separate study by Ma et al. (2007) showing that *E. faecium* is more heat-tolerant when compared to *Salmonella* in ground beef, by about 17 times. However, the acquisition of heat resistance by these strains should be take into consideration, particularly among neonates in the NICU. Premature babies could be exposed to such organisms from contaminated milk, which includes ingestion of these organisms via feeding tubes. The risks of microorganisms carrying virulence genes, being highly resistant to a variety of antibiotics and involved in nosocomial infections, would seriously increase the concern to the neonate's health. A previous study by Khalkhali and Mojgani (2017) reported that virulence genes and biogenic amine genes were determined among *E. faecium* TA0033 and *E. faecalis* TA102 isolated from human milk. Although, some enterococci are used as probiotics, which is still controversial among this group, however, a number of human infections have been associated with species of the genus *Enterococcus*. Nevertheless, the frequent isolation of *E. faecium* from foods and human sources shows that ability of this organism to tolerate stresses during the processing of milk and food products or inside the human body.

Bacterial capsular polysaccharides are another important virulence of many microorganisms. They protect these microbes against environmental stress and allow bacteria to escape from the host immune system (Wilson et al., 2002; Ogrodzki and Forsythe, 2015). Bacterial capsule production was determined in the present study by colony appearance on two different growth media: soya and whey-based infant formula. The results showed *E. faecium* strains were unable to produce capsular material on both

types of media used in this study. Furthermore, they were able to produce a high amount of biofilm on the same media, although the results indicate no obvious link between biofilm formation in these strains and capsule production. Capsules may provide resistance to desiccation, serum activity, and play a significant role in evading phagocytic killing (Ogrodzki and Forsythe, 2015).

Another important virulence factor might include the ability of the organisms to attach to human epithelial cells. In this study, the attachment ability and invasion of human epithelial cells by *E. faecium* strains was assessed to determine the potential risk of infection to neonates in NICUs. Human epithelial cell lines are most commonly used as host models to examine host-pathogen interactions. The Caco-2 monolayer cell line was used to assess their adhesion and invasion potentials as it is one of the most commonly used as a model of the intestinal epithelium (Sambuy et al*.,* 2005). It has been used in a wide range of experimental bacterial infections. For instance, Aleksas et al*.* (2015) studied meningitic strains of *E. coli* K1 isolated from neonatal nasogastric feeding tubes using different cell lines, including Caco-2. In the present study, all strains from different sources showed high adherence to the Caco-2 cell line. This result is in agreement with Nami et al. (2015), who reported that *E. faecium* CM33 had high adhesive potential to Caco-2 cells. This indicates that the tested isolates possess a key factor to facilitate and contribute to adherence. Different components of surface molecules are required for mediating adhesion of microorganisms to the intestinal epithelium.

Adhesive matrix molecules, termed MSCRAMMs (microbial surface components recognizing adhesive matrix molecules), are recognised by several studies to play an important role of the cellular adhesion of *E. faecium*. For instance, the Acm collagen-binding adhesin of *E. faecium* contributes to the pathogenesis of experimental endocarditis. EcbA, *E. faecium* collagen binding protein A, and fibrinogen are able to recognise adhesive matrix molecules. Moreover, the *efaA* gene was hypothesised to be involved in cell wall adherence. (Hendrickx *et al.* 2009; Sava et al., 2010; Soheili et al., 2014, Yang et al., 2016). The present analysis showed that all of these genes were present in all *E. faecium* genomes. This suggests that these genes may play an important

role in their ability to adhere to Caco-2 cells, however, this isolate showed low invasion potential. Although slight variation in the ability of these isolates to invade Caco-2 cells was noted, no significant difference was observed. This suggests that these strains would be considered to have low invasive potential and their ability to attach to Caco-2 cells does not necessarily reflect their ability to invade cells.

These isolates demonstrated an ability to adhere to Caco-2 cells, produce high levels of biofilm, resist human serum and persist in low pH. Such properties may provide the reason explaining their persistence among the two neonates investigated during the period. Moreover, these strains harbour several virulence genes such as *acm, ebpA, ebpB, ebpC, ecbA, esp* and *sqrA*. These isolates compare to previously characterised pathogenic strains, including vancomycin-resistant *E. faecium* isolated from the bloodstream (Aus 0004), and a strain isolated from the blood of a patient with endocarditis (DO) and VREF bloodstream VREF isolate (Aus0085). The *E. faecium* strains isolated in this study were found to share similar virulence genes as mentioned previously, whilst these genes are not present in *E. faecium* T110, which is a probiotic strain.

This observation suggests that the existence of additional virulence could enhance the virulence, fitness and persistence of these isolates, as well as increase the incidence of infection. The acquisition of different virulence factors among multidrug-resistant strains possibly contributes to their emergence as prominent nosocomial pathogens, and furthermore, could be the reason behind their high prevalence among two neonates during the three-month period. Indeed, premature babies (twins) in neonatal intensive care units are more at risk of being colonised by multidrug-resistant strains. These neonates lack a developed immune system, have an undeveloped gut microflora, and are highly susceptible to infections. Moreover, prolonged hospitalisation of neonates in NICUs may present the opportunity of bacterial colonisation of feeding tubes. However, it was clear that *E. faecium* isolated in this study had the ability to colonise and persist in feeding tubes and faecal samples among both neonates over three months. This could contribute to hospital-acquired infections. Additionally, the SNP typing suggested that these strains had very low numbers of SNPs difference from each other, showed a

significant degree of homology between the strains. Profiling these isolates in this study also indicated that all isolates were typed as ST80 belonging to clonal complex (CC17) among both babies in the NICU. This finding strongly suggests that *E. faecium* isolated from feeding tubes and faecal samples from the two neonates had likely originated from the same source.

Chapter 6. Conclusions and Future Work

6.1. Conclusions

Preterm infants in Intensive Care Units (ICUs) are considered to be high-risk patients due to their increased susceptibility to infections during hospitalisation (Ramasethu, 2017) and exposure to harmful pathogens through various routes. There are several risk factors to neonatal infection, the most important of which would include the use of medical devices such as nasogastric enteral feeding tubes (NEFTs). These tubes may also contribute to colonisation of neonates by pathogenic microorganisms, as they are often required to feed new-borns (Hurrell et al., 2009a; Gómez et al., 2016). The colonisation of nasogastric enteral feeding tubes by opportunistic pathogens has not received much attention prior to this study. The accumulation of bacterial clumps inside feeding tubes from biofilm formation can act as a locus and it has been recognised as a possible source of infectious organisms. Ingestion of these organisms would likely increase the risk of neonatal infection.

- The first part of this study (chapter 3) involved profiling a unique clinical collection of *A. baumannii* isolated from neonatal feeding tubes from NICUs in two separate hospitals. Consequently, characterisation of a range of phenotypic and genotypic traits were applied to answer several key questions. This involved identifying the species, source and relatedness of these strains. Furthermore, it was determined whether some of the strains originated from common sources, such as being transferred to multiple patients within specific neonatal units. Moreover, we sought to evaluate the possible risk to neonates acquiring these organisms through feeding tubes.

All strains isolated from feeding tubes were identified as *A. baumannii* using *rpoB* sequencing. The representative strains were confirmed as *A. baumannii* through whole genome sequencing (WGS). The analyses revealed an agreement between the *rpoB* and WGS data, suggesting that the partial *rpoB* sequence is likely to be a reliable and rapid identification method for *Acinetobacter* species. The PFGE technique showed that all strains clustered into five different pulsotypes, with the majority of strains clustering with pulsotype Ac1. During a period of five months, strains of the same

pulsotype (Ac1) were isolated from both hospitals (KAH and PRH). This indicates the ability of these strains to survive and persist for long period of time, possibly in both biotic and abiotic environments. MLST analysis revealed 2 different STs (ST193 and ST113). However, PFGE differentiated the isolates into 5 distinct patterns, whereas MLST identified two different STs, suggesting that PFGE is a more discriminatory typing method than MLST for these isolates.

All strains showed a high level of biofilm formation and produced more biofilm at 37 °C than at 25 °C in infant formula. Biofilm formation in *A. baumannii* is therefore influenced by incubation temperature. This study also revealed that there was no correlation between biofilm formation and capsular material produced, as well as amount of cellulose produced. Multiple drug-resistant strains of *A. baumannii* were detected in this study. This illustrates the fact that the neonates were exposed to strains which were highly resistant to antibiotics. These strains also showed an ability to survive at low acidity (pH 3.5) for up to 2h, which may increase the risk of exposure to bacterial pathogens in neonates. Tolerance of desiccation, resistance to human serum, and high level of biofilm formation were also observed. These features enable the strains to cause infection as a result of ingestion. These results would indicate survival and persistence of these strains in hospitals where neonates are at high risk of nosocomial infection in NICUs.

The genome comparison study revealed that all sequenced ST113 isolates harboured genes of the *csu* cluster and were able to form biofilm and to adhere to the Caco2 cell line. Non ST113 strains lacked the *csu* locus and were poor biofilm formers with low Caco-2 attachment. There was a significant correlation between epithelial cell adherence and biofilm formation. Such traits may confer persistence and survival of these strains in neonatal intensive care units and colonisation in enteral feeding tubes. This could explain the ability of these isolates to successfully colonise neonatal feeding tubes and offers the opportunity for these isolates to survive and potentially cause subsequent infections in the neonate. The majority of these strains were able to persist and replicate inside macrophages for up to 72 hours of incubation and were observed to be moderately cytotoxic to Caco-2 cells. Such characteristics might enhance the ability

of these strains to evade the host immune response and cause bacteraemia. In addition, it can be expected that the pathogenic potential of *A. baumannii* could lead to infection of neonates in NICUs.

In general, these isolates were defined as multidrug-resistant and showed an ability to form significant amounts of biofilm. These isolates have also shown the ability to tolerate acidic conditions, desiccation, and survive in human serum. Furthermore, other important observations included their ability to attach and invade intestinal epithelial cells and persist inside macrophages. These features could allow them to survive and facilitate their persistence and transmission in the NICU. Additionally, contaminated neonatal feeding tubes could serve as reservoirs for pathogenic *A. baumannii*. This strongly indicates a possibility of neonatal infections resulting from the persistent exposure by ingestion in hospitals where the neonates are at high risk of nosocomial infection in NICUs.

- The second part of the study (chapter 4) aimed to profile a diverse clinical collection of *Enterobacter* species, in particular *E. hormaechei*, by applying a range of phenotyping and genotyping methods to elucidate the potential risk of these species to neonates in NICUs. Additionally, this part of the study aimed to evaluate possible correlations between *E. hormaechei* isolated from sepsis and neonatal feeding tubes and the frequency of virulence genes.

PFGE profiles indicates that all of the *Enterobacter* strains formed unique, distinguishable pulsotypes. These strains were isolated from an NICU during a 7-year period and were unlikely to be related to one another. *fusA* typing was able to identify *E. hormaechei* and *E. aerogenes*, but was not able to discriminate between the subspecies of *E. hormaechei*, while the rest of strains were not clearly identified and clustered within the *Enterobacter* genus. Misidentification is a problematic issue during clinical infection, particularly in NICUs, because it may result in inappropriate treatment of the disease and mislead the investigation and attribution of the source of infection. WGS was performed for accurate identification and typing of pathogens.

The quantity of biofilm formation was higher in infant formula when compared to TSB medium and higher at 37 °C compared to 25 °C. It was noted that the quantity of biofilm production was influenced by formula type and incubation temperature for the majority of tested isolates. Capsules and production of cellulose were confirmed to have no active role in biofilm production. In this study, the results also indicated no association between the morphology of *Enterobacter* isolates on Congo Red agar, which revealed Curli fimbriae expression.

The majority of *Enterobacter* species were able to produce capsular material on whey rather than soya-based infant formula. This finding may indicate the difference in composition of infant formula used possibly affects the production of capsular material. Protease and haemolysins were detected among the tested *Enterobacter* strains, which also, possess genes for enterobactin and aerobactin siderophore biosynthesis (*entABCD*E and *IucC*). This may enhance the ability of pathogenic bacteria to grow in a human host in conditions of low-level iron concentrations and facilitate replication during infections. All tested strains showed an ability to survive in human serum, suggesting this would be of increased clinical concern to neonates in the NICU, especially those with compromised immune systems, making them highly susceptible to infections.

Enterobacter were defined as multidrug-resistant and harboured resistance genes, such as *fosA*, *bla*ACT β-lactamase and efflux pump AcrAB-TolC. Among these isolates, only *E. hormaechei* subsp*. steigerwaltii* 1977 isolated from CSF showed resistance to all antibiotics used in this study and possessed more antimicrobial resistance genes. Such isolates raise concern, suggesting that more attention should be given to neonates in NICUs to control the spread of these MDR isolates, particularly among preterm and low birth-weight infants. These isolates demonstrated the ability to maintain their viability during a 14-day period of desiccation in infant formula. Furthermore, these isolates showed a clear resistance to exposure to pH 3.5 for 2 hours. All *E. hormaechei* isolates were found to harbour the *rpoS* gene which is associated with various stress responses. Moreover, isolates were shown to have different nucleotide sequences corresponding to this gene, possibly suggesting a positive correlation between bacterial acid tolerance and r*poS* activity.

This part of the study provided the first known investigation into the possible correlation between *E. hormaechei* isolated from sepsis and neonatal feeding tubes and how closely related they are. Also, it was essential to evaluate the potential risk to neonates acquiring organisms from enteral feeding tubes through ingestion of contaminated feed. This work has shown that feeding tube strains exhibited a similar behaviour to that observed in isolates from CSF and sepsis cases, as they have the ability to adhere to and invade Caco-2 and HBMEC cell lines. It was interesting to note that feeding tube isolates showed no significant difference when compared to sepsis isolates for their ability to attach to Caco-2. Also, these strains were able to persist and replicate inside macrophages for up to 72 hours of incubation and showed a similar level of MTT reduction to some sepsis strains. Other important observations included their ability to attach to and invade intestinal epithelial cells and persist inside macrophages. Feeding tube strains also shared the same virulence genes despite differing from each other by a very high number of SNPs (up to 66,582 SNPs). For example, curli fimbriae genes *csgABCD* have been detected in all assessed *E. hormaechei* strains. However, the CsgA protein is the major subunit of curlin, which has been linked to the ability of *E. hormaechei* to attach to Caco2 cell-lines. It is clear that these strains would have the ability to cause infections in neonates in NICUs. There are several reasons which may explain the potential risk of these species to neonates in NICUs. The isolates recovered from neonatal feeding tubes were defined as multidrug-resistant and showed an ability to form significant amounts of biofilm. These strains also showed an ability to tolerate acidic conditions, desiccation, and survive in human serum. It can be concluded these features could allow them to survive and facilitate their persistence and dispersal within the NICU. The results of this study indicate that these bacteria are potentially virulent and could present a significant risk to the health of premature neonates in the NICU.

- The third part of this study (chapter 5) focussed on analysis of samples collected from preterm infant twins during their hospitalisation in NICU. This longitudinal study of twin babies aimed to compare potentially pathogenic isolates within and between the feeding tubes and faeces of two twin babies over time. Their exposure to pathogens through different routes of transmission may result in a

potential infection. The level of exposure during the infant's hospitalisation is expected to influence the microbial colonisation in their gut.

The isolates collected from two premature babies were identified using 16S rRNA sequencing. Due to the limited resolution of this method, *E. faecium* isolates were selected as representative strains among the most common species isolated, and confirmed by Whole-genome sequencing to help provide a complete picture of diversity among the population.

SNP typing suggested that these strains had very low numbers of SNPs difference from each other and showed a significant degree of homology between the strains. Profiling the isolates in this part of the study revealed that all isolates were typed as ST80, belonging to clonal complex (CC17), among both babies in the NICU. This analysis showed that *E. faecium* isolated from feeding tubes and faecal samples from the two neonates had a remarkable degree of homology between them, suggesting that they likely originated from the same source.

All *E. faecium* strains demonstrated a high amount of biofilm formation. Indeed, the ability of bacteria to form biofilms inside feeding tubes are a major concern. It is believed that bacterial biofilms are able to persist on medical device surfaces and are an important risk factor for invasion and dissemination of microbes within the host, and therefore carries an increased risk of disease. All *E. faecium* strains in the present study had detectable lipolytic and proteolytic activity and were able to induce α-haemolysis. Additionally, these strains showed an ability to tolerate human serum. When taken together, these findings would suggest an increased clinical concern to neonates in NICUs with compromised immune systems, who are highly susceptible to infections.

Data from the present study indicated that all *E. faecium* strains harboured the *gltK* gene and were able to replicate in and resist conditions of low acidity. This suggests that this gene may contribute to resistance to acidic conditions and can be considered as a feature which may result in infections of neonates. Moreover, these strains showed an ability to tolerate heat at 55 °C for 30 minutes in liquid infant formula. These risks would seriously increase the concern to the neonate's health. Premature babies could be

exposed to such organisms from contaminated milk or the surrounding environment, which includes ingestion of these organisms via feeding tubes.

These isolates demonstrated an ability to adhere to Caco-2 cells, produce high levels of biofilm, resist human serum and persist in low pH conditions. Such properties may provide a reason to explain their persistence among the two neonates investigated during the study period. Moreover, these strains harboured several virulence-associated genes such as *acm, ebpA*, *ebpB, ebpC, ecbA, esp* and *sqrA*. This observation suggests that the existence of additional virulence factors could enhance the virulence, fitness and persistence of these isolates, as well as increase the incidence of infection. The acquisition of different virulence factors among multidrug-resistant strains could possibly contribute to their emergence as prominent nosocomial pathogens, and furthermore, could be the reason behind their high prevalence among the two neonates during the three-month period.

These results led to the conclusion that these strains isolated from different neonatal locations were indeed the same clone and were able to persist and be transferred between the two premature infants in the NICU. This suggests that the isolates recovered from feeding tubes and faecal samples among the two neonates were likely derived from the same local source. It was also concluded that the persistence of antibiotic-resistant *E. faecium*, acquired by premature infants during hospitalisation in the NICU, may lead to an increased risk among these neonates. The gut microbiota composition during the first 3 months may have important health consequences and may be linked to numerous problems later in life

In summary, the present study has provided additional further insight to support the existing knowledge of the important risk factors of these isolates with respect to neonatal infections in NICUs. This study has provided evidence of colonisation and persistence of opportunistic pathogens (belong to the ESKAPE group) in neonatal feeding tubes, which are important causes of nosocomial infection and dissemination of multidrug-resistant (MDR) strains. Feeding tubes act as a loci where bacterial cells can adhere, colonise, and produce multi-species biofilm. These bacteria are likely to be

resistant to different stress conditions and therefore pose a serious potential risk to neonates in NICUs.

6.2. Future directions

The present study focused on profiling a clinical collection of strains isolated from neonatal feeding tubes, faecal samples and sepsis samples from neonates in NICUs, to evaluate the important risk factors of these isolates with respect to neonatal infections in NICUs. However, further research is needed to extend our knowledge about physiological and virulence traits, and to find an effective method or policy that can prevent the spread of such strains among neonates in NICUs. Future work should focus on the following areas:

- Real-time qPCR analysis would be required to determine gene expression among examined isolates.
- Performing proteomic approaches to investigate the synthesis of virulence proteins and their mechanisms of action. Proteomic profiling may contribute to a better understanding of pathogen physiology.
- Knockout methods using gene deletion for studying genes to confirm the role of each gene in virulence-related traits.
- Characterisation of isolates obtained from samples taken from different sites in the hospital environment, as well as from carers and different milk samples, to identify potential reservoirs of infection.

The present PhD study has also provided important recommendations in controlling and preventing such strains from being transmitted between neonates, which may occur due to prolonged hospitalisation. For example, improving practices in the preparation of feed would be a significant step in controlling and preventing infection through milk sharing and contamination of equipment used in feed preparation. Additionally, heavy colonisation by pathogenic bacteria can be prevented by avoiding placement of enteral

feeding tubes for prolonged periods. Improved personal hygiene is also recommended, which is essential to preventing the growth and transmission of pathogens, and as a result, protect the babies in the NICU. Moreover, routine microbiological screening should be performed to detecting probable sources of pathogens, particularly in NICUs with preterm infants and low birthweight neonates in order to minimise and prevent the possible risk of pathogen transmission.

References

Abamecha, A., Wondafrash, B., & Abdissa, A. (2015). Antimicrobial resistance profile of *Enterococcus* species isolated from intestinal tracts of hospitalized patients in Jimma, Ethiopia. *BMC Research Notes*, *8*(1), 1–7. https://doi.org/10.1186/s13104-015-1200-2

Abdi-Ali, A., Hendiani, S., Mohammadi, P., & Gharavi, S. (2014). Assessment of biofilm formation and resistance to imipenem and ciprofloxacin among clinical isolates of *Acinetobacter baumannii* in Tehran. *Jundishapur Journal of Microbiology*, *7*(1), 1–5. https://doi.org/10.5812/jjm.8606

Abraham, C., & Medzhitov, R. (2011). Interactions between the host innate immune system and microbes in inflammatory bowel disease. *Gastroenterology*, *140*(6), 1729–1737. https://doi.org/10.1053/j.gastro.2011.02.012

Acker, J. Van, Smet, F. De, Muyldermans, G., Naessens, A., Lauwers, S., Muyldermans, T. a N., Smet, F. D. E. (2001). outbreak of necrotizing Enterocolitis associated with *Enterobacter sakazakii* in powdered milk formula. *Journal of Clinical Microbiology*, *39*(1), 7–12. https://doi.org/10.1128/JCM.39.1.293

Adesida, S. A., Ezenta, C. C., Adagbada, A. O., Aladesokan, A. A., & Coker, A. O. (2017). carriage of multidrug resistant *Enterococcus faecium* and *Enterococcus faecalis* among apparently healthy humans. *J. Infect. Dis*, *11*(2), 83–89. https://doi.org/10.21010/ajid.v11i2.11

Akineden, Ö., Heinrich, V., Gross, M., & Usleber, E. (2017). Reassessment of *Cronobacter* spp. originally isolated as *Enterobacter sakazakii* from infant food. *Food Microbiology*, *65*, 44–50. https://doi.org/10.1016/j.fm.2017.01.021

Al Samawi, M. S., Khan, F. Y., Eldeeb, Y., Almaslamani, M., Alkhal, A., Alsoub, H., … Hashim, S. (2016). *Acinetobacter* infections among adult patients in Qatar: A 2-year hospital-based study. *Canadian Journal of Infectious Diseases and Medical Microbiology*, 2016. https://doi.org/10.1155/2016/6873689

Alkeskas, A., Ogrodzki, P., Saad, M., Masood, N., Rhoma, N. R., Moore, K., … Forsythe, S. (2015). The molecular characterisation of *Escherichia coli* K1 isolated from neonatal nasogastric feeding tubes. *BMC Infectious Diseases*, 15, 449. https://doi.org/10.1186/s12879-015-1210-7

Allan, V. J. M., Callow, M. E., Macaskie, L. E., & Paterson-Beedle, M. (2002). Effect of nutrient limitation on biofilm formation and phosphatase activity of a *Citrobacter* sp. *Microbiology*, *148*(1), 277–288. https://doi.org/10.1099/00221287-148-1-277

Almajed, F. S., & Forsythe, S. J. (2016). *Cronobacter sakazakii* clinical isolates overcome host barriers and evade the immune response. *Microbial Pathogenesis*, *90*(11), 55–63. https://doi.org/10.1016/j.micpath.2015.11.014

Almasaudi, S. B. (2018). *Acinetobacter* spp. as nosocomial pathogens: Epidemiology and resistance features. *Saudi Journal of Biological Sciences*, *25*(3), 586–596. https://doi.org/10.1016/j.sjbs.2016.02.009

Alsan, M., & Klompas, M. (2010). *Acinetobacter baumannii*: An emerging and important pathogen. *Journal of Clinical Outcomes Management*, *17*(8), 27–35.

Álvarez-Ordóñez, A., Begley, M., & Hill, C. (2012). Polymorphisms in *rpos* and stress tolerance heterogeneity in natural isolates of *Cronobacter sakazakii*. *Applied and Environmental Microbiology*, *78*(11), 3975–3984. https://doi.org/10.1128/AEM.07835-11

Amábile-Cuevas, C. F., Arredondo-García, J. L., Cruz, A., & Rosas, I. (2010). Fluoroquinolone resistance in clinical and environmental isolates of *Escherichia coli* in Mexico City. *Journal of Applied Microbiology*, *108*(1), 158–162. https://doi.org/10.1111/j.1365-2672.2009.04401.x

Antunes, L. C. S., Imperi, F., Carattoli, A., & Visca, P. (2011). Deciphering the multifactorial nature of *Acinetobacter baumannii* pathogenicity. *PLoS ONE*, *6*(8). https://doi.org/10.1371/journal.pone.0022674

Antunes, L. C. S., Visca, P., & Towner, K. J. (2014). *Acinetobacter baumannii*: Evolution of a global pathogen. *Pathogens and Disease*, *71*(3), 292–301. https://doi.org/10.1111/2049-632X.12125

Aparna, M. S., & Yadav, S. (2008). Biofilms: microbes and disease. *Brazilian Journal of Infectious Diseases*, *12*(6), 526–530. https://doi.org/10.1590/S1413-86702008000600016

Asha, P., Jyothis, M., & Zacharia, S. (2005). A comparative study of virulence factors in enterococci from clinical and nonclinical sources. *Ournal of Pharmaceutical and Biomedical Sciences*, *26*(08), 304–307.

Araújo, B. C., Moraes, M. S., Costa, L. E. O., & Nascimento, J. S. (2015). Short communication: Multidrug-resistant *Acinetobacter baumannii-calcoaceticus* complex isolated from infant milk formula and utensils in a nursery in Rio de Janeiro, Brazil. *Journal of Dairy Science*, 98(4), 2303–2306. https://doi.org/10.3168/jds.2014-8825

Araújo, T. F., & Ferreira, C. L. de L. F. (2013). The genus *Enterococcus* as probiotic: Safety concerns. *Brazilian Archives of Biology and Technology*, *56*(3), 457–466. https://doi.org/10.1590/S1516-89132013000300014

Arenas, J. (2012). The role of bacterial lipopolysaccharides as immune modulator in vaccine and drug development. *Endocrine, Metabolic & Immune Disorders-Drug Targets*, *12*(3), 221–235. https://doi.org/10.2174/187153012802002884

Arias, C. A., & Murray, B. E. (2012). The rise of the *Enterococcus*: Beyond vancomycin resistance. *Nature Reviews Microbiology*, *10*(4), 266–278. https://doi.org/10.1038/nrmicro2761

Arowosegbe, A. O., Ojo, D. A., Dedeke, I. O., Shittu, O. B., & Akingbade, O. A. (2017). Neonatal sepsis in a Nigerian Tertiary Hospital: Clinical features, clinical outcome, aetiology and antibiotic susceptibility pattern. *Southern African Journal of Infectious Diseases*, *32*(4), 127–131. https://doi.org/10.1080/23120053.2017.1335962

Bahreini, Z., Hosseini, F., & Salehi, M. (2014). Molecular investigation of the factors Involved in the Biofilm Formation in clinical Isolates of *Acinetobacter* : *CsuA* / *BABCDE* Gene cluster. *Bulletin of Environment, Pharmacology and Life Sciences*, *3*(5), 37–42.

Balagurunathan, R., & Radhakrishnan, M. (2007). Microbial siderophores- gateway for iron removal. Envis Centre Newsletter, 5.

Bales, P. M., Renke, E. M., May, S. L., Shen, Y., & Nelson, D. C. (2013). Purification and characterization of biofilm-associated EPS Exopolysaccharides from ESKAPE organisms and other pathogens. *PLoS ONE*, *8*(6). https://doi.org/10.1371/journal.pone.0067950

Bardbari, A., Arabestani, M., Karami, M., Keramat, F Alikhani, Y., & Bagheri, K. (2017). Correlation between ability of biofilme formation with their responsible genes and MDR patterns in clinical and environmental *Acinetobacter baumannii* isolation. *Microbial pathogenesis*, 108(7), 122-128. https://doi: 10.1016/j.micpath.2017.04.039.

Barnhart, M. M., & Chapman, M. R. (2010). Curli biogenesis and function. *Annu Rev Microbiol*, *60*(1), 131–147. https://doi.org/10.1146/annurev.micro.60.080805.142106

Barwolff, S., Grundmann, H., Schwab, F., Tami, A., Behnke, M., Geffers, C., Halle, E., Göbel, U., & Gastmeier, P. (2005). Incidence of transmission of pathogens in intensive care units. *Anaesthesist*, 54(6), 560–6. https://doi.org/10.1007/s00101-005-0848-8

Barrett, E., Guinane, C. M., Ryan, C. A., Dempsey, E. M., Murphy, B. P., O'Toole, P. W., Stanton, C. (2013). Microbiota diversity and stability of the preterm neonatal ileum and colon of two infants. *MicrobiologyOpen*, *2*(2), 215–225. https://doi.org/10.1002/mbo3.64

Barron, J. C., & Forsythe, S. J. (2007). Dry stress and survival time of *Enterobacter sakazakii* and other Enterobacteriaceae in dehydrated powdered infant formula. *Journal of Food Protection*, *70*(9), 2111–7. https://doi.org/10.4315/0362-028X-70.9.2111

Basiri, B., Sabzehei, M. K., Shokouhi, M., & Moradi, A. (2015). Evaluating the Incidence and Risk Factors of Nosocomial Infection in Neonates Hospitalized in the Neonatal Intensive Care Unit of Fatemieh Hospital in Hamadan, Iran, 2012 - 2013. *Archives of Pediatric Infectious Diseases*, *3*(2). https://doi.org/10.5812/pedinfect.23327

Battesti, A., Majdalani, N., & Gottesman, S. (2011). The RpoS-mediated general stress response in *Escherichia coli*. *Annual Review of Microbiology*, *65*(1), 189–213. https://doi.org/10.1146/annurev-micro-090110-102946

Behnood, A., Farajnia, S., Moaddab, S. R., & Ahdi-khosroshahi, S. (2013). Tn5281 in *Enterococcus faecalis* and *Enterococcus faecium* isolates from Tabriz hospitals. *Iranian Journal of Microbiology*, *5*(3), 203–208.

Berne, C., Ducret, A., Hardy, G. G., & Brun, Y. V. (2015). Adhesins involved in attachment to abiotic surfaces by Gram- negative bacteria. *Microbiology Spectrum*, *3*(4), 1–45. https://doi.org/10.1128/microbiolspec.MB-0018-2015.

Beukers, A. G., Zaheer, R., Cook, S. R., Stanford, K., Chaves, A. V., Ward, M. P., & McAllister, T. A. (2015). Effect of in-feed administration and withdrawal of tylosin phosphate on antibiotic resistance in enterococci isolated from feedlot steers. *Frontiers in Microbiology*, *6*(MAY), 1–14. https://doi.org/10.3389/fmicb.2015.00483

Bezirtzoglou E., Tsiotsias A., Welling G. W. (2011). Microbiota profile in feces of breast- and formula-fed newborns by using fluorescence in situ hybridization (FISH). *Anaerobe,* 17(6):478-82. https://doi: 10.1016/j.anaerobe.2011.03.009.

Billington, E. O., Phang, S. H., Gregson, D. B., Pitout, J. D. D., Ross, T., Church, D. L., … Parkins, M. D. (2014). Incidence, risk factors, and outcomes for *Enterococcus* spp. blood stream infections: A population-based study. *International Journal of Infectious Diseases*, *26*, 76–82. https://doi.org/10.1016/j.ijid.2014.02.012

Biswas, P. P., Dey, S., Sen, A., & Adhikari, L. (2016). Molecular characterization of virulence genes in vancomycin-resistant and vancomycin-sensitive Enterococci. *Journal of Global Infectious Diseases*, 8(1), 16–24. http://doi.org/10.4103/0974-777X.176141

Blanco, P., Hernando-Amado, S., Reales-Calderon, J., Corona, F., Lira, F., Alcalde-Rico, M., Martinez, J. (2016). Bacterial multidrug efflux pumps: much more than antibiotic resistance determinants. *Microorganisms*, *4*(1), 14. https://doi.org/10.3390/microorganisms4010014

Blanco, N., Harris, A. D., Rock, C., Johnson, J. K., Pineles, L., Bonomo, R. A., … Thom, K. A. (2018). Risk factors and outcomes associated with multidrug-resistant *Acinetobacter baumannii* upon Intensive Care Unit Admission. *Antimicrobial Agents and Chemotherapy*, 62(1), e01631–17. http://doi.org/10.1128/AAC.01631-17

Boban, N., Jerončič, A., & Punda-Polič, V. (2011). Outbreak of nosocomial bacteremias, caused by enterobacter gergoviae and *enterobacter aerogenes*, in the neonatal intensive care unit, case-control study. *Signa Vitae*, 6(1), 27–32. https://doi.org/10.22514/SV61.052011.4

Bong, R. (2006). Pulse field gel electrophoresis for comparative studies of bacteria diversity. *Journal of Experimental Microbiology and Immunology*, 10(12), 50–55. Retrieved from https://www.microbiology.ubc.ca/sites/default/files/roles/drupal%7B_%7Dungrad/JEMI/10/10-50.pdf

Boucher, H. W., Talbot, G. H., Bradley, J. S., Edwards, J. E., Gilbert, D., Rice, L. B., … Bartlett, J. (2009). Bad Bugs, no drugs: no ESKAPE! an update from the Infectious Diseases Society of America. *Clinical Infectious Diseases*, *48*(1), 1–12. https://doi.org/10.1086/595011

Breeuwer, P., Lardeau, A., Peterz, M., & Joosten, H. M. (2003). Desiccation and heat tolerance of *Enterobacter sakazakii. Journal of Applied Microbiology*, *95*(5), 967–973. https://doi.org/10.1046/j.1365-2672.2003.02067.x

Brooks, B., Firek, B. A., Miller, C. S., Sharon, I., Thomas, B. C., Baker, R., … Banfield, J. F. (2014). Microbes in the neonatal intensive care unit resemble those found in the gut of premature infants. *Microbiome*, *in press*, 1–16.

Brossard, K. A., & Campagnari, A. A. (2012). The *Acinetobacter baumannii* biofilm-associated protein plays a role in adherence to human epithelial cells. *Infection and Immunity*, *80*(1), 228–233. https://doi.org/10.1128/IAI.05913-11

Brusselaers, N., Vogelaers, D., & Blot, S. (2011). The rising problem of antimicrobial resistance in the intensive care unit. *Annals of Intensive Care*, *1*(1), 47. https://doi.org/10.1186/2110-5820-1-47

Bull, M. J., & Plummer, N. T. (2014). Part 1: The Human Gut Microbiome in Health and Disease. *Integrative Medicine (Encinitas, Calif.)*, *13*(6), 17–22. https://doi.org/26770121

Byappanahalli, M. N., Nevers, M. B., Korajkic, A., Staley, Z. R., & Harwood, V. J. (2012). Enterococci in the environment. *Microbiology and Molecular Biology Reviews*, *76*(4), 685–706. https://doi.org/10.1128/MMBR.00023-12

Cahenzli, J., Köller, Y., Wyss, M., Geuking, M. B., & McCoy, K. D. (2013). Intestinal microbial diversity during early-life colonization shapes long-term IgE levels. *Cell Host and Microbe*, *14*(5), 559–570. https://doi.org/10.1016/j.chom.2013.10.004

Cai, B., Echols, R., Magee, G., Arjona Ferreira, J. C., Morgan, G., Ariyasu, M., … Nagata, T. "Den." (2017). Prevalence of carbapenem-resistant Gram-negative infections in the United States predominated by *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. *Open Forum Infectious Diseases*, *4*(3), 1–7. https://doi.org/10.1093/ofid/ofx176

Camacho-gonzalez, A., Spearman, P. W., Diseases, P. I., Stoll, B. J., Brumley, G. W., & Drive, U. (2015). Neonatal infections Disease: evuluation of neonatal sepsis. *Pediatric Clinical North America North America*, *60*(2), 367–389. https://doi.org/10.1016/j.pcl.2012.12.003.Neonatal

Camarena, L., Bruno, V., Euskirchen, G., Poggio, S., & Snyder, M. (2010). Molecular mechanisms

of ethanol-induced pathogenesis revealed by RNA-sequencing. *PLoS Pathogens*, *6*(4), 22-27. https://doi.org/10.1371/journal.ppat.1000834

Campos, L. C., Lobianco, L. F., Seki, L. M., Santos, M. R., & Asensi, M. D. (2007). Outbreak of *Enterobacter hormaechei* septicaemia in new-borns caused by contaminated parenteral nutrition in Brazil. J*ournal of Hospital Infection*, 66 (1), 95-97. https://doi.org/10.1016/j.jhin.2007.02.013

Carding, S., Verbeke, K., Vipond, D. T., Corfe, B. M., & Owen, L. J. (2015). Dysbiosis of the gut microbiota in disease. *Microbial Ecology in Health & Disease*, *26*(0), 1–9. https://doi.org/10.3402/mehd.v26.26191

Cattoir, V. & Giard, J. C. (2014). Antibiotic resistance in *Enterococcus faecium* clinical isolates. *Expert Review of Anti-Infective Therapy*, 2(12), 239-24. https://doi.org/10.1586/14787210.2014.870886

Carmona, F., Prado, S. I., Silva, M. F. I., Gaspar, G. G., Bellissimo-Rodrigues, F., Martinez, R., Carlotti, A. P. C. P. (2012). Vancomycin-resistant *Enterococcus* outbreak in a pediatric intensive care unit: report of successful interventions for control and prevention. *Brazilian Journal of Medical and Biological Research*, *45*(2), 158–162. https://doi.org/10.1590/S0100-879X2012007500005

Carvalho-Assef, A. P. D. A., Pereira, P. S., Albano, R. M., Berião, G. C., Tavares, C. P., Chagas, T. P. G., … Asensi, M. D. (2014). Detection of NDM-1-, CTX-M-15-, and qnrB4-producing *enterobacter hormaechei* isolates in Brazil. *Antimicrobial Agents and Chemotherapy*, *58*(4), 2475–2476. https://doi.org/10.1128/AAC.02804-13

Carver, T. J., Rutherford, K. M., Berriman, M., Rajandream, M. A., Barrell, B. G., & Parkhill, J. (2005). ACT: The Artemis comparison tool. *Bioinformatics*, 21(16), 3422–3423. https://doi.org/10.1093/bioinformatics/bti553

Castilho, S. R. A., Godoy, C. S. D. M., Guilarde, A. O., Cardoso, J. L., André, M. C. P., Junqueira-Kipnis, A. P., & Kipnis, A. (2017). *Acinetobacter baumannii* strains isolated from patients in intensive care units in Goiânia, Brazil: Molecular and drug susceptibility profiles. *PLoS ONE*, *12*(5), 1–13. https://doi.org/10.1371/journal.pone.0176790

Caubilla-barron, J. & Forsythe, S. (2007). Dry stress and survival time of *Enterobacter sakazakii* and other *Enterobacteriaceae*. *Journal of Food Protection*, 70(9), 2111- 2117.

Caubilla-Barron, J., Hurrell, E., Townsend, S., Cheetham, P., Loc-Carrillo, C., Fayet, O., … Forsythe, S. J. (2007). Genotypic and phenotypic analysis of *Enterobacter sakazakii* strains from an outbreak resulting in fatalities in a neonatal intensive care unit in France. *Journal of Clinical Microbiology*, *45*(12), 3979–3985. https://doi.org/10.1128/JCM.01075-07

Cauwerts, K., Decostere, A., De Graef, E. M., Haesebrouck, F., & Pasmans, F. (2007). High prevalence of tetracycline resistance in *Enterococcus* isolates from broilers carrying the *erm*(B) gene. *Avian Pathology*, *36*(5), 395–399. https://doi.org/10.1080/03079450701589167

Cayô, R., Rodrigues-Costa, F., Matos, A. P., Carvalhaes, C. G., Jové, T., & Gales, A. C. (2015). Identification of a new integron harboring *bla* $_{IMP-10}$ in carbapenem-resistant *Acinetobacter baumannii* clinical isolates. *Antimicrobial Agents and Chemotherapy*, *59*(6), 3687–3689. https://doi.org/10.1128/AAC.04991-14

Caza, M., & Kronstad, J. W. (2013). Shared and distinct mechanisms of iron acquisition by bacterial and fungal pathogens of humans. *Frontiers in Cellular and Infection Microbiology*,

3(11), 1–23. https://doi.org/10.3389/fcimb.2013.00080

Cetinkaya, Y., Falk, P., & Mayhall, C. G. (2000). Vancomycin-resistant enterococci. *Clinical Microbiology Reviews*, *13*(4), 686–707. https://doi.org/10.1128/CMR.13.4.686-707.2000

Chagnot, C., Zorgani, M. A., Astruc, T., & Desvaux, M. (2013). Proteinaceous determinants of surface colonization in bacteria: bacterial adhesion and biofilm formation from a protein secretion perspective. *Frontiers in Microbiology*, *4*(10), 1–26. https://doi.org/10.3389/fmicb.2013.00303

Chan, J. Z. M., Halachev, M. R., Loman, N. J., Constantinidou, C., & Pallen, M. J. (2012). Defining bacterial species in the genomic era: Insights from the genus *Acinetobacter*. *BMC Microbiology*, *12*(1), 1. https://doi.org/10.1186/1471-2180-12-302

Chang, K. M., Huang, W. C., Chiou, C. S., Shen, G. H., Huang, C. C., & Wen, F. S. (2013). Suitable restriction enzyme for standardization of pulsed-field gel electrophoresis protocol and interlaboratory comparison of *Acinetobacter baumannii*. *Journal of Microbiology, Immunology and Infection*, *46*(3), 195–201. https://doi.org/10.1016/j.jmii.2012.05.011

Chang, Y., Luan, G., Xu, Y., Wang, Y., Shen, M., Zhang, C., … Ling, B. (2015). Characterization of carbapenem-resistant *Acinetobacter baumannii* isolates in a Chinese teaching hospital. *Frontiers in Microbiology*, *6*(9), 1–9. https://doi.org/10.3389/fmicb.2015.00910

Chapartegui-González, I., Lá zaro-Díez, M., Bravo, Z., Navas, J., Icardo, J. M., & Ramos-Vivas, J. (2018). *Acinetobacter baumannii* maintains its virulence after long-time starvation. *PLoS ONE*, *13*(8), 1–14. https://doi.org/10.1371/journal.pone.0201961

Cheah, A. L. Y., Spelman, T., Liew, D., Peel, T., Howden, B. P., Spelman, D., … Kong, D. C. M. (2013). Enterococcal bacteraemia: Factors influencing mortality, length of stay and costs of hospitalization. *Clinical Microbiology and Infection*, *19*(4), 181–189. https://doi.org/10.1111/1469-0691.12132

Chen, H. L., Lu, J. H., Wang, H. H., Chen, S. J., Chen, C. J., Wu, K. G., & Tang, R. Bin. (2014). Clinical analysis of *Enterobacter* bacteremia in pediatric patients: a 10-year study. *Journal of Microbiology, Immunology, and Infection*, *47*(5), 381–386. https://doi.org/10.1016/j.jmii.2013.03.016

Chen, H. N., Lee, M. L., Yu, W. K., Lin, Y. W., & Tsao, L. Y. (2009). Late-Onset *Enterobacter cloacae* sepsis in very-low-birth-weight neonates: experience in a medical center. *Pediatrics and Neonatology*, *50*(1), 3–7. https://doi.org/10.1016/S1875-9572(09)60022-X

Chen, L., & Wen, Y. (2011). The role of bacterial biofilm in persistent infections and control strategies. *International Journal of Oral Science*, *3*(2), 66–73. https://doi.org/10.4248/IJOS11022

Chen, W., Fang, T., Zhou, X., Zhang, D., Shi, X., & Shi, C. (2016). IncHI2 plasmids are predominant in antibiotic-resistant *Salmonella* isolates. *Frontiers in Microbiology*, *7*(9), 1–10. https://doi.org/10.3389/fmicb.2016.01566

Chen, Y., Frazzitta, A. E., Litvintseva, A. P., Fang, C., Mitchell, T. G., Springer, D. J., … Perfect, J. R. (2015). Next generation multilocus sequence typing (NGMLST) and the analytical software program MLSTEZ enable efficient, cost-effective, high-throughput, multilocus sequencing typing. *Fungal Genetics and Biology*, *75*, 64–71. https://doi.org/10.1016/j.fgb.2015.01.005

Cho, G. S., Li, B., Rostalsky, A., Fiedler, G., Rösch, N., Igbinosa, E., … Franz, C. M. A. P. (2018). Diversity and antibiotic susceptibility of *Acinetobacter* strains from milk powder produced

in Germany. *Frontiers in Microbiology*, *9*(3), 1–12. https://doi.org/10.3389/fmicb.2018.00536

Choi, A. H. K., Slamti, L., Avci, F. Y., Pier, G. B., & Maira-Litrán, T. (2009). The pgaABCD locus of *Acinetobacter baumannii* encodes the production of poly-β-1-6-N-acetylglucosamine, which is critical for biofilm formation. *Journal of Bacteriology*, *191*(19), 5953–5963. https://doi.org/10.1128/JB.00647-09

Choi, C. H., Lee, E. Y., Lee, Y. C., Park, T. I., Kim, H. J., Hyun, S. H., … Lee, J. C. (2005). Outer membrane protein 38 of *Acinetobacter baumannii* localizes to the mitochondria and induces apoptosis of epithelial cells. *Cellular Microbiology*, *7*(8), 1127–1138. https://doi.org/10.1111/j.1462-5822.2005.00538.x

Chollet, R., Bollet, C., Chevalier, J., Malléa, M., Pagès, J., & Davin-regli, A. (2002). *mar* operon involved in multidrug resistance of *Enterobacter aerogenes. Antimicrobial Agents and Chemotherapy, 46*(4), 1093–1097. https://doi.org/10.1128/AAC.46.4.1093

Chong, C. Y. L., Bloomfield, F. H., & O'Sullivan, J. M. (2018). Factors affecting gastrointestinal microbiome development in neonates. *Nutrients*, *10*(3), 1–17. https://doi.org/10.3390/nu10030274

Christaki, E., & Giamarellos-Bourboulis, E. J. (2014). The complex pathogenesis of bacteremia: From antimicrobial clearance mechanisms to the genetic background of the host. *Virulence*, *5*(1), 57–65. https://doi.org/10.4161/viru.26514

Christina, N., Ioanna, P., & George, L. (2015). Risk factors for nosocomial infections in neonatal intensive care units. *Health science journal*, *9*(29), 11–14.

Cilo, B. D., Ağca, H., Efe, K., Sınırtaş, M., Çelebi, S., Özkan, H., … Özakın, C. (2014). Investigation of vancomycin resistant *Enterococcus faecium* outbreak in neonatal intensive care unit. *International Journal of Clinical and Experimental Medicine*, *7*(12), 5342–5347.

Cordeiro, M. A., Werle, C. H., Milanez, G. P., & Yano, T. (2016). Curli fimbria: An *E. coli* adhesin associated with human cystitis. *Brazilian Journal of Microbiology*, *47*(2), 414–416. https://doi.org/10.1016/j.bjm.2016.01.024

Cortese, F., Scicchitano, P., Gesualdo, M., Filaninno, A., De Giorgi, E., Schettini, F., Ciccone, M. M. (2016). Early and late infections in newborns: where do we stand a review. *Pediatrics and Neonatology*, *57*(4), 265–273. https://doi.org/10.1016/j.pedneo.2015.09.007

Cossey, V., Jeurissen, A., Thelissen, M. J., Vanhole, C., & Schuermans, A. (2011). Expressed breast milk on a neonatal unit: A hazard analysis and critical control points approach. *American Journal of Infection Control*, *39*(10), 832–838. https://doi.org/10.1016/j.ajic.2011.01.019

Cox, C. R., Coburn, P. S., & Gilmore, M. S. (2005). Enterococcal cytolysin: a novel two component peptide system that serves as a bacterial defense against eukaryotic and prokaryotic cells. *Current Protein & Peptide Science*, *6*(1), 77–84. https://doi.org/10.2174/1389203053027557

Craven, H. M., McAuley, C. M., Duffy, L. L., & Fegan, N. (2010). Distribution, prevalence and persistence of *Cronobacter* (*Enterobacter sakazakii*) in the nonprocessing and processing environments of five milk powder factories. *Journal of Applied Microbiology*, *109*(3), 1044–1052. https://doi.org/10.1111/j.1365-2672.2010.04733.x

Cress, B. F., Englaender, J. A., He, W., Kasper, D., Linhardt, R. J., & Koffas, M. A. G. (2014). Masquerading microbial pathogens: Capsular polysaccharides mimic host-tissue molecules.

FEMS Microbiology Reviews, *38*(4), 660–697. https://doi.org/10.1111/1574-6976.12056

Culp, E., & Wright, G. D. (2017). Bacterial proteases, untapped antimicrobial drug targets. *Journal of Antibiotics*, *70*(4), 366–377. https://doi.org/10.1038/ja.2016.138

Daniel, D. S., Lee, S. M., Dykes, G. A., & Rahman, S. (2015). Public health risks of multiple-drug-resistant *Enterococcus* spp. in Southeast Asia. *Applied and Environmental Microbiology*, *81*(18), 6090–6097. https://doi.org/10.1128/AEM.01741-15

Davin-Regli, A., & Pagès, J. M. (2015). *Enterobacter aerogenes* and *Enterobacter cloacae*; Versatile bacterial pathogens confronting antibiotic treatment. *Frontiers in Microbiology*, *6*(MAY), 1–10. https://doi.org/10.3389/fmicb.2015.00392

Davis, M. a, Hancock, D. D., Besser, T. E., & Call, D. R. (2003). Evaluation of pulsed-field gel electrophoresis as a tool for determining the degree of genetic relatedness between strains of *Escherichia coli* O157 : H7. *Journal of Clinical Microbiology*, *41*(5), 1843–1849. https://doi.org/10.1128/JCM.41.5.1843

De Maayer, P., & Cowan, D. A. (2016). Flashy flagella: Flagellin modification is relatively common and highly versatile among the Enterobacteriaceae. *BMC Genomics*, *17*(1), 1–13. https://doi.org/10.1186/s12864-016-2735-x

Deplano, A., Denis, O., Nonhoff, C., Rost, F., Byl, B., Jacobs, F., … Struelens, M. J. (2007). Outbreak of hospital-adapted clonal complex-17 vancomycin-resistant *Enterococcus faecium* strain in a haematology unit: Role of rapid typing for early control. *Journal of Antimicrobial Chemotherapy*, *60*(4), 849–854. https://doi.org/10.1093/jac/dkm270

De Tejada, G. M., Heinbockel, L., Ferrer-Espada, R., Heine, H., Alexander, C., Bárcena-Varela, S., … Brandenburg, K. (2015). Lipoproteins/peptides are sepsis-inducing toxins from bacteria that can be neutralized by synthetic anti-endotoxin peptides. *Scientific Reports*, *5*(1), 1–15. https://doi.org/10.1038/srep14292

Deshpande, L. M., Fritsche, T. R., Moet, G. J., Biedenbach, D. J., & Jones, R. N. (2007). Antimicrobial resistance and molecular epidemiology of vancomycin-resistant enterococci from North America and Europe: a report from the SENTRY antimicrobial surveillance program. *Diagnostic Microbiology and Infectious Disease*, *58*(2), 163–170. https://doi.org/10.1016/j.diagmicrobio.2006.12.022

Deurenberg, R. H., Bathoorn, E., Chlebowicz, M. A., Couto, N., Ferdous, M., García-Cobos, S., Rossen, J. W. A. (2017). Application of next generation sequencing in clinical microbiology and infection prevention. *Journal of Biotechnology*, *243*, 16–24. https://doi.org/10.1016/j.jbiotec.2016.12.022

DiazGranados, C. A., & Jernigan, J. A. (2005). Impact of vancomycin resistance on mortality among patients with neutropenia and enterococcal bloodstream infection. *The Journal of Infectious Diseases*, *191*(4), 588–95. https://doi.org/10.1086/427512

Dobrić, H., Milas, V., & Milas, K. (2016). Late onset perinatal sepsis in the neonatology intensive care unit – risk factors. *Signa Vitae*, 11(2), 57–60. https://doi.org/10.22514/SV112.062016.12

Dong, Y., & Speer, C. P. (2015). Late-onset neonatal sepsis:Recent developments. *Archives of Disease in Childhood: Fetal and Neonatal Edition*, *100*(3), 257–263. https://doi.org/10.1136/archdischild-2014-306213

Doran, K. S., Banerjee, A., Disson, O., & Lecuit, M. (2013). Crossing host barriers. *Cold Spring*

Harb Perspect Med, *10*, 1–19. https://doi.org/doi: 10.1101/cshperspect.a010090

Drell, T., Lutsar, I., Štšepetova, J., Parm, Ü., Metsvaht, T., Ilmoja, M. L., Sepp, E. (2014). The development of gut microbiota in critically ill extremely low birth weight infants assessed with 16S rRNA gene based sequencing. *Gut Microbes*, *5*(3), 304-312. https://doi.org/10.4161/gmic.28849

Drudy, D., Mullane, N. R., Quinn, T., Wall, P. G., & Fanning, S. (2006). *Enterobacter sakazakii*: An emerging pathogen in powdered infant formula. *Clinical Infectious Diseases*, *42*(7), 996–1002. https://doi.org/10.1086/501019

Du, X.-J., Zhang, X., Li, P., Xue, R., & Wang, S. (2016). Screening of genes involved in interactions with intestinal epithelial cells in Cronobacter sakazakii. *AMB Express*, *6*(1), 16-24. https://doi.org/10.1186/s13568-016-0246-4

Edelson-Mammel, S., Porteous, M. & Buchanan, R. (2006). Acid resistance of twelve strains of *Enterobacter sakazakii*, and the impact of habituating the cells to an acidic environment. *Journal of Food Science*, 71(6), 201-207.

Eijkelkamp, B. A., Hassan, K. A., Paulsen, I. T., & Brown, M. H. (2011). Investigation of the human pathogen *Acinetobacter baumannii* under iron limiting conditions. *BMC Genomics*, *12*(1), 126-130. https://doi.org/10.1186/1471-2164-12-126

El Ashkar, S., Osman, M., Rafei, R., Mallat, H., Achkar, M., Dabboussi, F., & Hamze, M. (2017). Molecular detection of genes responsible for macrolide resistance among *Streptococcus pneumoniae* isolated in North Lebanon. *Journal of Infection and Public Health*, *10*(6), 745–748. https://doi.org/10.1016/j.jiph.2016.11.014

El-Mahallawy, H. A., Hassan, S. S., El-Wakil, M., & Moneer, M. M. (2016). Bacteremia due to ESKAPE pathogens: An emerging problem in cancer patients. *Journal of the Egyptian National Cancer Institute*, *28*(3), 157–162. https://doi.org/10.1016/j.jnci.2016.05.002

El-Shazly, S., Dashti, A., Vali, L., Bolaris, M., & Ibrahim, A. S. (2015). Molecular epidemiology and characterization of multiple drug-resistant (MDR) clinical isolates of *Acinetobacter baumannii*. *International Journal of Infectious Diseases*, *41*, 42–49. https://doi.org/10.1016/J.IJID.2015.10.016

Engür, D., Çakmak, C. C.,Türkmen,K. M., Telli, M., Eyigör, M., & Güzünler, M. (2014). A milk pump as a source for spreading *Acinetobacter baumannii* in a neonatal intensive care unit. *Breastfeed Medicice*, 9(10), 551–554. https://doi.10.1089/bfm.2014.0054

Espinal, P., Martí, S., & Vila, J. (2012). Effect of biofilm formation on the survival of *Acinetobacter baumannii* on dry surfaces. *Journal of Hospital Infection*, *80*(1), 56–60. https://doi.org/10.1016/j.jhin.2011.08.013

European Centre for Disease Prevention and Control. (2017). *Surveillance of antimicrobial resistance in Europe 2016. Annual report of the European Antimicrobial REsistance Surveillance Network (EARS-Net)*. https://doi.org/10.2900/296939

European Centre for Disease Prevention and Control. (2018). Multi-country outbreak of *Salmonella* Agona infections linked to infant formula. *EFSA Supporting Publications*, *15*(1). https://doi.org/10.2903/sp.efsa.2018.EN-1365

Evans, N., & Rutter, N. (1986). Development of the epidermis in the newborn. *Neonatology*, 49 (2), 74-80. https://dio:10.1159/000242513

Faron, M. L., Ledeboer, N. A., & Buchan, B. W. (2016). Resistance Mechanisms, Epidemiology,

and Approaches to Screening for Vancomycin-Resistant *Enterococcus* in the Health Care Setting. *Journal of Clinical Microbiology*, *54*(10), 2436–2447. https://doi.org/10.1128/JCM.00211-16.

FAO/WHO. (2004). *Enterobacter sakazakii* and other microorganisms in powderedinfant formula milk, (2), 98–101.

FAO/WHO. (2006). 10 Informe de reunión.*Enterobacter sakazakii* and *Salmonella* in powdered infant formula. Retrieved from http://www.fao.org/3/a-a0707e.pdf

Fei, P., Jiang, Y., Feng, J., Forsythe, S. J., Li, R., Zhou, Y., & Man, C. (2017). Antibiotic and desiccation resistance of *Cronobacter sakazakii* and *C. malonaticus* isolates from powdered infant formula and processing environments. *Frontiers in Microbiology*, *8*(3), 1–8. https://doi.org/10.3389/fmicb.2017.00316

Feizabadi, M. M., Fathollahzadeh, B., Taherikalani, M., Rasoolinejad, M., Sadeghifard, N., Aligholi, M., … Mohammadi-Yegane, S. (2008). Antimicrobial susceptibility patterns and distribution of *blaOXA* genes among *Acinetobacte*r spp. Isolated from patients at Tehran hospitals. *Japanese Journal of Infectious Diseases*, *61*, 274–278.

Ferguson, D. M., Talavera, G. N., Hernández, L. A. R., Weisberg, S. B., Ambrose, R. F., & Jay, J. A. (2016). Virulence genes among *Enterococcus faecalis* and *Enterococcus faecium* isolated from coastal beaches and human and nonhuman sources in southern California and Puerto Rico. *Journal of Pathogens*, 21(3), 1–7. https://doi.org/10.1155/2016/3437214

Ferreirinha, P., Pérez-Cabezas, B., Correia, A., Miyazawa, B., França, A., Carvalhais, V., … Vilanova, M. (2016). Poly- N -acetylglucosamine production by *Staphylococcus epidermidis* cells increases their in vivo proinflammatory effect. *Infection & Immunity*, *84*(10), 2933–2943. https://doi.org/10.1128/IAI.00290-16.Editor

Figueroa, J., and Densen, P. (1991). Infectious diseases associated with complement deficiencies. *Clinical Microbiology Reviews*, 4 (3), 359-395.

Finlay, B. B., & McFadden, G. (2006). Anti-immunology: Evasion of the host immune system by bacterial and viral pathogens. *Cell*, *124*(4), 767–782. https://doi.org/10.1016/j.cell.2006.01.034

Fisher, K., & Phillips, C. (2009). The ecology, epidemiology and virulence of *Enterococcus*. *Microbiology*, 155(6), 1749–1757. https://doi.org/10.1099/mic.0.026385-0

Foulquie, M. R., Sarantinopoulos P., Tsakalidou E., & De Vuyst L. (2006). The role and application of enterococci in food and health. *Intternational Journals Food Microbiology,* 106(1):1-24 https://doi.10.1016/j.ijfoodmicro.2005.06.026

Francolini, I., & Donelli, G. (2010). Prevention and control of biofilm-based medical-device-related infections. *FEMS Immunology and Medical Microbiology*, *59*(3), 227–238. https://doi.org/10.1111/j.1574-695X.2010.00665.x

Freitas, A. R., Tedim, A. P., Francia, M. V., Jensen, L. B., Novais, C., Peixe, L., … Coque, T. M. (2016). Multilevel population genetic analysis of *vanA* and *vanB Enterococcus faecium* causing nosocomial outbreaks in 27 countries (1986-2012). *Journal of Antimicrobial Chemotherapy*, *71*(12), 3351–3366. https://doi.org/10.1093/JAC/DKW312

Froh, E. B., Vanderpool, J., & Spatz, D. L. (2018). Best practices to limit contamination of donor milk in a milk bank. *JOGNN - Journal of Obstetric, Gynecologic, and Neonatal Nursing*, *47*(4), 547–555. https://doi.org/10.1016/j.jogn.2017.12.002

Furumura, M. T., Figueiredo, P. M. S., Carbonell, G. V., Da Costa Darini, A. L., & Yano, T. (2006). Virulence-associated characteristics of *Enterococcus faecalis* strains isolated from clinical sources. *Brazilian Journal of Microbiology*, *37*(3), 230–236. https://doi.org/10.1590/S1517-83822006000300007

Gaddy, J. A., Tomaras, A. P., & Actis, L. A. (2009). The *Acinetobacter baumannii* 19606 OmpA protein plays a role in biofilm formation on abiotic surfaces and in the interaction of this pathogen with eukaryotic cells. *Infection and Immunity*, *77*(8), 3150–3160. https://doi.org/10.1128/IAI.00096-09

Gallego, L. (2016). *Acinetobacter baumannii*: Factors involved in its high adaptability to adverse environmental conditions. *Journal of Microbiology & Experimentation*, *3*(2), 2–5. https://doi.org/10.15406/jmen.2016.03.00085

Gao, L., Lyu, Y., & Li, Y. (2017). Trends in drug resistance of *Acinetobacter baumannii* over a 10-year period: Nationwide data from the China surveillance of antimicrobial resistance program. *Chinese Medical Journal*, *130*(6), 659–664. https://doi.org/10.4103/0366-6999.201601

Gastmeier, P., Loui, A., Stamm-Balderjahn, S., Hansen, S., Zuschneid, I., Sohr, D., … Rüden, H. (2007). Outbreaks in neonatal intensive care units-They are not like others. *American Journal of Infection Control*, *35*(3), 172–176. https://doi.org/10.1016/j.ajic.2006.07.007

Garg, s., Mohan, B., & Taneja, N. (2017). Biofilm formation capability of enterococcal strains causing urinary tract infection vis-a-vis colonisation and correlation with enterococcal surface protein gene. *Journal of Medical Microbiology*, 35(1): 48–52. https://doi.10.4103/ijmm.IJMM_16_102

Garrido, M. A., Gálvez, A., & Pérez Pulido, R. (2014). Antimicrobial resistance in Enterococci. *J Infect Dis Ther*, *2*(4), 1-7. https://doi.org/10.4172/2332-0877.1000150

Gellatly, S. L., & Hancock, R. E. W. (2013). *Pseudomonas aeruginosa*: New insights into pathogenesis and host defenses. *Pathogens and Disease*, *67*(3), 159–173. https://doi.org/10.1111/2049-632X.12033

Ghafourian, S., Sadeghifard, N., Soheili, S., & Sekawi, Z. (2014). Extended spectrum beta-lactamases: Definition, classification and epidemiology. *Current Issues in Molecular Biology*, *17*(1), 11–22. https://doi.org/10.1016/B978-1-4377-1738-9.00102-X

Ghanavati, R., Emaneini, M., Kalantar-Neyestanaki, D., Maraji, A. S., Dalvand, M., Beigverdi, R., & Jabalameli, F. (2018). Clonal relation and antimicrobial resistance pattern of extended-spectrum β-lactamase- and AmpC β-lactamase-producing *enterobacter* spp. Isolated from different clinical samples in Tehran, Iran. *Revista Da Sociedade Brasileira de Medicina Tropical*, *51*(1), 88–93. https://doi.org/10.1590/0037-8682-0227-2017

Ghareeb, K., Awad, W. a., Mohnl, M., Porta, R., Biarnes, M., Bohm, J., & Schatzmayr, G. (2012). Evaluating the efficacy of an avian-specific probiotic to reduce the colonization of *Campylobacter jejuni* in broiler chickens. *Poultry Science*, *91*(3), 1825–1832. https://doi.org/10.3382/ps.2012-02168

Giammanco, G. M., Aleo, A., Guida, I., & Mammina, C. (2011). molecular epidemiological survey of *Citrobacter freundii* Misidentified as *Cronobacter* spp. (*Enterobacter sakazakii*) and *Enterobacter hormaechei* isolated from powdered infant milk formula. *Foodborne Pathogens and Disease*, *8*(4), 517–525. https://doi.org/10.1089/fpd.2010.0719

Girlich, D., Damaceno, Q. S., Oliveira, A. C., & Nordmann, P. (2014). OXA-253, a variant of the

carbapenem-hydrolyzing class d β-lactamase OXA-143 in *Acinetobacter baumannii*. *Antimicrobial Agents and Chemotherapy*, *58*(5), 2976–2978. https://doi.org/10.1128/AAC.02640-13

Goller, C., Wang, X., Itoh, Y., & Romeo, T. (2006). The cation-responsive protein NhaR of *Escherichia coli* activates pgaABCD transcripiton, required for the production of the biofilm adhesin poly beta-1,6-N-acetyl-D-glucosamine. *Journal of bacteriology*, 18(2), 8022–8032.

Gómez, M., Moles, L., Melgar, A., Ureta, N., Bustos, G., Fernández, L., & Jiménez, E. (2016). Early gut colonization of preterm infants: Effect of enteral feeding tubes. *Journal of Pediatric Gastroenterology and Nutrition*, *62*(6), 893–900. https://doi.org/10.1097/MPG.0000000000001104

Gonzalez, A. M., & Garduno, V. (2016). Antibiotic-resistant *Acinetobacter baumannii* increasing success remains a challenge as a nosocomial pathogen. *Journal of Pathogens*, *2016*, 1–10. https://doi.org/10.1155/2016/7318075

Gophna, U., Barlev, M., Seijffers, R., Oelschlager, T. a, & Ron, E. Z. (2001). Curli fibers mediate internalization of *Escherichia coli* by eukaryotic cells. *Infection and Immunity*, *69*(4), 2659–2665. https://doi.org/10.1128/IAI.69.4.2659

Graves, A. K., & Weaver, R. W. (2010). Characterization of enterococci populations collected from a subsurface flow constructed wetland. *Journal of Applied Microbiology*, *108*(4), 1226–1234. https://doi.org/10.1111/j.1365-2672.2009.04516.x

Groer, M. W., Luciano, A. A., Dishaw, L. J., Ashmeade, T. L., Miller, E., & Gilbert, J. A. (2014). Development of the preterm infant gut microbiome: a research priority. *Microbiome*, *2*, 38. https://doi.org/10.1186/2049-2618-2-38

Guaraldi, F., & Salvatori, G. (2012). Effect of breast and formula feeding on gut microbiota shaping in newborns. *Frontiers in Cellular and Infection Microbiology*, *2*(10), 1–4. https://doi.org/10.3389/fcimb.2012.00094

Guérin, F., Lallement, C., Isnard, C., Dhalluin, A., Cattoir, V., & Giard, J. (2016). Pumps in *Enterobacter cloacae* Complex, *60*(4), 2373–2382. https://doi.org/10.1128/AAC.02840-15.Address

Gundi, V. a K. B., Dijkshoorn, L., Burignat, S., Raoult, D., & La Scola, B. (2009). Validation of partial rpoB gene sequence analysis for the identification of clinically important and emerging *Acinetobacter* species. *Microbiology (Reading, England)*, *155*(7), 33–41. https://doi.org/10.1099/mic.0.026054

Haiko, J., & Westerlund-Wikström, B. (2013). The role of the bacterial flagellum in adhesion and virulence. *Biology*, *2*(4), 1242–1267. https://doi.org/10.3390/biology2041242

Hammerum, A. M. (2012). Enterococci of animal origin and their significance for public health. *Clinical Microbiology and Infection*, *18*(7), 619–625. https://doi.org/10.1111/j.1469-0691.2012.03829

Hampton, T. (2013). Report reveals scope of US antibiotic resistance threat. *JAMA*, 310(16), 1661–1663. https://doi.10.1001/jama.2013.280695

Hartmann, I., Carranza, P., Lehner, A., Stephan, R., Eberl, L., & Riedel, K. (2010). Genes involved in *Cronobacter sakazakii* biofilm formation. *Applied and Environmental Microbiology*, *76*(7), 2251–2261. https://doi.org/10.1128/AEM.00930-09

Hasan, T., Choi, C. H., & Oh, M. H. (2015). Genes involved in the biosynthesis and transport of Acinetobactin in *Acinetobacter baumannii*. *Genomics & Informatics*, *13*(1), 2–6. https://doi.org/10.5808/GI.2015.13.1.2

He, X., Lu, F., Yuan, F., Jiang, D., Zhao, P., Zhu, J., … Lu, G. (2015). Biofilm formation caused by clinical *Acinetobacter baumannii* isolates is associated with overexpression of the AdeFGH efflux pump. *Antimicrobial Agents and Chemotherapy*, *59*(8), 4817–4825. https://doi.org/10.1128/AAC.00877-15

Hegstad, K., Mikalsen, T., Coque, T. M., Werner, G., & Sundsfjord, A. (2010). Mobile genetic elements and their contribution to the emergence of antimicrobial resistant *Enterococcus faecalis* and *Enterococcus* faecium. *Clinical Microbiology and Infection*, *16*(6), 541–554. https://doi.org/10.1111/j.1469-0691.2010.03226.x

Heikens, E., Bonten, M. J. M., & Willems, R. J. L. (2007). Enterococcal surface protein esp is important for biofilm formation of *Enterococcus faecium* E1162. *Journal of Bacteriology*, *189*(22), 8233–8240. https://doi.org/10.1128/JB.01205-07

Hendrickx, A. P. A., Van Luit-Asbroek, M., Schapendonk, C. M. E., Van Wamel, W. J. B., Braat, J. C., Wijnands, L. M., … Willems, R. J. L. (2009). SgrA, a nidogen-binding LPXTG surface adhesin implicated in biofilm formation, and EcbA, a collagen binding MSCRAMM, are two novel adhesins of hospital-acquired *Enterococcus faecium*. *Infection and Immunity*, *77*(11), 5097–5106. https://doi.org/10.1128/IAI.00275-09

Hensel, K. O., Van Den Bruck, R., Klare, I., Heldmann, M., Ghebremedhin, B., & Jenke, A. C. (2017). Nursing staff fluctuation and pathogenic burden in the NICU - Effective outbreak management and the underestimated relevance of non-resistant strains. *Scientific Reports*, *7*(9), 1–7. https://doi.org/10.1038/srep45014

Hewitt, K. M., Mannino, F. L., Gonzalez, A., Chase, J. H., Caporaso, J. G., Knight, R., & Kelley, S. T. (2013). Bacterial diversity in two neonatal intensive care units (NICUs). *PLoS ONE*, *8*(1). 152-157 https://doi.org/10.1371/journal.pone.0054703

Higgins, P. G., Pérez-Llarena, F. J., Zander, E., Fernández, A., Bou, G., & Seifert, H. (2013). OXA-235, a novel class D β-lactamase involved in resistance to carbapenems in *Acinetobacter baumannii*. *Antimicrobial Agents and Chemotherapy*, *57*(5), 2121–2126. https://doi.org/10.1128/AAC.02413-12

Higgins, P. G., Wisplinghoff, H., Krut, O., & Seifert, H. (2007). A PCR-based method to differentiate between *Acinetobacter baumannii* and *Acinetobacter* genomic species 13TU. *Clinical Microbiology and Infection*, *13*(12), 1200–1201. https://doi.org/10.1111/j.1469-0691.2007.01819.x

Higuita, N. I. A., & Huycke, M. M. (2014). Enterococcal disease , epidemiology , and implications for treatment. Enterococci: *From Commensals to Leading Causes of Drug Resistant Infection*, 4(1) 1–27.

Ho, P. L., Shek, R. H. L., Chow, K. H., Duan, R. S., Mak, G. C., Lai, E. L., … Lai, W. M. (2005). Detection and characterization of extended-spectrum β-lactamases among bloodstream isolates of *Enterobacter* spp. in Hong Kong, 2000-2002. *Journal of Antimicrobial Chemotherapy*, *55*(3), 326–332. https://doi.org/10.1093/jac/dki010

Hoffmann, H., Stindl, S., Ludwig, W., Stumpf, A., Mehlen, A., Monget, D., … Schleifer, K. H. (2005). steigerwaltii subsp . nov ., Three new subspecies of clinical importance. *Journal of Clinical Microbiology*, *43*(7), 3297–3303. https://doi.org/10.1128/JCM.43.7.3297

Holden, V. I., & Bachman, M. A. (2015). Diverging roles of bacterial siderophores during infection. *Metallomics*, *7*(6), 986–995. https://doi.org/10.1039/c4mt00333k

Hollenbeck, B. L., & Rice, L. B. (2012). Intrinsic and acquired resistance mechanisms in *Enterococcus*. *Virulence*, *3*(5), 421–433. https://doi.org/10.4161/viru.21282

Holý, O., & Forsythe, S. (2014). *Cronobacter* spp. as emerging causes of healthcare-associated infection. *Journal of Hospital Infection*, *86*(3), 169–177. https://doi.org/10.1016/j.jhin.2013.09.011

Hou, C., & Yang, F. (2015). Drug-resistant gene of *bla*OXA-23, *bla*OXA-24, *bla*OXA-51 and *bla*OXA-58 in *Acinetobacter baumannii*. *International Journal of Clinical and Experimental Medicine*, *8*(8), 13859–13863. https://doi.org/10.4158/EP10367.OR

Howard, A., O'Donoghue, M., Feeney, A., & Sleator, R. D. (2012). *Acinetobacter baumannii*: an emerging opportunistic pathogen. *Virulence*, *3*(3), 243–50. https://doi.org/10.4161/viru.19700

Hsu, A. J., & Tamma, P. D. (2014). Treatment of multidrug-resistant gram-negative infections in children. *Clinical Infectious Diseases*, *58*(10), 1439–1448. https://doi.org/10.1093/cid/ciu069

Hu, L., Grim, C. J., Franco, A. A., Jarvis, K. G., Sathyamoorthy, V., Kothary, M. H., (2015). Analysis of the cellulose synthase operon genes, *bcsA*, *bcsB* and *bcsC* in *Cronobacter* species. Prevalence among species and their roles in biofilm formation and cell-cell aggregation. *Food Microbio,* 52(12), 97–105. https://doi.10.1016/j.fm.2015.07.007.

Hufnagel, M., Liese, C., Loescher, C., Kunze, M., Proempeler, H., Berner, R., & Krueger, M. (2007). Enterococcal colonization of infants in a neonatal intensive care unit: Associated predictors, risk factors and seasonal patterns. *BMC Infectious Diseases*, *7*, 1–11. https://doi.org/10.1186/1471-2334-7-107

Hunter, C. J., Singamsetty, V. K., Chokshi, N. K., Boyle, P., Camerini, V., Grishin, A. V, … Prasadarao, N. V. (2008). *Enterobacter sakazakii* enhances epithelial cell injury by inducing apoptosis in a rat model of necrotizing enterocolitis. *The Journal of Infectious Diseases*, *198*(4), 586–93. https://doi.org/10.1086/590186

Hurrell, E., Kucerova, E., Loughlin, M., Caubilla-Barron, J., & Forsythe, S. J. (2009a). Biofilm formation on enteral feeding tubes by *Cronobacter sakazakii, Salmonella serovars* and other *Enterobacteriaceae*. *International Journal of Food Microbiology*, *136*(2), 227–231. https://doi.org/10.1016/j.ijfoodmicro.2009.08.007

Hurrell, E., Kucerova, E., Loughlin, M., Caubilla-Barron, J., Hilton, A., Armstrong, R., Forsythe, S. (2009b). Neonatal enteral feeding tubes as loci for colonisation by members of the *Enterobacteriaceae*. *BMC Infectious Diseases*, *9*(2), 146. https://doi.org/10.1186/1471-2334-9-146

Husain, I., Sharma, A., Kumar, S., & Malik, F. (2016). Purification and characterization of glutaminase free asparaginase from *enterobacter cloacae*: In-vitro evaluation of cytotoxic potential against human myeloid leukemia HL-60 cells. *PLoS ONE*, *11*(2), 1–27. https://doi.org/10.1371/journal.pone.0148877

Hussey, S., Wall, R., Gruffman, E., O'Sullivan, L., Ryan, C. A., Murphy, B., Ross, R. P. (2011). Parenteral antibiotics reduce bifidobacteria colonization and diversity in neonates. *International Journal of Microbiology*, 2011*,* https://doi.org/10.1155/2011/130574

Ispirli, H., Demirbaş, F., & Dertli, E. (2015). Characterization of functional properties of *Enterococcus faecium* strains isolated from human gut. *Canadian Journal Of microbiology*. Retrieved from http://www.nrcresearchpress.com/doi/abs/10.1139/cjm-2015-0446

Itoh, Y., Wang, X., Hinnebusch, B. J., Iii, J. F. P., & Romeo, T. (2005). Depolymerization of b -1 , 6-N -Acetyl- D -Glucosamine disrupts the integrity of diverse bacterial biofilms. *Journal of Bacteriology*, *187*(1), 382–387. https://doi.org/10.1128/JB.187.1.382

Iversen, C., Mullane, N., McCardell, B., Tall, B. D., Lehner, A., Fanning, S., … Joosten, H. (2008). Cronobacter gen. nov., a new genus to accommodate the biogroups of *Enterobacter sakazakii*, and proposal of *Cronobacter sakazakii* gen. nov., comb. nov., *Cronobacter malonaticus* sp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., Cro. *International Journal of Systematic and Evolutionary Microbiology*, *58*(6), 1442–1447. https://doi.org/10.1099/ijs.0.65577-0

Jacobs, A. C., Hood, I., Boyd, K. L., Olson, P. D., Morrison, J. M., Carson, S., … Dunman, P. M. (2010). Inactivation of phospholipase D diminishes *Acinetobacter baumannii* pathogenesis. *Infection and Immunity*, *78*(5), 1952–1962. https://doi.org/10.1128/IAI.00889-09

Jacqueline, C., & Caillon, J. (2014). Impact of bacterial biofilm on the treatment of prosthetic joint infections. *Journal of Antimicrobial Chemotherapy*, *69*(1), 37–40. https://doi.org/10.1093/jac/dku254

Jackson, E. E., Flores, J. P., Fernández-Escartín, E., & Forsythe, S. J. (2015). Reevaluation of a suspected *Cronobacter sakazakii* outbreak in Mexico. *Journal of Food Protection*, *78*(6), 1191–1196. https://doi.org/10.4315/0362-028X.JFP-14-563

Jamal, M., Ahmad, W., Andleeb, S., Jalil, F., Imran, M., Nawaz, M. A., … Kamil, M. A. (2018). Bacterial biofilm and associated infections. *Journal of the Chinese Medical Association*, *81*(1), 7–11. https://doi.org/10.1016/j.jcma.2017.07.012

Jandhyala, S. M., Talukdar, R., Subramanyam, C., Vuyyuru, H., Sasikala, M., & Reddy, D. N. (2015). Role of the normal gut microbiota. *World Journal of Gastroenterology*, *21*(29), 8836–8847. https://doi.org/10.3748/wjg.v21.i29.8787

Jesumirhewe, C., Ogunlowo, P. O., Olley, M., Springer, B., Allerberger, F., & Ruppitsch, W. (2016). Accuracy of conventional identification methods used for *Enterobacteriaceae* isolates in three Nigerian hospitals. *PeerJ*, *4*, e2511. https://doi.org/10.7717/peerj.2511

Ji, K., Wang, W., Zeng, B., Chen, S., Zhao, Q., Chen, Y., Ma, T. (2016). Bacterial cellulose synthesis mechanism of facultative anaerobe *Enterobacter* sp. FY-07. *Scientific Reports*, *6*(2), 1–12. https://doi.org/10.1038/srep21863

Jo, H. Y., Kim, Y., Park, H. W., Moon, H. E., Bae, S., Kim, J., … Paek, S. H. (2015). The unreliability of mtt assay in the cytotoxic test of primary cultured glioblastoma cells. *Experimental Neurobiology*, *24*(3), 235-241. https://doi.org/10.5607/en.2015.24.3.235

Jolley, K. A., Bray, J. E., & Maiden, M. C. J. (2017). A RESTful application programming interface for the PubMLST molecular typing and genome databases. *Database : The Journal of Biological Databases and Curation*, *2017*(2), 1–7. https://doi.org/10.1093/database/bax060

Joly-Guillou, M. L. (2005). Clinical impact and pathogenicity of Acinetobacter. *Clinical Microbiology and Infection*, *11*(11), 868–873. https://doi.org/10.1111/j.1469-0691.2005.01227

Joo, H.-S., & Otto, M. (2013). Molecular basis of in-vivo biofilm formation by bacterial pathogens. *Chemistry & Biology*, *19*(12), 1503–1513. https://doi.org/10.1016/j.chembiol.2012.10.022.

Joseph, C. J., Lian, W. Bin, & Yeo, C. L. (2012). Nosocomial infections (late onset sepsis) in the neonatal intensive care unit (NICU). *Proceedings of Singapore Healthcare*, *21*(4), 238–244. https://doi.org/10.1177/201010581202100404

Juma, N.A., Manning, G. & Forsythe, S.J. (2016). Desiccation survival of *Acinetobacter* spp. in infant formula. *Food Control*, 68(10), 162-166. http://doi.org/10.1016/j.foodcont.2016.03.043

Kadam, S. R., den, H., Heidy, M. W., van, S., Stijn, Z., Marcel, H., Moezelaar, R., & Abee, T. (2013).Diversity assessment of *Listeria monocytogenes* biofilm formation: Impact of growth condition, serotype and strain origin. *International journal of food microbiology*, 165(3), 259-64 https://doi.org/10.1016/j.ijfoodmicro.2013.05.025

Kafil, H. S., & Mobarez, A. M. (2015). Assessment of biofilm formation by enterococci isolates from urinary tract infections with different virulence profiles. *Journal of King Saud University - Science*, *27*(4), 312–317. https://doi.org/10.1016/j.jksus.2014.12.007

Kafilzadeh, F., & Farsimadan, F. (2016). Investigating multidrug efflux pumps in relation to the antibiotic resistance pattern in *Escherichia coli* strains from patients in Iran. *Biomedical Research*, *27*(4), 1130–1135.

Kalyantanda, G., Shumyak, L., & Archibald, L. K. (2015). *Cronobacter* Species Contamination of Powdered Infant Formula and the Implications for Neonatal Health. *Frontiers in Pediatrics*, *3*(7), 1–5. https://doi.org/10.3389/fped.2015.00056

Karlowsky, J. A., Hoban, D. J., Hackel, M. A., Lob, S. H., & Sahm, D. F. (2017). Resistance among Gram-negative ESKAPE pathogens isolated from hospitalized patients with intra-abdominal and urinary tract infections in Latin American countries: SMART 2013–2015. *Brazilian Journal of Infectious Diseases*, *21*(3), 343–348. https://doi.org/10.1016/j.bjid.2017.03.006

Kamolvit, W., Sidjabat, H. E., & Paterson, D. L. (2015). Molecular Epidemiology and Mechanisms of Carbapenem Resistance of *Acinetobacter* spp. in Asia and Oceania. *Microbial Drug Resistance*, *21*(4), 424–434. https://doi.org/10.1089/mdr.2014.0234

Kartali, G., Tzelepi, E., Pournaras, S., Kontopoulou, C., Kontos, F., Sofianou, D., … Tsakris, A. (2002). Outbreak of infections caused by Enterobacter cloacae producing the integron-associated β-lactamase IBC-1 in a neonatal intensive care unit of a Greek hospital. *Antimicrobial Agents and Chemotherapy*, *46*(5), 1577–1580. https://doi.org/10.1128/AAC.46.5.1577-1580.2002

Kato, H., Ide, K., Fukase, F., Shimura, Y., Yasuda, S., Goto, H., … Nakajima, H. (2018). Polymerase chain reaction-based open reading frame typing (POT) method analysis for a methicillin-resistant *Staphylococcus aureus* (MRSA) outbreak through breast-feeding in the neonatal intensive care unit. *IDCases*, *12*(2), 1–3. https://doi.org/10.1016/j.idcr.2018.02.005

Keim, S. A., Kulkarni, M. M., McNamara, K., Geraghty, S. R., Billock, R. M., Ronau, R., … Kwiek, J. J. (2015). Cow's milk contamination of human milk purchased via the internet. *Pediatrics*, *135*(5), 1157–1162. https://doi.org/10.1542/peds.2014-3554

Khalkhali, S., & Mojgani, N. (2017). Bacteriocinogenic potential and virulence traits of *Enterococcus faecium* and *E. faecalis* isolated from human milk. *Iranian Journal of*

Microbiology, *9*(4), 224–233.

Khan, H. A., Ahmad, A., & Mehboob, R. (2015). Nosocomial infections and their control strategies. *Asian Pacific Journal of Tropical Biomedicine*, *5*(7), 509–514. https://doi.org/10.1016/j.apjtb.2015.05.001

Khelissa, S. O., Abdallah, M., Jama, C., Faille, C., & Chihib, N. E. (2017). Bacterial contamination and biofilm formation on abiotic surfaces and strategies to overcome their persistence. *Journal of Materials and Environmental Science*, *8*(9), 3326–3346.

Khosravi, A. D., Sadeghi, P., Shahraki, A. H., Heidarieh, P., & Sheikhi, N. (2015). Molecular methods for identification of *acinetobacter* species by partial sequencing of the *rpoB* and 16S rRNA genes. *Journal of Clinical and Diagnostic Research*. https://doi.org/10.7860/JCDR/2015/13867.6188

Kikuchi, T., Mizunoe, Y., Takade, A., Naito, S., & Yoshida, S. I. (2005). Curli fibers are required for development of biofilm architecture in *Escherichia coli* K-12 and enhance bacterial adherence to human uroepithelial cells. *Microbiology and Immunology*, *49*(9), 875–884. https://doi.org/10.1111/j.1348-0421.2005.tb03678.x

Kim, K., Kim, K. P., Choi, J., Lim, J. A., Lee, J., Hwang, S., & Ryu, S. (2010). Outer membrane proteins A (OmpA) and X (OmpX) are essential for basolateral invasion of *Cronobacter sakazakii*. *Applied and Environmental Microbiology*, *76*(15), 5188–5198. https://doi.org/10.1128/AEM.02498-09

Kim, S. M., Lee, H. W., Choi, Y. W., Kim, S. H., Lee, J. C., Lee, Y. C., Kim, J. (2012). Involvement of curli fimbriae in the biofilm formation of *Enterobacter cloacae*. *Journal of Microbiology*, *50*(1), 175–178. https://doi.org/10.1007/s12275-012-2044-2

Kim, S. W., Choi, C. H., Moon, D. C., Jin, J. S., Lee, J. H., Shin, J. H., Lee, J. C. (2009). Serum resistance of *Acinetobacter baumannii* through the binding of factor H to outer membrane proteins. *FEMS Microbiology Letters*, *301*(2), 224–231. https://doi.org/10.1111/j.1574-6968.2009.01820.x

Kim, S. W., Oh, M. H., Jun, S. H., Jeon, H., Kim, S. Il, Kim, K., … Lee, J. C. (2016). Outer membrane protein a plays a role in pathogenesis of *Acinetobacter nosocomialis*. *Virulence*, *7*(4), 413–426. https://doi.org/10.1080/21505594.2016.1140298

Kivanç, S. A., Kivanç, M., & Yiğit, T. (2016). Antibiotic susceptibility, antibacterial activity and characterisation of *Enterococcus faecium* strains isolated from breast milk. *Experimental and Therapeutic Medicine*, *12*(3), 1732–1740. https://doi.org/10.3892/etm.2016.3545

Klaassen, C. H. W., van Haren, H. A., & Horrevorts, A. M. (2002). Molecular fingerprinting of Clostridium difficile isolates: pulsed-field gel electrophoresis versus amplified fragment length polymorphism. *Journal of Clinical Microbiology*, *40*(1), 101–4. https://doi.org/10.1128/JCM.40.1.101

Knols, B. G., Smallegange, R. C., Tacconelli, E., Magrini, N., Kahlmeter, G., & Singh, N. (2016). Global priority list of antibiotic-resistant bacteria to guide research, Discovery, And Development Of New Antibiotics. *The Lancet Infectious Diseases*, *9*(9), 535–536. https://doi.org/10.1016/S1473-3099(09)70222-1

Kong, C., Neoh, H. M., & Nathan, S. (2016). Targeting *Staphylococcus aureus* toxins: A potential form of anti-virulence therapy. *Toxins*, *8*(3), 1–21. https://doi.org/10.3390/toxins8030072

Kose, S., Ozer, E., Gulay, Z., Akkoclu, G., Tokgoz, H., Agus, N., … Ozturk, R. (2016). *Enterobacter*

Cloacae sepsis outbreak in neonatal intensive care unit due to contaminated total parenteral nutrition solution. *Journal of Pediatric Research*, *3*(2), 109–112. https://doi.org/10.4274/jpr.20143

Kostakioti, M., Hadjifrangiskou, M., & Hultgren, S. J. (2013). Bacterial biofilms: Development, dispersal, and therapeutic strategies in the dawn of the postantibiotic era. *Cold Spring Harbor Perspectives in Medicine*, *3*(4), 1–23. https://doi.org/10.1101/cshperspect.a010306

Koziel, J., & Potempa, J. (2013). Protease-armed bacteria in the skin. *Cell and Tissue Research*, *351*(2), 325–337. https://doi.org/10.1007/s00441-012-1355-2

Krachler, A. M., & Orth, K. (2013). Targeting the bacteria-host interface strategies in anti-adhesion therapy. *Virulence*, *4*(4), 284–294. https://doi.org/10.4161/viru.24606

Krzymińska, S., Mokracka, J., Koczura, R., & Kaznowski, A. (2009). Cytotoxic activity of *Enterobacter cloacae* human isolates. *FEMS Immunology and Medical Microbiology*, *56*(3), 248–252. https://doi.org/10.1111/j.1574-695X.2009.00572

Krzymińska, S., Koczura, R., Mokracka, J., & Kaznowski, A. (2010). Isolates of the *Enterobacter cloacae* complex induce apoptosis of human intestinal epithelial cells. *Microbial Pathogenesis*, 49(3), 83-89. https://doi.10.1016/j.micpath.2010.04.003

Kuehn, B. M. CDC (2014). Hospital antibiotic use promotes resistance: checklist can improve practics. JAMA, 31(5), 1485-61492.https://doi:10.1001/jama.2014.2847

Kumar, S., Shankar, B., Arya, S., Deb, M., & Chellani, H. (2018). Healthcare associated infections in neonatal intensive care unit and its correlation with environmental surveillance. *Journal of Infection and Public Health*, *11*(2), 275–279. https://doi.org/10.1016/j.jiph.2017.08.005

Kwon, K. H., Hwang, S. Y., Moon, B. Y., Park, Y. K., Shin, S., Hwang, C. Y., & Park, Y. H. (2012). Occurrence of antimicrobial resistance and virulence genes, and distribution of enterococcal clonal complex 17 from animals and human beings in Korea. *Journal of Veterinary Diagnostic Investigation*, *24*(5), 924–931. https://doi.org/10.1177/1040638712455634

Kwong, J. C., Mccallum, N., Sintchenko, V., & Howden, B. P. (2015). Whole genome sequencing in clinical and public health microbiology. *Pathology*, *47*(3), 199–210. https://doi.org/10.1097/PAT.0000000000000235

Lago, M., Monteil, V., Douche, T., Guglielmini, J., Criscuolo, A., Maufrais, C., … Norel, F. (2017). Proteome remodelling by the stress sigma factor RpoS/σS in *Salmonella*: Identification of small proteins and evidence for post-transcriptional regulation. *Scientific Reports*, *7*(1), 1–15. https://doi.org/10.1038/s41598-017-02362-3

Lam, M. M. C., Seemann, T., Tobias, N. J., Chen, H., Haring, V., Moore, R. J., … Stinear, T. P. (2013). Comparative analysis of the complete genome of an epidemic hospital sequence type 203 clone of vancomycin-resistant *Enterococcus faecium*. *BMC Genomics*, *14*(1), 1. https://doi.org/10.1186/1471-2164-14-595

Lasaro, M. A., Salinger, N., Zhang, J., Wang, Y., Zhong, Z., Goulian, M., & Zhu, J. (2009). F1C fimbriae play an important role in biofilm formation and intestinal colonization by the *Escherichia coli* commensal strain nissle 1917. *Applied and Environmental Microbiology*, *75*(1), 246–251. https://doi.org/10.1128/AEM.01144-08

Lăzureanu, V., Poroşnicu, M., Gândac, C., Moisil, T., Bădiţoiu, L., Laza, R., … Marinescu, A. R.

(2016). Infection with *Acinetobacter baumannii* in an intensive care unit in the Western part of Romania. *BMC Infectious Diseases*, *16*(1). https://doi.org/10.1186/s12879-016-1399-0

Leavis, H., Top, J., Shankar, N., Borgen, K., Bonten, M., Embden, J. Van, & Willems, R. J. L. (2004). A novel putative Enterococcal pathogenicity island linked to the *esp* virulence gene of *Enterococcus faecium* and associated with epidemicity. *Journal of Bacteriology*, *186*(3), 672–682. https://doi.org/10.1128/JB.186.3.672

Lebreton, F., Manson, A. L., Saavedra, J. T., Straub, T. J., Earl, A. M., & Gilmore, M. S. (2017). Tracing the Enterococci from paleozoic origins to the hospital. *Cell*, *169*(5), 849–861.e13. https://doi.org/10.1016/j.cell.2017.04.027

Lebreton, F., Willems, R. J. L., & Gilmore, M. S. (2014). *Enterococcus* diversity, origins in nature, and gut colonization. Enterococci*: From Commensals to Leading Causes of Drug Resistant Infection*, 1–56. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/24649513

Lee, B. Y., Yilmaz, S. L., Wong, K. F., Bartsch, S. M., Eubank, S., Song, Y., … Huang, S. S. (2013). Modeling the regional spread and control of vancomycin-resistant enterococci. *American Journal of Infection Control*, *41*(8), 668–673. https://doi.org/10.1016/j.ajic.2013.01.013

Lee, H. W., Koh, Y. M., Kim, J., Lee, J. C., Lee, Y. C., Seol, S. Y., & Cho, D. T. (2008). Capacity of multidrug-resistant clinical isolates of Acinetobacter baumannii to form biofilm and adhere to epithelial cell surfaces. *Clinical Microbiology and Infection*, *14*(1), 49–54. https://doi.org/10.1111/j.1469-0691.2007.01842.x

Lee, J. J., Kim, J. H., Kim, D. G., Kim, D. H., Simborio, H. L., & Min, W. G. (2014). Characterization of betaine aldehyde dehydrogenase (BetB) as an essential virulence factor of *Brucella abortus*. *Vet. Microbiol*. 168(1), 131–140. https://doi.10.1016/j.vetmic.2013.10.007

Lee, W. G., Ahn, S. H., Jung, M. K., Jin, H. Y., & Park, I. J. (2012). Characterization of a vancomycin-resistant *Enterococcus faecium* outbreak caused by 2 genetically different clones at a neonatal intensive care unit. *Annals of Laboratory Medicine*, *32*(1), 82–86. https://doi.org/10.3343/alm.2012.32.1.82

Lee, Y. (2017). Biofilm formation and antimicrobial resistance in *Enterococcus*. *Infection & Chemotherapy*, 49(3), 236–237. https://doi.org/https://doi.org/10.3947/ic.2017.49.3.236

Legeay, C., Bourigault, C., Lepelletier, D., & Zahar, J. R. (2015). Prevention of healthcare-associated infections in neonates: Room for improvement. *Journal of Hospital Infection*, *89*(4), 319–323. https://doi.org/10.1016/j.jhin.2015.02.003

Leski, T. A., Bangura, U., Jimmy, D. H., Ansumana, R., Lizewski, S. E., Li, R. W., … Vora, G. J. (2013). Identification of *bla*OXA-51-like, *bla*OXA-58, *bla*DIM-1, and *bla*VIM carbapenemase genes in hospital *Enterobacteriaceae* isolates from Sierra Leone. *Journal of Clinical Microbiology*, 51(7), 2435–2438. https://doi.org/10.1128/JCM.00832-13

Lester, C. H., Sandvang, D., Olsen, S. S., Schønheyder, H. C., Jarløv, J. O., Bangsborg, J., … Hammerum, A. M. (2008). Emergence of ampicillin-resistant *Enterococcus faecium* in Danish hospitals. *Journal of Antimicrobial Chemotherapy*, *62*(6), 1203–1206. https://doi.org/10.1093/jac/dkn360

Limoli, D. H., Jones, C. J., Wozniak, D. J., & Cruz, S. (2015). Bacterial extracellular polysaccharides in biofilm formation and function. *Microbiol Spectr.*, *3*(3), 1–30. https://doi.org/10.1128/microbiolspec.MB-0011-2014.Bacterial

Lin, J., Zhao, F., Feng, Y., & Zong, Z. (2017). Crossm draft genome sequence of a high-level colistin-resistant clinical strain of the *Enterobacter cloacae* complex. *Genome Announcements*, *5*(14), 4–5. https://doi.org/10.1128/ genomeA.00131-17

Lindholm, M., & Oscarsson, J. (2017). Role of outer membrane proteins in serum resistance of *A. actinomycetemcomitans* and *A. aphrophilus*. *Journal of Oral Microbiology*, *9*(sup1), 1325204. https://doi.org/10.1080/20002297.2017.1325204

Liu, W.-Y., Wong, C.-F., Chung, K. M.-K., Jiang, J.-W., & Leung, F. C.-C. (2013). Comparative Genome Analysis of *Enterobacter cloacae*. *PLoS ONE*, *8*(9), e74487. https://doi.org/10.1371/journal.pone.0074487

Llaca-Díaz, J. M., Mendoza-Olazarán, S., Camacho-Ortiz, A., Flores, S., & Garza-González, E. (2013). One-year surveillance of eskape pathogens in an intensive care unit of Monterrey, Mexico. *Chemotherapy*, *58*(6), 475–481. https://doi.org/10.1159/000346352

Longo, F., Vuotto, C., & Donelli, G. (2014). Biofilm formation in *Acinetobacter baumannii*. *The New Microbiologica*, *37*(2), 119–27. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/24858639

Luo, L. M., Wu, L. J., Xiao, Y. L., Zhao, D., Chen, Z. X., Kang, M., … Xie, Y. (2015). Enhancing pili assembly and biofilm formation in *Acinetobacter baumannii* ATCC19606 using non-native acyl-homoserine lactones. *BMC Microbiology*, *15*(1), 1–7. https://doi.org/10.1186/s12866-015-0397-5

Lyman, M. (2017). *Acinetobacter baumannii* Outbreak in Nicu at the Colonial War Memorial Hospital Suva , fiji , December 2016 – july 2017. *Technical Report*, (July).

Lyu, L. D., Tang, B. K., Fan, X. Y., Ma, H., & Zhao, G. P. (2013). Mycobacterial MazG safeguards genetic stability via housecleaning of 5-OH-dCTP. *PLoS Pathogens*, *9*(12), 1–11. https://doi.org/10.1371/journal.ppat.1003814

Ma, L., Kornacki, J. L., Zhang, G., Lin, C. M., & Doyle, M. P. (2007). Development of thermal surrogate microorganisms in ground beef for in-plant critical control point validation. *Journal of Food Protection*, 70(4), 952–957.

Magiorakos, a, Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., … Hindler, J. F. (2011). Bacteria : an international expert proposal for interim standard definitions for acquired resistance. *Microbiology*, *18*(3), 268–281. https://doi.org/10.1111/j.1469-0691.2011.03570.x

Martin, C., Ling, P.-R., & Blackburn, G. (2016). Review of infant feeding: key features of breast milk and infant formula. *Nutrients*, *8*(5), 279. https://doi.org/10.3390/nu8050279

Martinsen, T. C., Bergh, K., & Waldum, H. L. (2005). Gastric juice: A barrier against infectious diseases. *Basic and Clinical Pharmacology and Toxicology*, *96*(2), 94–102. https://doi.org/10.1111/j.1742-7843.2005.pto960202.x

McAuley, C. M., Gobius, K. S., Britz, M.L., & Craven, H. M. (2012). Heat resistance of thermoduric enterococci isolated from milk. *Journal of Food Microbiology*, 154(3): 162–168. https://doi.10.1016/j.ijfoodmicro.2011.12.033.

McNeill, K., & Hamilton, I. R. (2003). Acid tolerance response of biofilm cells of *Streptococcus mutans*. *FEMS Microbiology Letters*, *221*(1), 25–30. https://doi.org/10.1016/S0378-1097(03)00164-2

Mendes, R. E., Castanheira, M., Farrell, D. J., Flamm, R. K., Sader, H. S., & Jones, R. N. (2016).

Longitudinal (2001-14) analysis of enterococci and VRE causing invasive infections in European and US hospitals, including a contemporary (2010-13) analysis of oritavancin in vitro potency. *Journal of Antimicrobial Chemotherapy*, *71*(12), 3453–3458. https://doi.org/10.1093/jac/dkw319

Méndez-Vilas, A., & Pereira, M. O. (2013). Microbial pathogens and strategies for combating them science, technology and education. *Microbial Pathogens and Strategies for Combating Them : Science, Technology and Education*, *3*(1), 1429–1438. Retrieved from https://repositorium.sdum.uminho.pt/handle/1822/33030

Mense, L., Röler, S., Hanusch, R., Roberg, C., & Rüdiger, M. (2014). Bacterial contamination of mechanically extracted breast milk. *American Journal of Perinatology*, *31*(4), 293–298. https://doi.org/10.1055/s-0033-1348028

Miajlovic, H., & Smith, S. G. (2014). Bacterial self-defence: How *Escherichia coli* evades serum killing. *FEMS Microbiology Letters*, *354*(1), 1–9. https://doi.org/10.1111/1574-6968.12419

Migura, L., Liebana, E., & Jensen, L. B. (2007). Transposon characterization of vancomycin-resistant *Enterococcus faecium* (VREF) and dissemination of resistance associated with transferable plasmids. *Journal of Antimicrobial Chemotherapy*, *60*(2), 263–268. https://doi.org/10.1093/jac/dkm186

Mihara, K., Tanabe, T., Yamakawa, Y., Funahashi, T., Nakao, H., Narimatsu, S., & Yamamoto, S. (2004). Identification and transcriptional organization of a gene cluster involved in biosynthesis and transport of acinetobactin, a siderophore produced by *Acinetobacter baumannii* ATCC 19606. *Jurnal of Miceobiology*, 150(8), 2587-2597. https://doi. 10.1099/mic.0.27141-0

Mikalsen, T., Pedersen, T., Willems, R., Coque, T. M., Werner, G., Sadowy, E., … Hegstad, K. (2015). Investigating the mobilome in clinically important lineages of *Enterococcus faecium* and *Enterococcus* faecalis. *BMC Genomics*, *16*(1), 1–16. https://doi.org/10.1186/s1264-015-1407-6

Mittal, R., Wang, Y., Hunter, C., Gonzalez-Gomez, I., & Prasadarao, N. (2009). Brain damage in newborn rat model of meningitis by *Enterobacter sakazakii*: a role for outer membrane protein A. *Laboratory Investigation; a Journal of Technical Methods and Pathology*, *89*(3), 263–277. https://doi.org/10.1038/labinvest.2008.164

Moemen, D., Tawfeek, D., & Badawy, W. (2015). Healthcare-associated vancomycin resistant *Enterococcus faecium* infections in the Mansoura university hospitals intensive care units, Egypt. *Brazilian Journal of Microbiology*, *46*(3), 777–783. https://doi.org/10.1590/S1517-838246320140403

Mogensen, T. H. (2009). Pathogen recognition and inflammatory signaling in innate immune defenses. *Clinical Microbiology Reviews*, *22*(2), 240–273. https://doi.org/10.1128/CMR.00046-08

Mohammed, D., & El Seifi, O. S. (2014). Bacterial nosocomial infections in neonatal intensive care unit, Zagazig University Hospital, Egypt. *Egyptian Pediatric Association Gazette*, *62*(3–4), 72–79. https://doi.org/10.1016/j.epag.2014.10.001

Mohseny, A. B., van Velze, V., Steggerda, S. J., Smits-Wintjens, V. E. H. J., Bekker, V., & Lopriore, E. (2018). Late-onset sepsis due to urinary tract infection in very preterm neonates is not uncommon. *European Journal of Pediatrics*, *177*(1), 33–38. https://doi.org/10.1007/s00431-017-3030-9

Mokracka, J., Koczura, R., & Kaznowski, A. (2004). Yersiniabactin and other siderophores produced by clinical isolates of *Enterobacter* spp. and *Citrobacter* spp. *FEMS Immunology and Medical Microbiology*, *40*(1), 51–55. https://doi.org/10.1016/S0928-8244(03)00276-1

Moles, L., Gómez, M., Jiménez, E., Fernández, L., Bustos, G., Chaves, F., … del Campo, R. (2015). Preterm infant gut colonization in the neonatal ICU and complete restoration 2 years later. *Clinical Microbiology and Infection*, *21*(10), 936.e1-936.e10. https://doi.org/10.1016/j.cmi.2015.06.003

Morand, P. C., Billoet, A., Rottman, M., Sivadon-Tardy, V., Eyrolle, L., Jeanne, L., … Dumaine, V. (2009). Specific distribution within the *Enterobacter cloacae* complex of strains isolated from infected orthopedic implants. *Journal of Clinical Microbiology*, *47*(8), 2489–2495. https://doi.org/10.1128/JCM.00290-09

Mortensen, B. L., & Skaar, E. P. (2013). The contribution of nutrient metal acquisition and metabolism to *Acinetobacter baumannii* survival within the host. *Frontiers in Cellular and Infection Microbiology*, *3*(12), 1–10. https://doi.org/10.3389/fcimb.2013.00095

Mubarak, Z., & Soraya, C. (2018). The acid tolerance response and pH adaptation of *Enterococcus* faecalis in extract of lime Citrus aurantiifolia from Aceh Indonesia. *F1000Research*, *7*(0), 1–15. https://doi.org/10.12688/f1000research.13990.2

Mutters, N. T., Mersch-Sundermann, V., Mutters, R., Brandt, C., Schneider-Brachert, W., & Frank, U. (2013). Control of vancomycin-resistant enterococci in hospitals. *Deutsches Arzteblatt International*, *110*(43), 725–732. https://doi.org/10.3238/arztebl.2013.0725

Muytjens, H. L., Roelofs-Willemse, H., & Jaspar, G. H. J. (1988). Quality of powdered substitutes for breast milk with regard to members of the family *Enterobacteriaceae*. *Journal of Clinical Microbiology*, *26*(4), 743–746.

Nagpal, R., & Yamashiro, Y. (2017). Cesarean-section and the dysbiosis of neonatal gut microbiome: ill-effects, side-effects, or just effects. *International Journal of Probiotics and Prebiotics*, *12*(3), 103–108. Retrieved from http://www.nchpjournals.com/manuscript/uploads/article_871.pdf

Nash, M. J., Frank, D. N., & Friedman, J. E. (2017). Early microbes modify immune system development and metabolic homeostasis-the "Restaurant" hypothesis revisited. *Frontiers in Endocrinology*, *8*(12), 1–7. https://doi.org/10.3389/fendo.2017.00349

Nami, Y., Haghshenas, B., Haghshenas, M., & Khosroushahi, A. Y. (2015). Antimicrobial activity and the presence of virulence factors and bacteriocin structural genes in *Enterococcus faecium* CM33 isolated from ewe colostrum. *Frontiers in Microbiology*, *6*(7), 1–10. https://doi.org/10.3389/fmicb.2015.00782

Navidinia, M. (2016). The clinical importance of emerging ESKAPE pathogens in nosocomial infections. *Journal of Paramedical Sciences (JPS)*, *7(3), 43-57*. https://doi.org/10.22037/jps.v7i3.12584

Novovic, K., Mihajlovic, S., Vasiljevic, Z., Filipic, B., Begovic, J., & Jovcic, B. (2015). Carbapenem-resistant *Acinetobacter baumannii* from Serbia: Revision of CarO classification. *PLoS ONE*, *10*(3), 1–16. https://doi.org/10.1371/journal.pone.0122793

O'Driscoll, T., & Crank, C. W. (2015). Vancomycin-resistant enterococcal infections: Epidemiology, clinical manifestations, and optimal management. *Infection and Drug Resistance*, *8*, 217–230. https://doi.org/10.2147/IDR.S54125

O'Hara, C. M., Steigerwalt, A. G., Hill, B. C., Farmer, J. J., Fanning, G. R., & Brenner, D. J. (1989). *Enterobacter hormaechei*, a new species of the family *Enterobacteriaceae* formerly known as Enteric Group 75. *Journal of Clinical Microbiology*, *27*(9), 2046–2049.

Ogasawara, H., Yamamoto, K., & Ishihama, A. (2010). Regulatory role of MlrA in transcription activation of csgD, the master regulator of biofilm formation in *Escherichia coli*. *FEMS Microbiology Letters*, *312*(2), 160–168. https://doi.org/10.1111/j.1574-6968.2010.02112.x

Ogrodzki, P., Cheung, C. S., Saad, M., Dahmani, K., Coxill, R., Liang, H., & Forsythe, S. J. (2017). Rapid in situ imaging and whole genome sequencing of biofilm in neonatal feeding tubes: A clinical proof of concept. *Scientific Reports*, *7*(1), 1–8. https://doi.org/10.1038/s41598-017-15769-9

Ogrodzki, P., & Forsythe, S. (2015). Capsular profiling of the Cronobacter genus and the association of specific *Cronobacter sakazakii* and *C. malonaticus* capsule types with neonatal meningitis and necrotizing enterocolitis. *BMC Genomics*, *16*(1), 1–15. https://doi.org/10.1186/s12864-015-1960-z

Ohad, S., Block, C., Kravitz, V., Farber, A., Pilo, S., Breuer, R., & Rorman, E. (2014). Rapid identification of *Enterobacter hormaechei* and *Enterobacter cloacae* genetic cluster III. *Journal of Applied Microbiology*, 116(5), 1315-1321. https://doi.org/10.1111/jam.12439

Oranges, T., Dini, V., & Romanelli, M. (2015). Skin physiology of the neonate and infant: clinical implications. *Advances in Wound Care*, *4*(10), 587–595. https://doi.org/10.1089/wound.2015.0642

Osaili, T. M., Shaker, R. R., Ayyash, M. M., Al-Nabulsi, A. A., & Forsythe, S. J. (2009). Survival and growth of *Cronobacter* species (*Enterobacter sakazakii*) in wheat-based infant follow-on formulas. *Letters in Applied Microbiology*, *48*(4), 408–412. https://doi.org/10.1111/j.1472-765X.2008.02541.x

Özsoy, S., & İlki, A. (2017). Detection of vancomycin-resistant enterococci (VRE) in stool specimens submitted for *Clostridium difficile* toxin testing. *Brazilian Journal of Microbiology*, *48*(3), 489–492. https://doi.org/10.1016/j.bjm.2016.12.012

Öztürk, H., Ozkirimli, E., & Özgür, A. (2015). Classification of beta-lactamases and Penicillin Binding Proteins using ligand-centric network models. *PLoS ONE*, *10*(2), 1–23. https://doi.org/10.1371/journal.pone.0117874

Paauw, A., Caspers, M. P. M., Leverstein-van Hall, M. A., Schuren, F. H. J., Montijn, R. C., Verhoef, J., & Fluit, A. C. (2009). Identification of resistance and virulence factors in an epidemic *Enterobacter hormaechei* outbreak strain. *Microbiology*, *155*(5), 1478–1488. https://doi.org/10.1099/mic.0.024828-0

Paauw, A., Caspers, M. P. M., Schuren, F. H. J., Leverstein-van Hall, M. A., Delétoile, A., Montijn, R. C., … Fluit, A. C. (2008). Genomic diversity within the *Enterobacter cloacae* complex. *PLoS ONE*, *3*(8), e3018. https://doi.org/10.1371/journal.pone.0003018

Paauw, A., Leverstein-van Hall, M. A., Verhoef, J., & Fluit, A. C. (2010). Evolution in quantum leaps: Multiple combinatorial transfers of HPI and other genetic modules in *Enterobacteriaceae*. *PLoS ONE*, *5*(1), e8662. https://doi.org/10.1371/journal.pone.0008662

Pakharukova, N., Tuittila, M., Paavilainen, S., Malmi, H., Parilova, O., Teneberg, S., … Zavialov, A. V. (2018). Structural basis for *Acinetobacter baumannii* biofilm formation. *Proceedings of the National Academy of Sciences*, *115*(21), 5558–5563.

https://doi.org/10.1073/pnas.1800961115

Palmer, K. L., Kos, V. N., & Gilmore, M. S. (2011). Horizontal gene transfer and the genomics of enterococcal antibiotic resistance. *Current Opinion Microbiology*, 13(5), 632–639. https://doi.org/10.1016/j.mib.2010.08.004.Horizontal

Parra-Flores, J., Cerda-Leal, F., Contreras, A., Valenzuela-Riffo, N., Rodríguez, A., & Aguirre, J. (2018). *Cronobacter sakazakii* and microbiological parameters in dairy formulas associated with a food alert in Chile. *Frontiers in Microbiology*, *9*(7), 1–9. https://doi.org/10.3389/fmicb.2018.01708

Patel, V. (2015). Child mortality in England and Wales: 2015, 1–14. https://www.ons.gov.uk/peoplepopulationandcommunity/birthsdeathsandmarriages/deaths/bulletins/childh

Pavlovic, M., Konrad, R., Iwobi, A. N., Sing, A., Busch, U., & Huber, I. (2012). A dual approach employing MALDI-TOF MS and real-time PCR for fast species identification within the *Enterobacter cloacae* complex. *FEMS Microbiology Letters*, *328*(1), 46–53. https://doi.org/10.1111/j.1574-6968.2011.02479.x

Peleg, A. Y., Seifert, H., & Paterson, D. L. (2008). *Acinetobacter baumannii*: emergence of a successful pathogen. *Clinical Microbiology Reviews*, *21*(3), 538–82. https://doi.org/10.1128/CMR.00058-07

Penwell, W. F., Arivett, B. A., & Actis, L. A. (2012). The *Acinetobacter baumannii* enta gene located outside the acinetobactin cluster is critical for siderophore production, iron acquisition and virulence. *PLoS ONE*, *7*(5). https://doi.org/10.1371/journal.pone.0036493

Percival, S. L., Suleman, L., Vuotto, C., & Donelli, G. (2015). Healthcare-Associated infections, medical devices and biofilms: Risk, tolerance and control. *Journal of Medical Microbiology*, *64*(4), 323–334. https://doi.org/10.1099/jmm.0.000032

Pereira, C. A. P., Marra, A. R., Camargo, L. F. A., Pignatari, A. C. C., Sukiennik, T., Behar, P. R. P., … Edmond, M. B. (2013). Nosocomial bloodstream infections in Brazilian Pediatric patients: Microbiology, epidemiology, and clinical features. *PLoS ONE*, *8*(7), 8–13. https://doi.org/10.1371/journal.pone.0068144

Perez-Muñoz, M. E., Arrieta, M. C., Ramer-Tait, A. E., & Walter, J. (2017). A critical assessment of the “sterile womb” and “in utero colonization” hypotheses: Implications for research on the pioneer infant microbiome. *Microbiome*, *5*(1), 1–19. https://doi.org/10.1186/s40168-017-0268-4

Perez, A., Poza, M., Fernández, A., Del Carmen Fernández, M., Mallo, S., Merino, M., … Bou, G. (2012). Involvement of the AcrAB-TolC efflux pump in the resistance, fitness, and virulence of *Enterobacter cloacae*. *Antimicrobial Agents and Chemotherapy*, *56*(4), 2084–2090. https://doi.org/10.1128/AAC.05509-11

Petersen, S. M., Greisen, G., & Krogfelt, K. A. (2016). Nasogastric feeding tubes from a neonatal department yield high concentrations of potentially pathogenic bacteria -even 1 d after insertion. *Pediatric Research*, *80*(3), 395–400. https://doi.org/10.1038/pr.2016.86

Petrik, M., Zhai, C., Haas, H., & Decristoforo, C. (2017). Siderophores for molecular imaging applications. *Clinical and Translational Imaging*, *5*(1), 15–27. https://doi.org/10.1007/s40336-016-0211-x

Phoon, H., Hussin, H., Hussain B., Lim, S. Y., Woon, J. J., Er Y. X., & Thong, K. L. (2018).

Distribution, genetic diversity and antimicrobial resistance of clinically important bacteria from the environment of a tertiary hospital in Malaysia. *Journal of Global Antimicrobial Resistance,* 2 (14)132-140. https://doi.org/10.1016/j.jgar.2018.02.022. Epub 2018 Mar 11.

Pizarro-Cerdá, J., & Cossart, P. (2006). Bacterial adhesion and entry into host cells. *Cell*, *124*(4), 715–727. https://doi.org/10.1016/j.cell.2006.02.012

Polin, R. a, & Saiman, L. (2003). Nosocomial infections in the neonatal intensive care unit CONS : negative pathogens are also Group B streptococci. *Neo Reviews*, *4*(3), 81–89. https://doi.org/10.1542/neo.4-3-e81

Poyart, C., Pellegrini, E., Gaillot, O., Baptista, M., Trieu-cuot, P., & Boumaila, C. (2001). Contribution of mn-cofactored superoxide dismutase (SodA) to the virulence of *Streptococcus agalactiae*. *Infection and Immunty*, *69*(8), 5098–5106. https://doi.org/10.1128/IAI.69.8.5098

Qin, X., Galloway-Pẽa, J. R., Sillanpaa, J., Roh, J. H., Nallapareddy, S. R., Chowdhury, S., … Murray, B. E. (2012). Complete genome sequence of *Enterococcus faecium* strain TX16 and comparative genomic analysis of *Enterococcus faecium* genomes. *BMC Microbiology*, *12*(1), 1. https://doi.org/10.1186/1471-2180-12-135

Ramasethu, J. (2017). Prevention and treatment of neonatal nosocomial infections. *Maternal Health, Neonatology and Perinatology*, *3*(1), 5-11. https://doi.org/10.1186/s40748-017-0043-3

Rameshwarnath, S., & Naidoo, S. (2018). Risk factors associated with nosocomial infections in the Neonatal Intensive Care Unit at Mahatma Gandhi Memorial hospital between 2014 and 2015. *Southern African Journal of Infectious Diseases*, 33(4), 1–8. https://doi.org/10.1080/23120053.2018.1453641

Ramsey, M., & Zilberberg, D. (2009) Secular trends of hospitalization with vancomycin-resistant *Enterococcus* infection in the United States, 2000–2006. *Infection Control & Hospital Epidemiology*, 30(2), 184-189. https:// doi: 10.1086/593956

Raymond, K. N., Allred, B. E., & Sia, A. K. (2015). coordination chemistry of microbial iron transport. *Accounts of Chemical Research*, *48*(9), 2496–2505. https://doi.org/10.1021/acs.accounts.5b00301

Re, S. Da, & Ghigo, J. (2006). A csgd-independent pathway for cellulose production and biofilm formation in *Escherichia coli*. *Journal of Bacteriology*, *188*(8), 3073–3087. https://doi.org/10.1128/JB.188.8.3073

Reena, A. A. A., Subramaniyan, A., & Kanungo, R. (2017). Biofilm formation as a virulence factor of *Acinetobacter baumannii*: An emerging pathogen in critical care units A. *Journal of Current Research in Scientific Medicine*, 3(11), 74–78. https://doi.org/10.4103/jcrsm.jcrsm

Reichhardt, C., Jacobson, A. N., Maher, M. C., Uang, J., McCrate, O. A., Eckart, M., & Cegelski, L. (2015). Congo red interactions with curli-producing *E. coli* and native curli amyloid fibers. *PLoS ONE*, 10(10), e0140388. https://doi.org/10.1371/journal.pone.0140388

Revdiwala, S., Rajdev, B. M., & Mulla, S. (2012). Characterization of bacterial etiologic agents of biofilm formation in medical devices in critical care setup. *Critical Care Research and* Practice, 2012. https://doi.org/10.1155/2012/945805

Ribet, D., & Cossart, P. (2015). How bacterial pathogens colonize their hosts and invade deeper tissues. *Microbes and Infection*, *17*(3), 173–183.

https://doi.org/10.1016/j.micinf.2015.01.004

Richards, M. J., Edwards, J. R., Culver, D. H., & Gaynes, R. P. (1999). Nosocomial infections in pediatric intensive care units in the United States. *Pediatrics*, *103*(4), 39. https://doi.org/10.1542/peds.103.4.e39

Ring, A., Braun, J. S., Pohl, J., Nizet, V., Stremmel, W., & Shenep, J. L. (2002). Group B Streptococcal β-hemolysin induces mortality and liver injury in experimental sepsis. *The Journal of Infectious Diseases*, *185*(12), 1745–1753. https://doi.org/10.1086/340818

Riss, T. L., Moravec, R. A., Niles, A. L., Duellman, S., Benink, H. A., Worzella, T. J., & Minor, L. (2013). Cell viability assays. *Assay Guidance Manual*, *114*(8), 785–796. https://doi.org/10.1016/j.acthis.2012.01.006

Rodríguez, J. M., Murphy, K., Stanton, C., Ross, R. P., Kober, O. I., Juge, N., … Collado, M. C. (2015). The composition of the gut microbiota throughout life, with an emphasis on early life. *Microbial Ecology in Health & Disease*, *26*(0), 1–17. https://doi.org/10.3402/mehd.v26.26050

Rodríguez, J., Herrera, S., Echeita, A., Soler, P., Simon, F., & Mateo, S. (2010). Nationwide outbreak of *Salmonella* serotype Kedougou associated with infant formula, Spain, 2008. *Eurosurveillance*, *15*(22), 1-5. https://doi.org/19582 [pii]

Rodulfo, H., Martínez, D., & De Donato, M. (2016). Molecular identification of multidrug resistant enterobacter hormaechei in venezuela. *Investigacion Clinica (Venezuela)*, *57*(4), 402–408.

Römling, U., & Balsalobre, C. (2012). Biofilm infections, their resilience to therapy and innovative treatment strategies. *Journal of Internal Medicine*, *272*(6), 541–561. https://doi.org/10.1111/joim.12004

Rosvoll, T. C. S., Lindstad, B. L., Lunde, T. M., Hegstad, K., Aasnæs, B., Hammerum, A. M., … Pedersen, T. (2012). Increased high-level gentamicin resistance in invasive *Enterococcus faecium* is associated with aac(6')Ie-aph(2')Ia-encoding transferable megaplasmids hosted by major hospital-adapted lineages. *FEMS Immunology and Medical Microbiology*, *66*(2), 166–176. https://doi.org/10.1111/j.1574-695X.2012.00997.x

Rosvoll, T. C. S., Pedersen, T., Sletvold, H., Johnsen, P. J., Sollid, J. E., Simonsen, G. S., … Sundsfjord, A. (2010). PCR-based plasmid typing in *Enterococcus faecium* strains reveals widely distributed pRE25-, pRUM-, pIP501- and pHTβ-related replicons associated with glycopeptide resistance and stabilizing toxin-antitoxin systems. *FEMS Immunology and Medical Microbiology*, *58*(2), 254–268. https://doi.org/10.1111/j.1574-695X.2009.00633.x

Russo, T. A., Luke, N. R., Beanan, J. M., Olson, R., Sauberan, S. L., MacDonald, U., … Campagnari, A. A. (2010). The K1 capsular polysaccharide of *Acinetobacter baumannii* strain 307-0294 is a major virulence factor. *Infection and Immunity*, *78*(9), 3993–4000. https://doi.org/10.1128/IAI.00366-10

Russo, T. A., MacDonald, U., Beanan, J. M., Olson, R., MacDonald, I. J., Sauberan, S. L., … Umland, T. C. (2009). Penicillin-Binding Protein 7/8 Contributes to the survival of *Acinetobacter baumannii* in vitro and in vivo. *The Journal of Infectious Diseases*, *199*(4), 513–521. https://doi.org/10.1086/596317

Ryu, S. Y., Baek, W.-K., & Kim, H. A. (2017). Association of biofilm production with colonization among clinical isolates of *Acinetobacter baumannii*. *Journal of Central South University. Medical Sciences,* , *32*(2), 345–351.

https://doi.org/https://dx.doi.org/10.3904/kjim.2015.287

Sadowska-Krawczenko, I., Jankowska, A., & Kurylak, A. (2012). Healthcare-associated infections in a neonatal intensive care unit. *Archives of Medical Science*, *8*(5), 854–858. https://doi.org/10.5114/aoms.2012.31412

Saffari, F., Dalfardi, M. S., Mansouri, S., & Ahmadrajabi, R. (2017). Survey for correlation between biofilm formation and virulence determinants in a collection of pathogenic and fecal *Enterococcus faecalis* isolates. *Infection and Chemotherapy*, *49*(3), 176–183. https://doi.org/10.3947/ic.2017.49.3.176

Sambuy, Y., Angelis, I., Ranaldi, G., Scarino, M. L., Stammati. A., & Zucco, F. (2005). The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biology and Toxicology*, 21(1), 1-26. https://doi.10.1007/s10565-005-0085-6

Sanchez-Larrayoz, A. F., Elhosseiny, N. M., Chevrette, M. G., Fu, Y., Giunta, P., Spallanzani, R. G., … Maira-Litrán, T. (2017). Complexity of complement resistance factors expressed by *Acinetobacter baumannii* needed for survival in human serum. *The Journal of Immunology*, ji1700877. https://doi.org/10.4049/jimmunol.1700877

Sani, N. A., Ghassem, M., Babji, A. S., Kupusamy, U. P., & Jaafar, N. (2014). Incidence of *cronobacter sakazakii* in powdered infant formula milk available in Malaysia. *Sains Malaysiana*, *43*(12), 1855–1863.

Sankar, M. J., Natarajan, C. K., Das, R. R., Agarwal, R., Chandrasekaran, A., & Paul, V. K. (2016). When do newborns die? A systematic review of timing of overall and cause-specific neonatal deaths in developing countries. *Journal of Perinatology*, *36*(S), 1–11. https://doi.org/10.1038/jp.2016.27

Santagati, M., Campanile, F., & Stefani, S. (2012). Genomic diversification of enterococci in hosts: The role of the mobilome. *Frontiers in Microbiology*, *3*(3), 1–9. https://doi.org/10.3389/fmicb.2012.00095

Santajit, S., & Indrawattana, N. (2016). Mechanisms of Antimicrobial Resistance in ESKAPE Pathogens. *BioMed Research International*, *2016*. https://doi.org/10.1155/2016/2475067

Sava, I. G., Heikens, E., & Huebner, J. (2010). Pathogenesis and immunity in enterococcal infections. *Clinical Microbiology and Infection*, *16*(6), 533–540. https://doi.org/10.1111/j.1469-0691.2010.03213.x

Scatamburlo Moreira, M. A., De Souza, E. C., & De Moraes, C. A. (2004). Multidrug efflux systems in gram-negative bacteria. *Brazilian Journal of Microbiology*, *35*(1–2), 19–28. https://doi.org/10.1590/S1517-83822004000100003

Schleifer, K. H., & Kilpper-Balz, R. (1984). Transfer of *Streptococcus faecalis* and *Streptococcus faecium* to the Genus *Enterococcus* nom. rev. as *Enterococcus faecalis* comb. nov. and *Enterococcus faecium* comb. nov. *International Journal of Systematic Bacteriology*, *34*(1), 31–34. https://doi.org/10.1099/00207713-34-1-31

Schneditz, G., Rentner, J., Roier, S., Pletz, J., Herzog, K. A. T., Bucker, R., … Zechner, E. L. (2014). Enterotoxicity of a nonribosomal peptide causes antibiotic-associated colitis. *Proceedings of the National Academy of Sciences*, *111*(36), 13181–13186. https://doi.org/10.1073/pnas.1403274111

Scola, B. La, Gundi, V. a K. B., Khamis, A., & Raoult, D. (2006). The principles of DNA Sequencing.

Society, *44*(3), 827–832. https://doi.org/10.1128/JCM.44.3.827

Scott, Q., Jordy P. M. C., Sacha A. F. T., van Hijum, c, d., Martijn, A. H., Willem, J. G., Melchers, W., & van Schaik, H. F. L. W. (2017). Whole-genome sequencing of bacterial pathogens: the future of nosocomial outbreak analysis. *Clinical Microbiology Reviews*, *30*(4), 1015–1063. https://doi.org/10.1128/ CMR.00016-17

Seifi, M., Ghasemi, A., Raeisi, S., & Heidarzadeh, S. (2017). Application of next-generation sequencing in clinical molecular diagnostics. *Brazilian Archives of Biology and Technology*, *60*(12), 1–15. https://doi.org/10.1590/1678-4324-2017160414

Selasi, G. N., Nicholas, A., Jeon, H., Na, S. H., Kwon, H. Il, Kim, Y. J., … Lee, J. C. (2016). Differences in biofilm mass, expression of biofilm-associated genes, and resistance to desiccation between epidemic and sporadic clones of carbapenem-resistant *Acinetobacter baumannii* sequence type 191. *PLoS ONE*, *11*(9), 1–11. https://doi.org/10.1371/journal.pone.0162576

Shah, B. A., & Padbury, J. F. (2014). Neonatal sepsis an old problem with new insights. *Virulence*, *5*(1), 163–171. https://doi.org/10.4161/viru.26906

Shantala, G. B., Nagarathnamma, T., Pooja, D. R., Harsha, T. R., & Karthik, R. (2014). Neonatal septicaemia caused by vancomycin resistant *Enterococcus faecium*-a case report. *Journal of Clinical and Diagnostic Research*, *8*(11), 3-4. https://doi.org/10.7860/JCDR/2014/10284.5220

Sharma, C. M., Agrawal, R. P., Sharan, H., Kumar, B., Sharma, D., & Bhatia, S. S. (2013). "Neonatal sepsis": Bacteria & their susceptibility pattern towards antibiotics in neonatal intensive care unit. *Journal of Clinical and Diagnostic Research*, *7*(11), 2511–2513. https://doi.org/10.7860/JCDR/2013/6796.3594

Sharma, V. K., Kudva, I. T., Bearson, B. L., & Stasko, J. A. (2016). Contributions of EspA filaments and curli fimbriae in cellular adherence and biofilm formation of enterohemorrhagic *Escherichia coli* O157:H7. *PLoS ONE*, *11*(2), 1–23. https://doi.org/10.1371/journal.pone.0149745

Shin, S.H., Lim, Y., Lee, S.E., Yang, N.W. and Rhee, J.H. (2001). CAS agar diffusion assay for the measurement of siderophores in biological fluids. *Journal of Microbiological Methods,* 44(1), 89-95.

Sievert, D. M., Ricks, P., Edwards, J. R., Schneider, A., Patel, J., Srinivasan, A., … Fridkin, S. (2013). Antimicrobial-resistant pathogens associated with healthcare-associated infections summary of data reported to the national healthcare safety network at the Centers for Disease Control and Prevention, 2009–2010. *Infection Control & Hospital Epidemiology*, *34*(01), 1–14. https://doi.org/10.1086/668770

Sileem, A. E., Said, A. M., & Meleha, M. S. (2017). *Acinetobacter baumannii* in ICU patients: A prospective study highlighting their incidence, antibiotic sensitivity pattern and impact on ICU stay and mortality. *Egyptian Journal of Chest Diseases and Tuberculosis*, *66*(4), 693–698. https://doi.org/10.1016/j.ejcdt.2017.01.003

Sillanpaa, J., Chang, C., Singh, K. V., Montealegre, M. C., Nallapareddy, S. R., Harvey, B. R., … Murray, B. E. (2013). Contribution of individual Ebp pilus subunits of *Enterococcus faecalis* OG1RF to pilus biogenesis, biofilm formation and urinary tract infection. *PLoS ONE*, *8*(7), 1–20. https://doi.org/10.1371/journal.pone.0068813

Sillanpaa, J., Nallapareddy, S. R., Singh, K. V, Prakash, V. P., Fothergill, T., Ton-That, H., & Murray, B. E. (2010). Characterization of the ebp(fm) pilus-encoding operon of *Enterococcus*

faecium and its role in biofilm formation and virulence in a murine model of urinary tract infection. *Virulence*, *1*(4), 236–246. https://doi.org/10.4161/viru.1.4.11966

Silva, D. M., Menezes, E. M. N., Silva, E. V., & Lamounier, C., T. A. (2017). Prevalence and antimicrobial susceptibility profile of ESKAPE pathogens from the Federal District. *Brazilian Journal of Pathology and Laboratory Medicine*, (8), 240–245. https://doi.org/10.5935/1676-2444.20170037

Simjee, S., White, D. G., McDermott, P. F., Wagner, D. D., Zervos, M. J., Donabedian, S. M., … Walker, R. D. (2002). Characterization of Tn 1546 in vancomycin-resistant *Enterococcus faecium* isolated from canine urinary tract infections : evidence of gene exchange between human and animal enterococci. *Journal of Clinical Microbiology*, *40*(12), 4659–4665. https://doi.org/10.1128/JCM.40.12.4659

Singamsetty, V. K., Wang, Y., Shimada, H., & Prasadarao, N. V. (2008). Outer membrane protein A expression in *Enterobacter sakazakii* is required to induce microtubule condensation in human brain microvascular endothelial cells for invasion. *Microbial Pathogenesis*, *45*(3), 181–191. https://doi.org/10.1016/j.micpath.2008.05.006

Singh, S., Karagas, M. R., Mueller, N. T., Street, W., Building, R., Ranjan, R., … Campus, A. M. (2017). Analysis of the microbiome: Advantages of whole genome shotgun versus 16S amplicon sequencing Ravi. *Biochem Biophys Res Commun*, *17*(5), 895–906. https://doi.org/10.1016/j.bbrc.2015.12.083.Analysis

Sirijan, S., & Nitaya, I. (2006). Mechanisms of antimicrobial resistance in bacteria in ESKAPE pahogens. *BioMed Research International*, *2016*, 1–8. https://doi.org/10.1016/j.ajic.2006.05.219

Sletvold, H., Johnsen, P. J., Wikmark, O. G., Simonsen, G. S., Sundsfjord, A., & Nielsen, K. M. (2010). Tn1546 is part of a larger plasmid-encoded genetic unit horizontally disseminated among clonal *Enterococcus faecium* lineages. *Journal of Antimicrobial Chemotherapy*, *65*(9), 1894–1906. https://doi.org/10.1093/jac/dkq219

Sligl, W., Taylor, G., & Brindley, P. G. (2006). Five years of nosocomial Gram-negative bacteremia in a general intensive care unit: epidemiology, antimicrobial susceptibility patterns, and outcomes. *International Journal of Infectious Diseases*, *10*(4), 320–325. https://doi.org/10.1016/j.ijid.2005.07.003

Smani, Y., McConnell, M. J., & Pachón, J. (2012). Role of fibronectin in the adhesion of *Acinetobacter baumannii* to host cells. *PLoS ONE*, *7*(4). https://doi.org/10.1371/journal.pone.0033073

Smilowitz, J., O'Sullivan, A., & Farver, M. (2015). The influence of early infant-feeding practices on the intestinal microbiome and body composition in infants. *Nutrition and Metabolic Insights*, *8*, 1. https://doi.org/10.4137/NMI.S29530

Smith, D. R., Price, J. E., Burby, P. E., Blanco, L. P., Chamberlain, J., & Chapman, M. R. (2017). The production of curli amyloid fibers is deeply integrated into the biology of *Escherichia coli*. *Biomolecules*, *7*(4). https://doi.org/10.3390/biom7040075

Soares, R. O., Fedi, A. C., Reiter, K. C., Caierão, J., & d'Azevedo, P. A. (2014). Correlation between biofilm formation and *gelE* , *esp* , and *agg* genes in *Enterococcus* spp. clinical isolates. *Virulence*, *5*(5), 634–637. https://doi.org/10.4161/viru.28998

Softic, I., Tahirovic´, H., Skokic´, F., Tihic´, N., Ciommo, V. Di, & Auriti, C. (2013). An outbreak of nosocomial infection with *Acinetobacter baumannii* in the neonatal intensive care unit of

the Department of Paediatrics, University Clinical Centre Tuzla, Bosnia and Herzegovina. *Paediatrics Today*, *9*(2), 163–169. https://doi.org/10.5457/p2005-114.71

Soheili, S., Ghafourian, S., Sekawi, Z., Neela, V., Sadeghifard, N., Ramli, R., & Hamat, R. A. (2014). Wide distribution of virulence genes among *Enterococcus faecium* and *Enterococcus faecalis* clinical isolates. *Scientific World Journal*, *2014*. https://doi.org/10.1155/2014/623174

Solheim, M., Aakra, Å., Snipen, L. G., Brede, D. A., & Nes, I. F. (2009). Comparative genomics of *Enterococcus faecalis* from healthy Norwegian infants. *BMC Genomics*, *10*, 1–11. https://doi.org/10.1186/1471-2164-10-194

Soni, R., & English, S. (2015). Prevention of late-onset sepsis in the neonate. *Infant*, *11*(2), 42–6.

Sood, S., Malhotra, M., Das, B. K., & Kapil, A. (2008). Enterococcal infections & antimicrobial resistance. *Indian Journal of Medical Research*, *128*(2), 111–121.

Soto, G. E., & Hultgren, S. J. (1999). Bacterial Adhesins : Common Themes and Variations in Architecture and Assembly. *Journal of Bacteriology*, *181*(4), 1059–1071.

Sotolongo, J., Ruiz, J., & Fukata, M. (2012). The role of innate immunity in the host defense against intestinal bacterial pathogens. *Current Infectious Disease Reports*, *14*(1), 15–23. https://doi.org/10.1007/s11908-011-0234-4

Spironello, R. A., Botura-Amado, C., Balensiefer, T. K., de Barros Carvalho, M. D., Corrêa, D. A. M., & Cuman, R. K. N. (2015). A report on antibiotic management of neonatal sepsis caused by *enterobacter* spp. *Tropical Journal of Pharmaceutical Research*, *14*(11), 2131–2134. https://doi.org/10.4314/tjpr.v14i11.26

Squires, A. H., Atas, E., & Meller, A. (2015). Genomic pathogen typing using solid-state nanopores. *PLoS ONE*, *10*(11), 1–16. https://doi.org/10.1371/journal.pone.0142944

Stahl, J., Bergmann, H., Göttig, S., Ebersberger, I., & Averhoff, B. (2015). *Acinetobacter baumannii* virulence is mediated by the concerted action of three phospholipases D. *PLoS ONE*, *10*(9), 1–19. https://doi.org/10.1371/journal.pone.0138360

Stasic, A.J., Wong, A.C.L., & Kaspar, C.W. (2012). Osmotic and desication tolerance in *Escherichia coli* O157:H7 requries rpos. *Spring-Verlag*, 25(6), 660-665. https://doi.org/10.1007/s00284-012-0210-8

Stockwell, V. O., & Loper, J. E. (2005). The sigma factor RpoS is required for stress tolerance and environmental fitness of *Pseudomonas fluorescens* Pf-5. *Microbiology*, *151*(9), 3001–3009. https://doi.org/10.1099/mic.0.28077-0

Stoesser, N., Sheppard, A. E., Shakya, M., Sthapit, B., Thorson, S., Giess, A., … Crook, D. W. (2014). Dynamics of MDR *Enterobacter cloacae* outbreaks in a neonatal unit in Nepal: Insights using wider sampling frames and next-generation sequencing. *Journal of Antimicrobial Chemotherapy*, *70*(4), 1008–1015. https://doi.org/10.1093/jac/dku521

Stones, D. H., & Krachler, A. M. (2016). Against the tide: the role of bacterial adhesion in host colonization. *Biochemical Society Transactions*, *44*(6), 1571–1580. https://doi.org/10.1042/BST20160186

Subhadra, B., Hwan Oh, M., & Hee Choi, C. (2016). Quorum sensing in *Acinetobacter*: with special emphasis on antibiotic resistance, biofilm formation and quorum quenching. *AIMS Microbiology*, *2*(1), 27–41. https://doi.org/10.3934/microbiol.2016.1.27

Szakacs, T. A., Kalan, L., McConnell, M. J., Eshaghi, A., Shahinas, D., McGeer, A., … Patel, S. N. (2014). Outbreak of vancomycin-susceptible *Enterococcus faecium* containing the wild-type vanA gene. *Journal of Clinical Microbiology*, *52*(5), 1682–1686. https://doi.org/10.1128/JCM.03563-13

Tajeddin, E., Rashidan, M., Razaghi, M., Javadi, S. S. S., Sherafat, S. J., Alebouyeh, M., … Zali, M. R. (2016). The role of the intensive care unit environment and health-care workers in the transmission of bacteria associated with hospital acquired infections. *Journal of Infection and Public Health*, *9*(1), 13–23. https://doi.org/10.1016/j.jiph.2015.05.010

Tan, M. S. F., White, A. P., Rahman, S., & Dykes, G. A. (2016). Role of fimbriae, flagella and cellulose on the attachment of *Salmonella Typhimurium* ATCC 14028 to plant cell wall models. *PLoS ONE*, *11*(6), 1–13. https://doi.org/10.1371/journal.pone.0158311

Tanaka, M., & Nakayama, J. (2017). Development of the gut microbiota in infancy and its impact on health in later life. *Allergology International*, *66*(4), 515–522. https://doi.org/10.1016/j.alit.2017.07.010

Taylor, J. D., Zhou, Y., Salgado, P. S., Patwardhan, A., McGuffie, M., Pape, T., … Matthews, S. J. (2011). Atomic resolution insights into curli fiber biogenesis. *Structure*, *19*(9), 1307–1316. https://doi.org/10.1016/j.str.2011.05.015

Tedim, A. P., Ruiz-Garbajosa, P., Corander, J., Rodrï guez, C. M., Cantï, R., Willems, R. J., … Coque, T. M. (2015). Population biology of intestinal *Enterococcus* Isolates from hospitalized and nonhospitalized individuals in different age groups. *Applied and Environmental Microbiology*, *81*(5), 1820–1831. https://doi.org/10.1128/AEM.03661-14

Teixeira, A. B., Martins, A. F., Barin, J., Hermes, D. M., Pitt, C. P., & Barth, A. L. (2013). First report of carbapenem-resistant *Acinetobacter nosocomialis* isolates harboring ISAba1-blaOXA-23 genes in Latin America. *Journal of Clinical Microbiology*, *51*(8), 2739–2741. https://doi.org/10.1128/JCM.00469-13

Tenover, F. C., Arbeit, R. D., Goering, R. V, Mickelsen, P. a, Murray, B. E., Persing, D. H., & Swaminathan, B. (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis : criteria for bacterial strain typing: Crite. *Journal of Clinical Microbiology*, *33*(9), 2233–2239. https://doi.org/0095-1137/

Thumu, S. C. R., & Halami, P. M. (2014). Phenotypic expression, molecular characterization and transferability of erythromycin resistance genes in *Enterococcus* spp. isolated from naturally fermented food. *Journal of Applied Microbiology*, *116*(3), 689–699. https://doi.org/10.1111/jam.12386

Thursby, E., & Juge, N. (2017). Introduction to the human gut microbiota. *Biochemical Journal*, *474*(11), 1823–1836. https://doi.org/10.1042/BCJ20160510

Tilley, D., Law, R., Warren, S., Samis, J. A., & Kumar, A. (2014). CpaA a novel protease from *Acinetobacter baumannii* clinical isolates deregulates blood coagulation. *FEMS Microbiology Letters*, *356*(1), 53–61. https://doi.org/10.1111/1574-6968.12496

Toleman, M. A., Bennett, P. M., Walsh, T. R., Nemec, A., Dolzani, L., Brisse, S., … Tolmasky, M. E. (2011). NIH public access. *sciences-New York*, *13*(6), 151–171. https://doi.org/10.1016/j.drup.2010.08.003.Aminoglycoside

Tomaras, A. P., Dorsey, C. W., Edelmann, R. E., & Actis, L. A. (2003). Attachment to and biofilm formation on abiotic surfaces by *Acinetobacter baumannii*: Involvement of a novel chaperone-usher pili assembly system. *Microbiology*, *149*(12), 3473–3484.

https://doi.org/10.1099/mic.0.26541-0

Tomaschek, F., Higgins, P. G., Stefanik, D., Wisplinghoff, H., & Seifert, H. (2016). Head-to-head comparison of two multi-locus sequence typing (MLST) schemes for characterization of *acinetobacter baumannii* outbreak and sporadic isolates. *PLOS ONE*, *11*(4), e0153014. https://doi.org/10.1371/journal.pone.0153014

Top, J., Paganelli, F. L., Zhang, X., van Schaik, W., Leavis, H. L., van Luit-Asbroek, M., … Willems, R. J. L. (2013). The *Enterococcus faecium* Enterococcal biofilm regulator, EbrB, regulates the esp operon and is implicated in biofilm formation and intestinal colonization. *PLoS ONE*, *8*(5). https://doi.org/10.1371/journal.pone.0065224

Top, J., Willems, R., Van Der Velden, S., Asbroek, M., & Bonten, M. (2008). Emergence of clonal complex 17 *Enterococcus faecium* in the Netherlands. *Journal of Clinical Microbiology*, *46*(1), 214–219. https://doi.org/10.1128/JCM.01351-07

Torkaman, M., Afsharpaiman, S. H., Hoseini, M. J., Moradi, M., Mazraati, A., Amirsalari, S., & Kavehmanesh, Z. (2009). Platelet count and neonatal sepsis, a high prevalence of *Enterobacter* spp. *Singapore Medical Journal, 50*(5), 482–485.

Towner, K. J., Levi, K., Vlassiadi, M., Gould, I. M., MacKenzie, F. M., Struelens, M. J., … Van Looveren, M. (2008). Genetic diversity of carbapenem-resistant isolates of *Acinetobacter baumannii* in Europe. *Clinical Microbiology and Infection*, *14*(2), 161–167. https://doi.org/10.1111/j.1469-0691.2007.01911.x

Townsend, S., Hurrell, E., & Forsythe, S. (2008a). Virulence studies of *Enterobacter sakazakii* isolates associated with a neonatal intensive care unit outbreak. *BMC Microbiology*, *8*, 1–9. https://doi.org/10.1186/1471-2180-8-64

Townsend, S. M., Hurrell, E., Caubilla-Barron, J., Loc-Carrillo, C., & Forsythe, S. J. (2008b). Characterization of an extended-spectrum betalactamase *Enterobacter hormaechei* nosocomial outbreak, and other *Enterobacter hormaechei* misidentified as *Cronobacter* (*Enterobacter*) *sakazakii*. *Microbiology*, *154*(12), 3659–3667. https://doi.org/10.1099/mic.0.2008/021980-0

Townsend, S. M., Hurrell, E., Gonzalez-Gomez, I., Lowe, J., Frye, J. G., Forsythe, S., & Badger, J. L. (2007a). *Enterobacter sakazakii* invades brain capillary endothelial cells, persists in human macrophages influencing cytokine secretion and induces severe brain pathology in the neonatal rat. *Microbiology*, *153*(10), 3538–3547. https://doi.org/10.1099/mic.0.2007/009316-0

Townsend, S., Caubilla, B., Juncal; Loc-Carrillo, C., &; Forsythe, S. (2007b). The presence of endotoxin in powdered infant formula milk and the influence of endotoxin and *Enterobacter sakazakii* on bacterial translocation in the infant rat. *Food microbiology*, 1(24), 67-74. https://doi.org/10.1016/j.fm.2006.03.009

Tracey, K. J. (2014). Hypertension: An immune disorder. *Immunity*, *41*(5), 673–674. https://doi.org/10.1016/j.immuni.2014.11.007

Turabelidze, G., Lawrence, S. J., Gao, H., Sodergren, E., Weinstock, G. M., Abubucker, S., … Tarr, P. I. (2013). Precise dissection of an *Escherichia coli* O157:H7 outbreak by single nucleotide polymorphism analysis. *Journal of Clinical Microbiology*, *51*(12), 3950–3954. https://doi.org/10.1128/JCM.01930-13

Turton, J. F., Woodford, N., Glover, J., Yarde, S., Kaufmann, M. E., & Pitt, T. L. (2006). Identification of *Acinetobacter baumannii* by detection of the *bla* OXA-51-like

carbapenemase gene intrinsic to this species. *Journal of Clinical Microbiology*, *44*(8), 2974–2976. https://doi.org/10.1128/JCM.01021-06

Ubeda, C., Taur, Y., Jenq, R. R., Equinda, M. J., Son, T., Samstein, M., … Pamer, E. G. (2010). Vancomycin-resistant Enterococcus domination of intestinal microbiota is enabled by antibiotic treatment in mice and precedes bloodstream invasion in humans. Journal of Clinical Investigation, 120(12). https://doi.org/10.1172/JCI43918DS1

Uhlich, G. A., Cooke, P. H., & Solomon, E. B. (2006). Analyses of the Red-Dry-Rough Phenotype of an *Escherichia coli* O157 : H7 Strain and Its Role in Biofilm Formation and Resistance to Antibacterial Agents Analyses of the Red-Dry-Rough Phenotype of an *Escherichia coli* O157 : H7 Strain and Its Role in Biofil. *Applied and Enviromental Microbiology*, *72*(4), 2564–2562. https://doi.org/10.1128/AEM.72.4.2564

Ünal, N., Aşkar, Ş., & Yildirim, M. (2017). Antibiotic resistance profile of *Enterococcus faecium* and *Enterococcus faecalis* isolated from broiler cloacal samples. *Turkish Journal of Veterinary and Animal Sciences*, *41*(2), 199–203. https://doi.org/10.3906/vet-1607-26

Unemo, M., & Dillon, J. A. R. (2011). Review and international recommendation of methods for typing neisseria gonorrhoeae isolates and their implications for improved knowledge of gonococcal epidemiology, treatment, and biology. *Clinical Microbiology Reviews*, *24*(3), 447–458. https://doi.org/10.1128/CMR.00040-10

UNICEF. (2017). Committing to Child Survival: A promise renewed – progress report 2015. Retrieved August 8, 2017, from https://www.unicef.org/publications/index_83078.html

United Nations Sustainable Development. (2017). Health-united nations sustainable development. retrieved July 12, 2017, from http://www.un.org/sustainabledevelopment/health/

Uwingabiye, J., Lemnouer, A., Baidoo, S., Frikh, M., Kasouati, J., Maleb, A., … Elouennass, M. (2017). Intensive care unit-acquired *Acinetobacter baumannii* infections in a Moroccan teaching hospital: epidemiology, risk factors and outcome. *Germs*, *7*(4), 193–205. https://doi.org/10.18683/germs.2017.1126

Van Houdt, R., & Michiels, C. W. (2010). Biofilm formation and the food industry, a focus on the bacterial outer surface. *Journal of Applied Microbiology*, *109*(4), 1117–1131. https://doi.org/10.1111/j.1365-2672.2010.04756.x

van Schaik, W., Top, J., Riley, D. R., Boekhorst, J., Vrijenhoek, J. E. P., Schapendonk, C. M. E., … Willems, R. J. L. (2010). Pyrosequencing-based comparative genome analysis of the nosocomial pathogen *Enterococcus faecium* and identification of a large transferable pathogenicity island. *BMC Genomics*, *11*(1). https://doi.org/10.1186/1471-2164-11-239

Van Tyne, D., Martin, M. J., & Gilmore, M. S. (2013). Structure, function, and biology of the *Enterococcus faecalis* cytolysin. *Toxins*, *5*(5), 895–911. https://doi.org/10.3390/toxins5050895

Van Wamel, W. J. B., Hendrickx, A. P. A., Bonten, M. J. M., Top, J., Posthuma, G., & Willems, R. J. L. (2007). Growth condition-dependent Esp expression by *Enterococcus faecium* affects initial adherence and biofilm formation. *Infection and Immunity*, *75*(2), 924–931. https://doi.org/10.1128/IAI.00941-06

Vaziri, F., Peerayeh, S. N., Nejad, Q. B., & Farhadian, A. (2011). The prevalence of aminoglycoside-modifying enzyme genes (aac (6')-I, aac (6')-II, ant (2")-I, aph (3')-VI) in *Pseudomonas aeruginosa*. *Clinics (São Paulo, Brazil)*, *66*(9), 1519–22.

https://doi.org/10.1590/S1807-59322011000900002

Venkateswaran, S., Gwynne, P. J., Wu, M., Hardman, A., Lilienkampf, A., Pernagallo, S., … Gallagher, M. P. (2016). High-throughput Identification of Bacteria Repellent Polymers for Medical Devices. *Journal of Visualized Experiments*, (117), 1–10. https://doi.org/10.3791/54382

Villalon, P., Valdezate, S., Medina-Pascual, M. J., Rubio, V., Vindel, A., & Saez-Nieto, J. A. (2011). Clonal diversity of nosocomial epidemic *Acinetobacter baumannii* strains isolated in Spain. *Journal of Clinical Microbiology*, 49(3), 875–882. https://doi.org/10.1128/JCM.01026-10

Von-Dolinger de Brito, D., Oliveira, E. J., Abdallah, V. O. S., da Costa Darini, A. L., & Filho, P. P. G. (2005). An outbreak of *Acinetobacter baumannii* septicemia in a neonatal intensive care unit of a university hospital in Brazil. *The Brazilian Journal of Infectious Diseases : An Official Publication of the Brazilian Society of Infectious Diseases*, *9*(4), 301–9. https://doi.org//S1413-86702005000400006

Vu, B., Chen, M., Crawford, R. J., & Ivanova, E. P. (2009). Bacterial extracellular polysaccharides involved in biofilm formation. *Molecules*, *14*(7), 2535–2554. https://doi.org/10.3390/molecules14072535

Wang, H., Wilksch, J. J., Strugnell, R. A., & Gee, M. L. (2015). Role of capsular polysaccharides in biofilm formation: An AFM nanomechanics Study. *Environ Microbiol*, 17(5), 1560-73. https://doi.10.1111/1462-2920.12578.

Wassilew, N., Seth-Smith, H. M. B., Rolli, E., Fietze, Y., Casanova, C., Führer, U., … Buetti, N. (2018). Outbreak of vancomycin-resistant *Enterococcus faecium* clone ST796, Switzerland, December 2017 to April 2018. *Eurosurveillance*, *23*(29), 1–5. https://doi.org/10.2807/1560-7917.ES.2018.23.29.1800351

Webb, S. A., & Kahler, C. M. (2008). Bench-to-bedside review: Bacterial virulence and subversion of host defences. *Critical Care*, *12*(6), 234. https://doi.org/10.1186/cc7091

Weems, M. F., Dereddy, N. R., & Arnold, S. R. (2015). Mother's milk as a source of *Enterobacter cloacae* sepsis in a preterm infant. *Breastfeeding Medicine*, *10*(10), 503–504. https://doi.org/10.1089/bfm.2015.0146

Wei, H. M., Hsu, Y. L., Lin, H. C., Hsieh, T. H., Yen, T. Y., Lin, H. C., … Hwang, K. P. (2015). Multidrug-resistant *Acinetobacter baumannii* infection among neonates in a neonatal intensive care unit at a medical center in central Taiwan. *Journal of Microbiology, Immunology and Infection*, *48*(5), 531–539. https://doi.org/10.1016/j.jmii.2014.08.025

Weiser, J. N., & Gotschlich, E. C. (1991). Outer membrane protein A (OmpA) contributes to serum resistance and pathogenicity of *Escherichia coli* K-1. *Infection and Immunity*, *59*(7), 2252–2258.

Wenger, P. N., Tokars, J. I., Brennan, P., Samel, C., Bland, L., Miller, M., … Jarvis, W. (1997). An outbreak of *Enterobacter hormaechei* infection and colonization in an intensive care nursery. *Clinical Infectious Diseases*, *24*(6), 1243–4. https://doi.org/10.1086/513650

Werner, G., Coque, T. M., Hammerum, A. M., Hope, R., Hryniewicz, W., Johnson, A., … Kristinsson, K. G. (2008). Emergence and spread of vancomycin resistance among enterococci. *Eurosurveillance, 13*(74), 1–10. https://doi.org/10.1007/978-3-8349-8149-3

Willems, R. J. L., Top, J., Van Santen, M., Robinson, D. A., Coque, T. M., Baquero, F., … Bonten, M. J. M. (2005). Global spread of vancomycin-resistant *Enterococcus faecium* from distinct

nosocomial genetic complex. *Emerging Infectious Diseases*, *11*(6), 821–828. https://doi.org/10.3201/1106.041204

Williams, B. J., Morlin, G., Valentine, N., & Smith, A. L. (2001). Serum resistance in an invasive, nontypeable *Haemophilus influenzae* strain. *Infection and Immunity*, *69*(2), 695–705. https://doi.org/10.1128/IAI.69.2.695-705.2001

Wilson, J. W., Schurr, M. J., Leblanc, C. L., Ramamurthy, R., Buchanan, K.L., & Nickerson, C.A. (2002). Mechanisms of bacterial pathogenicity. *Postgraduate Medical Journal*, 78 (918), 216-224. https://doi: 10.1136/pmj.78.918.216

Wisplinghoff, H., Edmond, M. B., Pfaller, M. A., Jones, R. N., Wenzel, R. P., & Seifert, H. (2000). Nosocomial bloodstream infections caused by *Acinetobacter* species in United States hospitals: Clinical features, molecular epidemiology, and antimicrobial susceptibility. *Clinical Infectious Diseases*, *31*(3), 690–697. https://doi.org/10.1086/314040

World Health Organization. (2010). The burden of health care-associated infection worldwide: A summary. *World Health Organization*, 3. Retrieved from http://www.who.int/gpsc/country_work/summary_20100430_en.pdf

World Health Organization. (2011). World Health Day 2011: Policy package to combat antimicrobial resistance, 2011. Retrieved from http://www.who.int/world-health-day/2011/presskit/WHDIntrototobriefs.pdf?ua=1

World Health Organization. (2017). Child mortality 2017. *Unicef for Every Child*, 44. https://doi.org/10.1016/S0140-6736(10)60703-9

World Health Organization. (2018). Newborns: reducing mortality. [online] Available at: http://www.who.int/news-room/fact-sheets/detail/newborns-reducing-mortality

Wroblewska, M. M., Sawicka-Grzelak, A., Marchel, H., Luczak, M., & Sivan, A. (2008). Biofilm production by clinical strains of *Acinetobacter baumannii* isolated from patients hospitalized in two tertiary care hospitals. *FEMS Immunology and Medical Microbiology*, *53*(1), 140–144. https://doi.org/10.1111/j.1574-695X.2008.00403.x

Wu, H., Moser, C., Wang, H. Z., Høiby, N., & Song, Z. J. (2015). Strategies for combating bacterial biofilm infections. *International Journal of Oral Science*, *7*(7), 1–7. https://doi.org/10.1038/ijos.2014.65

Xie, R., Zhang, X. D., Zhao, Q., Peng, B., & Zheng, J. (2018). Analysis of global prevalence of antibiotic resistance in *Acinetobacter baumannii* infections disclosed a faster increase in OECD countries. *Emerging Microbes & Infections*, *7*(1), 31. https://doi.org/10.1038/s41426-018-0038-9

Yang, J., Jiang, Y., Guo, L., Ye, Li., Ma, Y., & Luo, Y. (2016). Prevalence of diverse clones of vancomycin-resistant *Enterococcus faecium* ST78 in a Chinese Hospital. *Microbial Drug Resistance*, *22*(4), 294–300. https://doi.org/10.1089/mdr.2015.0069

Yasufuku, T., Shigemura, K., Shirakawa, T., Matsumoto, M., Nakano, Y., Tanaka, K., … Fujisawa, M. (2011). Correlation of overexpression of efflux pump genes with antibiotic resistance in *Escherichia coli* strains clinically isolated from urinary tract infection patients. *Journal of Clinical Microbiology*, *49*(1), 189–194. https://doi.org/10.1128/JCM.00827-10

Yusef, D., Shalakhti, T., Awad, S., Algharaibeh, H., & Khasawneh, W. (2018). Clinical characteristics and epidemiology of sepsis in the neonatal intensive care unit in the era of multi-drug resistant organisms: A retrospective review. *Pediatrics and Neonatology*, *59*(1),

35–41. https://doi.org/10.1016/j.pedneo.2017.06.001

Zarrilli, R., Casillo, R., Di Popolo, A., Tripodi, M. F., Bagattini, M., Cuccurullo, S., … Utili, R. (2007). Molecular epidemiology of a clonal outbreak of multidrug-resistant *Acinetobacter baumannii* in a university hospital in Italy. *Clinical Microbiology and Infection*, *13*(5), 481–489. https://doi.org/10.1111/j.1469-0691.2006.01675.x

Zhao, W., & Hu, Z. (2013). pidemiology and genetics of CTX-M extended-spectrum β -lactamases in Gram-negative bacteria. *Informa Healthcare*, *39*(3), 79–101. https://doi.org/10.3109/1040841X.2012.691460

Zhang, G., Meredith, T. C., & Kahne, D. (2013a). On the essentiality of lipopolysaccharide to Gram-
negative bacteria. *Current Opinion in Microbiology*, 16(6), 779–785. http://doi.org/10.1016/j.mib.2013.09.007

Zhang, X., Bierschenk, D., Top, J., Anastasiou, I., Bonten, M. J. M., Willems, R. J. L., & van Schaik, W. (2013b). Functional genomic analysis of bile salt resistance in *Enterococcus faecium*. *BMC Genomics*, *14*(1), 1-9. https://doi.org/10.1186/1471-2164-14-299

Zhang, X., de Maat, V., Guzmán Prieto, A. M., Prajsnar, T. K., Bayjanov, J. R., de Been, M., … van Schaik, W. (2017b). RNA-seq and Tn-seq reveal fitness determinants of vancomycin-resistant *Enterococcus faecium* during growth in human serum. *BMC Genomics*, *18*(1), 1–12. https://doi.org/10.1186/s12864-017-4299-9

Zhang, Y., Du, M., Chang, Y., Chen, L. an, & Zhang, Q. (2017a). Incidence, clinical characteristics, and outcomes of nosocomial *Enterococcus* spp. bloodstream infections in a tertiary-care hospital in Beijing, China: A four-year retrospective study. *Antimicrobial Resistance and Infection Control*, *6*(1), 1–11. https://doi.org/10.1186/s13756-017-0231-y19

Zhang, Y., Lei, Y., Khammanivong, A., & Herzberg, M. C. (2004). Identification of a novel two-component system in *Streptococcus gordonii* V288 involved in biofilm formation. *Infection and Immunity*, *72*(6), 3489–3494. https://doi.org/10.1128/IAI.72.6.3489-3494.2004

Zhu, W., Murray, P. R., Huskins, W. C., Jernigan, J. A., McDonald, L. C., Clark, N. C., … Patel, J. B. (2010). Dissemination of an *Enterococcus* Inc18-like vanA plasmid associated with vancomycin-resistant Staphylococcus aureus. *Antimicrobial Agents and Chemotherapy*, *54*(10), 4314–4320. https://doi.org/10.1128/AAC.00185-10

Zieve, D., & Eltz, D. (2011). Platelet Count. *Medline Plus*, *50*(5), 482–485. Retrieved from http://www.nlm.nih.gov/medlineplus/ency/article/003647.htm

Zou, J., Shankar, N. (2016). Surface protein Esp enhances pro-inflammatory cytokine expression through NF-κB activation during enterococcal infection. Innate Immunity 22(1), 31–39. https://doi.org/10.1177/1753425915611237.